FUNCTIONAL CHARACTERISATION OF SMALL SIGNALLING PEPTIDES IN THE REGULATION OF *ARABIDOPSIS THALIANA* DEVELOPMENT

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ABSTRACT

Root architecture governs the uptake of water and nutrients from the soil and thus is essential for plant growth and survival. The control of lateral root branching is a crucial aspect of determining root architecture, and is a process largely controlled by the phytohormone auxin, which promotes lateral root organogenesis from founder cells within the vasculature and also subsequent lateral root elongation. A small posttranslationally modified peptide, C-TERMINALLY ENCODED PEPTIDE1 (CEP1) is a bioactive 15-amino acid peptide cleaved from a larger precursor protein previously shown to negatively affect primary root elongation and lateral root branching in *Arabidopsis thaliana*.

Within this thesis, genetic, biochemical and pharmacological approaches are used to demonstrate that another member of the CEP family, CEP5, reduces primary root length and negatively regulates lateral root branching in *Arabidopsis thaliana* with minor effects on aboveground architecture. CEP5 is also demonstrated to have an antagonistic relationship with auxin, with increased abundance of CEP5 leading to downstream stabilisation of the auxin signalling repressor AUXIN/INDOLE ACETIC ACID (AUX/IAA) proteins. Correspondingly, reduced abundance of CEP5 increases auxin responses, including enhanced lateral root progression. These studies suggest CEP5 regulates lateral root branching through attenuation of auxin responses.

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List of abbreviations used in this thesis:

AA: Amino acid

CCP: Circumferential Cell Proliferation

CEP: C-terminally Encoded Peptide

CEPR: C-terminally Encoded Peptide Receptor

Cf.: Compared to

DII: Domain II (from an AUX/IAA protein)

DAG: Days After Germination

DR5: Direct Repeat 5 promoter

GOF: Gain-of-Function

Hyp: Hydroxyproline

IAA: Indole-3-Acetic Acid

LC-MS: Liquid Chromatography-Mass Spectrometry

LR: Lateral Root

LRFC: Lateral Root Founder Cell

LOF: Loss-of-Function

MALDI-TOF: Matrix-Assisted Laser Desorption/Ionization-Time Of-Flight

mCEP5: Mutated CEP5 sequence

MS: Murashige and Skoog media

NAA: 1-Naphthaleneacetic Acid

NMR: Nuclear Magnetic Resonance

NE: Nuclear extract

OE: Overexpression (i.e. increased expression line)

PRL: Primary Root Length

PPP: Phloem Pole Pericycle

QC: Quiescent Centre

RID: Raw Integrated Density

RKN: Root Knot Nematode

RNAi: RNA interference (i.e. expression knockdown line)

SD or STDEV: Standard Deviation

SE or STERR: Standard Error

Spp.: Species (plural)

TDIF: Tracheary element Differentiation Inhibitory Factor

TDR: Tracheary element Differentiation Inhibitory Factor Receptor

TP: Total Protein

XIP: Xylem Intermixed with Phloem

XPP: Xylem Pole Pericycle

1. INTRODUCTION

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1.1 Root architecture

Plants are undeniably important to humans and indeed, most life on earth. The benefits to humankind provided by plants include food, fuel, fibres, medicines and materials as well as ornamental and leisure uses. It is easy to think of plants purely in terms of the clearly visible, aboveground parts of plants - the leaves, flowers, fruits and stems; however, this is only half the story. Plants also demonstrate a considerable degree of variability in the less visible underground elements: root systems. Root system architecture varies between species and also within species, subject to genotype and environment. The importance of root systems combined with the inherent difficulty of studying them has led to roots being described as 'the hidden half' of the plant' (Waisel, Eshel *et al*. 2002).

Roots are important organs in plants for a wide variety of processes, including nutrient and water uptake, anchoring and mechanical support. In addition, many plants use roots for storage functions, and some root structures

serve as human food sources- including root vegetables (such as carrots, parsnips and many others) and spices (such as ginger, arrowroot, liquorice and others). Roots serve as the major interface between the plant and various biotic and abiotic factors in the soil environment: by both sensing and responding to environmental cues, roots enable plants to overcome the challenges posed by their sessile status. This is seen in the many ways plants can dramatically alter their root architecture to optimise growth in a large variety of environmental and soil nutrient conditions. Root architecture has traditionally been largely ignored by plant breeders in terms of potential yield increases, and was not a major selection criterion as part of the crop development programmes of the 1960's green revolution (Waines and Ehdaie 2007). Understanding the development and architecture of roots thus holds potential for the exploitation and manipulation of root characteristics to both increase plant yield and optimise agricultural land use (Den Herder, Van Isterdael *et al*. 2010).

Root system architecture (RSA) –on a macro scale– describes the organisation of the primary and lateral roots and also accessory roots (including other root types found in cereals) where they are present; and is a key determinant of nutrient and water use efficiency in plants. On a micro scale, this includes root hairs that increase the surface area aiding with uptake of water and nutrients (Chandler and Brendel 2002, Davies 2003, Xiong, Wang *et al*. 2006, Denning, Kabambe *et al*. 2009, Godfray, Beddington *et al*. 2010). In both dicots and monocots, adventitious roots are also sometimes formed post-embryonically at the root-shoot junction, for example to explore and exploit the phosphorus-rich upper soil strata (Figure 1a and 1b).

1.2 Primary and Lateral Root Development

Primary roots are the first root to emerge in both dicots and monocots, and are derived from embryonically-formed meristematic tissue. The root itself broadly consists of xylem and phloem within a central vascular column and

pericycle to constitute the stele, surrounded by concentric layers of epidermal, cortical and endodermal tissues (Figure 1c–e).

In terms of vertical organisation, the root can be divided into three main developmental zones. Towards the root tip (rootward), both primary and mature lateral roots contain a meristematic tissue zone, which contains the root apical meristem – the basic stem cell pool for the other cell types in the root. Within the root apical meristem there is an area of rarely dividing cells, the quiescent centre, which signal to surrounding cells to organise and maintain the population of initial stem cells (Dolan, Janmaat *et al*. 1993, van den Berg, Willemsen *et al*. 1997). The meristematic zone also contains the root basal meristem, a shootward-orientated transition zone where cells formed by the division of cells in the apical meristem slow their division and begin to elongate. The earliest events in lateral root initiation occur in this zone (De Smet, Tetsumura *et al*. 2007). Immediately shootward of this zone is the aptly-named elongation zone, where proliferative cell division ceases and more rapid cell elongation occurs (Verbelen, De Cnodder *et al*. 2006). The third, most shootward zone is the differentiation zone, where cell elongation subsides and cell differentiation occurs, and lateral roots emerge from the parent root (Beeckman, Burssens *et al*. 2001, Dubrovsky, Gambetta *et al*. 2006). Thus, the youngest lateral roots are found the closest to the primary root tip, and maturing lateral roots become more distal as they transition away from the tip.

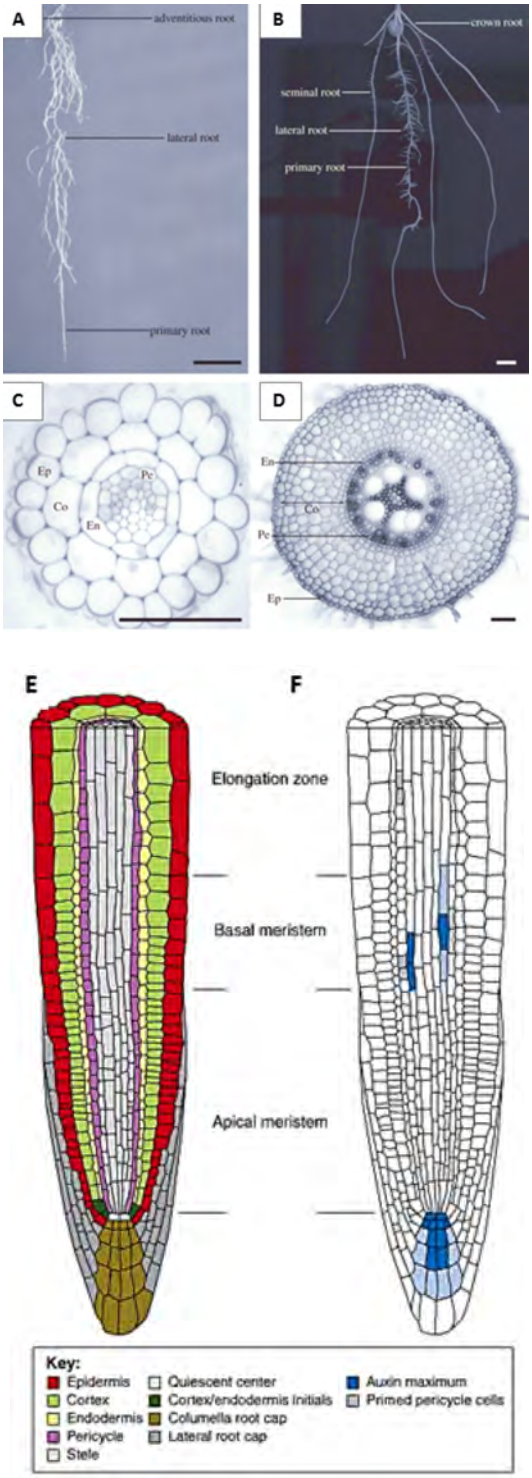

Figure 1. Macroscopic and microscopic organisation of roots. *Arabidopsis* and maize root system. **(A, B)** Macroscopic view of 14 day old *Arabidopsis* **(A)** and 10 day old maize root system **(B)**, with the major root types indicated. **(C, D)**. Transverse section through an *Arabidopsis* **(C)** and maize root **(D)**. The epidermis (Ep), cortex (Co), endodermis (En) and pericycle (Pe) are indicated. Maize pictures in **(B)** and **(D)** were kindly provided by Leen Jansen. Scale bars: **(A, B)** 1 cm and **(C, D)** 100 µm. **(E)** Schematic of the main tissue types and their distribution in the primary root. **(F)** The auxin signalling maximum present as reported by DR5::GUS marker line (De Smet *et al.* 2007). The sites of auxin accumulation in the protoxylem within the basal meristem region prime the adjacent pericycle cells to become competent to form lateral root initials. Figure 1E and 1F from Peret *et al.* 2009.

Root branching is essential to increase the surface area of the root system, enabling the plant to tap more distant reserves of water, aid nutrient foraging and improve soil anchorage. In contrast to primary roots, lateral roots are formed post-embryonically, a process which requires the re-specification of existing differentiated cells into lateral root founder cells (LRFCs). The formation of a properly organised and patterned lateral root from a limited number of founding cells requires tight temporal and spatial control of cell division. In *Arabidopsis* and most dicots, the xylem pole pericycle (a tissue layer located between the central vascular cylinder and the endodermis, proximal to the xylem) contains the cells which form the initials for lateral root branching: whilst in monocots such as maize, the phloem pole pericycle is the tissue where lateral roots initiate. Lateral root initiation can be divided into three main stages: priming, founder cell establishment and asymmetric cell division (De Smet 2012).

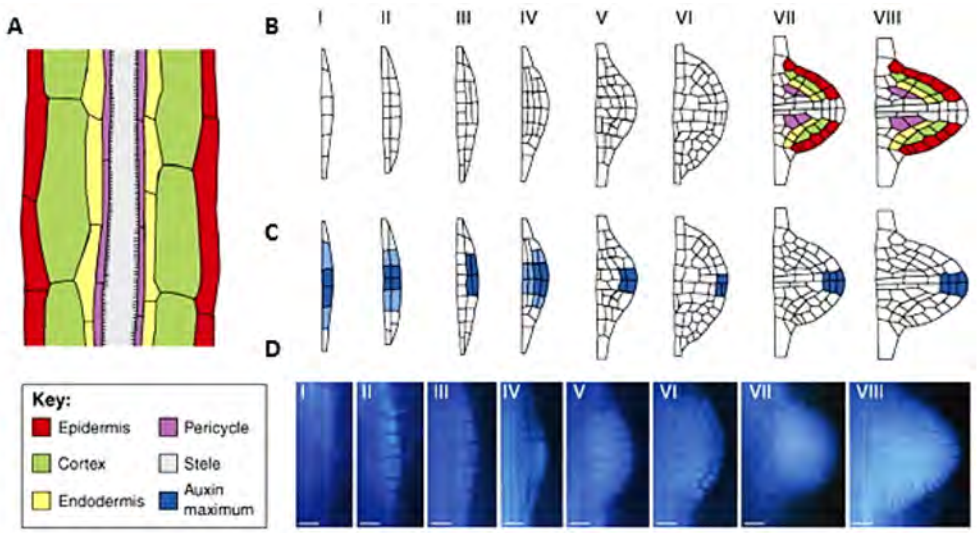

Figure 2. Morphological progression of a lateral root primordium during lateral root development. (A) Tissue organisation in the primary (parent) root. Lateral roots originate from the pericycle tissue (purple). **(B)** The stages of lateral root development from I–VIII. Tightly controlled cell divisions in both the anticlinal and periclinal division planes enable creation of a dome-like primordia structure following the initial creation of the lateral root founder cells (LRFCs, Stage I). As the nascent lateral root prepares to emerge (Stages VII-VIII) cells in the primordia differentiate into mature tissue types and the lateral root vasculature forms. **(C)** Infographic showing the distribution of auxin abundance in the primordia at each stage, as reported by DR5:GUS (darker blue representing areas of auxin maxima. **(D)**The cartoons were drawn from aniline-blue-stained roots for each stage of lateral root development; scale bars represent 20 µm.

Figure from Figure 1, Peret *et al*. 2009.

Lateral root initiation is the result of auxin-dependent cell cycle progression in a subset of pericycle cells which have specifically been made competent to become LRFCs; in *Arabidopsis* these are specific pairs of xylem pole pericycle (XPP) cells (Figure 1f). (Dubrovsky, Rost *et al*. 2001). These initial cells subsequently go through several rounds of anticlinal divisions to produce a single layered primordium (stage I) approximately ten cells long, before dividing periclinally to form two layers (stage II). Further rounds of anticlinal and periclinal cell division form a dome-shaped primordium which grows until it emerges perpendicularly to the parent primary root at stage VIII (Sabatini, Beis *et al*. 1999, De Smet, Vassileva *et al*. 2008) (Figure 2). In contrast to the formation of shoot lateral organs, the later stages of lateral root emergence require the *de novo* creation of a new apical meristem within the emerging lateral root.

1.3 Auxin in lateral root development

Lateral root initiation requires that primed pericycle cells become LRFCs and undergo several rounds of cell division to form the lateral root primordium, the basis of the new lateral root. The overriding influence in the cell cycle control required during root formation is the phytohormone auxin; indeed, simple exogenous application of auxin alone is sufficient to initiate lateral roots (Thimann 1936, Nagao 1942, Blakely, Blakely *et al*. 1988). Lateral root initiation sites are created following the concentration of auxin in defined pericycle regions; the pericycle cells encompassed by these maxima are then 'primed' to undergo the divisions necessary for lateral root primordia formation (Dubrovsky, Sauer *et al*. 2008) (Figure 2).

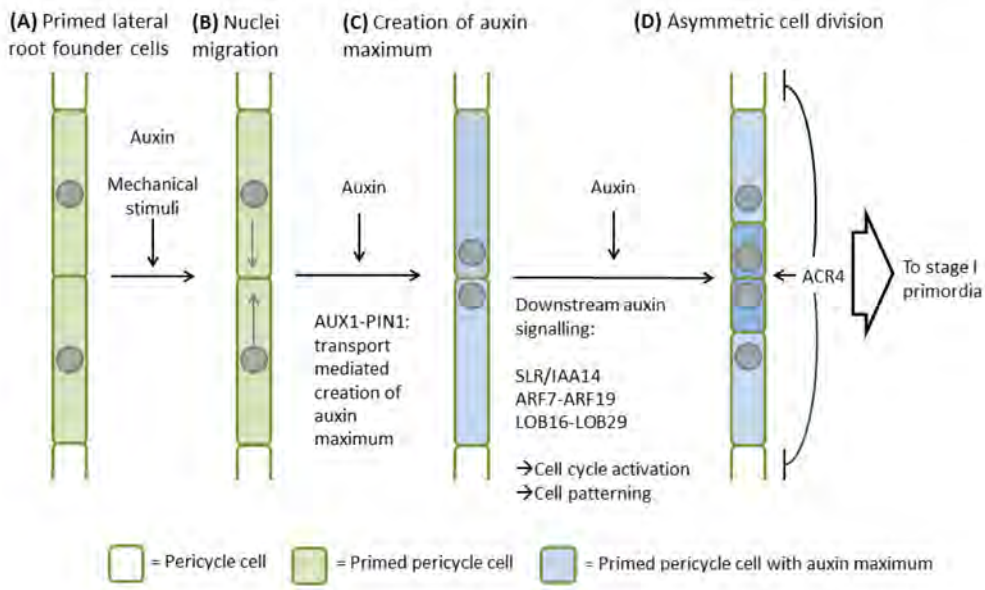

Figure 3. Lateral root initiation from primed pericycle cells. Lateral root founder cells (LRFCs) are formed from primed pericycle cells that have been made competent to respecificate and divide. Auxin and mechanical stimuli cause redistribution of AUX1 auxin influx carriers which further concentrate auxin within the LRFCs to form auxin maxima. Auxin then initiates a signal cascade that cases the degradation of SLR/IAA14 auxin-signalling repressor protein, freeing the ARF7 and ARF19 transcription factors to drive transcription of auxin-responsive genes involved in developing the new lateral root, including LATERAL ORGAN BOUNDARIES 16 and 29 (LOB16/LOB29). Meanwhile, the receptor-like kinase ACR4 helps pattern the new lateral root primordium by promoting formative cell divisions in the primordium whilst simultaneously repressing cell divisions in the flanking cells.

Figure based upon Figure 3 from Peret *et al*. 2009.

There are two main models to explain initiation of lateral root primordia: the ‘bending hypothesis’ and the ‘oscillation hypothesis’. The 'bending hypothesis' is based on observations that lateral roots initiate from concentrated regions of auxin maxima formed at the convex side of bends in the primary root, with these bends being induced by either gravity or mechanical bending from where a root encounters obstacles in growth. Conversely, the 'oscillation hypothesis' proposes that the expression of genes involved in primordium initiation naturally oscillate according to time, and it is this periodicity of expression that determines the site of lateral root primordia initiation. Increasingly, evidence suggests both models are correct, and integration of both signals is required to determine primordia initiation, spacing and development (Kircher & Schopfer 2015).

Nevertheless, the earliest events in lateral root initiation occur following oscillations of auxin concentration within the basal meristematic region of the root (De Smet, Tetsumura *et al*. 2007, De Smet, Lau *et al*. 2010). Observations using the synthetic auxin-driven reporter DR5 (see section 1.3.3 'Auxin signalling') suggests that auxin drives the oscillatory transcription of genes involved in specification of LRFCs and subsequent development of lateral roots, whilst concurrently providing a mechanism of regulating lateral root spacing (De Smet, Tetsumura *et al*. 2007, Moreno-Risueno, Van Norman *et al*. 2010). The expression of approximately 3400 genes oscillates in phase or antiphase to the oscillations of auxin: these include transcription factors which display corresponding initiation defects in lines where they have been mutated. Correspondingly, one of these is GATA23, a GATA-type transcription factor which is a marker for founder cell identity which requires the TIR1-AFB and AUX/IAA28 auxin signalling modules for its expression (Brady, Orlando *et al*. 2007, De Rybel, Vassileva *et al*. 2010, Moreno-Risueno, Van Norman *et al*. 2010).

However, these transcriptional oscillations, occurring approximately every six hours, do not always strictly correspond to formation of a lateral root; furthermore, the auxin oscillations do not display 'sidedness', whilst lateral roots typically emerge in a left-right alternating pattern (De Smet, Tetsumura *et al*. 2007). It is thus likely that the oscillation in gene expression does not necessarily require auxin oscillation and is rather a self-sustaining process governed by other unclear mechanisms (Moreno-Risueno, Van Norman *et al*. 2010).

Furthermore, the initial priming of cells to become competent to form LRFCs is likely decoupled from the subsequent developmental processes required to produce a fully formed lateral root (Van Norman, Xuan *et al*. 2013). In support of this, mutants for auxin transport or signalling (which typically show a reduced lateral root phenotype) have been demonstrated to be capable of forming lateral root primordia when mechanically stimulated, despite these sites not necessarily progressing into fully formed lateral roots (Ditengou, Teale *et al*. 2008, Richter, Monshausen *et al*. 2009). Furthermore, despite the fact that as many as 12 pericycle cells may transition through the

oscillation zone during the oscillation period, only a subset of these will undergo respecification to become LRFCs, suggesting the existence of further refinement mechanisms to focus the positioning of branch sites through restricting the number of pericycle cells undergoing respecification. One possible explanation for this discrepancy is the alternative hypothesis that rather than acting as the signal for the initial specification of LRFCs, the main role of auxin is simply to promote LRFC division following earlier specification of LRFCs through a different mechanism (Van Norman, Xuan *et al*. 2013).

1.3.1 Auxin synthesis

Indole-3-acetic acid (IAA) is the most well-known form of auxin native to plants (though other naturally occurring active forms of auxin such as 4-chloroindole-3-acetic acid: 4-Cl-IAA; and phenylacetic acid: PAA, also exist). IAA can be produced by either tryptophan dependent (IAOx, IAM and IPyA pathways) or tryptophan independent mechanisms, and is produced mainly from tryptophan precursors in young aerial tissues and transported to other organs (Woodward and Bartel 2005, Korasick, Enders *et al*. 2013). In the tryptophan dependent auxin synthesis pathway, tryptophan is converted to indole-3-pyruvic acid (IPA) by TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS (TAA) before finally being converted into indole acetic acid (IAA), the most common natural auxin, by YUCCA (YUC) family flavin monoxygenase enzymes (Mashiguchi, Tanaka *et al*. 2011, Won, Shen *et al*. 2011). Interestingly, the *YUCCA* and *TAA* enzymes are expressed in discrete regions, suggesting localised biosynthesis of auxin is also an important factor in generating precise auxin distributions within plants.

Furthermore, IAA can be stored in plants in the form of IAA conjugated with either sugars or amino acids; in particular the GRETCHEN HAGEN 3 (GH3) family of acyl acid amido synthetases are rapidly induced by auxin and catalyse the conjugation of amino acids to IAA (Staswick, Serban *et*

al. 2005). This conjugation can represent a means of controlling auxin levels: some of these conjugates can also be hydrolysed enzymatically to yield active IAA (Ludwig-Muller 2011, Ljung 2013). IAA can also be peroxisomally converted into indole-3-butyric acid (IBA), where free IAA is released upon either hydrolysis or β-oxidation of IBA respectively. The synthetic molecule 'non-auxin-like lateral root inducer', naxillin, acts upon an enoyl-CoA hydratase involved in a peroxisomal β-oxidative IBA to IAA conversion pathway, increasing the level of IAA two-fold in the root tip and increasing lateral root density. This specificity of the region of free IAA increase appears to be due to the dependence of naxillin activity on the IAA14 and ARF7-ARF19 signalling modules of the auxin signalling pathway (see below), and suggests the localised release of IAA from an IBA storage intermediate might be of particular importance in lateral root development (De Rybel, Audenaert *et al*. 2012). In support of this, higher-order mutants for ENOYL-COA HYDROTASE2 (ECH2) and a family of predicted dehydrogenase/reductases also involved in IBA β-oxidation, INDOLE-3-BUTYRIC ACID RESPONSE (IBR1, IBR3 and IBR10), have reduced free auxin levels and decreased lateral root numbers (Strader, Wheeler *et al*. 2011). Localised release of IAA from IBA storage intermediates in the root cap may therefore represent a localised mechanism of lateral root branch regulation and determine the spatiotemporal patterning of pre-branch sites (De Rybel, Audenaert *et al*. 2012, Xuan, Audenaert *et al*. 2015).

1.3.2 Auxin transport

IAA is redistributed from the synthesising tissues to other parts of the plant. Auxin can be transported over large distances in the plants through the vasculature, whilst on an intercellular level, protonated IAA (IAAH) can enter cells via simple lipophilic diffusion at apoplastic pH (pH ~5.5; IAA pKa = 4.6). This passive diffusion is thought to account for ~15% of auxin transport into cells (Zazimalova, Murphy *et al*. 2010). Once inside the cell, the cytosolic

pH (pH 7) causes IAAH to deprotonate to IAA^{-}, thus passive diffusion is not a viable method of auxin exit from cells.

The auxin carrier proteins (such as the influx carrier AUXIN RESISTANT1/LIKE AUX1 (AUX1/LAX1) and the PINFORMED (PIN) and the ATP-driven P-GLYCOPROTEIN (PGP)/MULTIDRUG RESISTANCE (MDR)/B-TYPE ATP BINDING CASSETTE (ABCB) efflux carriers) together can co-ordinate to produce a more targeted directional flow of auxin (Rubery and Sheldrake 1974, Raven 1975, Li, Yang *et al*. 2005, Spalding 2013).

1.3.2.1 AUX/LAX and NRT1.1 auxin influx carriers

In contrast to the passive mechanism involved in uptake of ionic IAA, influx carrier proteins actively mediate the uptake of anionic IAA into the cell. IAA uptake is predicted to be 15 times greater in cells expressing *AUX1* influx carriers than in cells relying on passive auxin influx alone (Swarup, Kramer *et al*. 2005, Kramer and Bennett 2006). In *Arabidopsis*, the AUX/LAX proteins comprise of a family of four amino acid transporter (ATF), permease-like H+ symporters: AUX1 and LIKE AUX1–3 (LAX1–3) (Maher and Martindale 1980, Evans, Ishikawa *et al*. 1994, Bennett, Marchant *et al*. 1996, Swarup, Kramer *et al*. 2005). AUX/LAX proteins have multiple transmembrane regions, with AUX1 containing 11 transmembrane segments (Swarup, Kargul *et al*. 2004). The similarity of AUX1/LAX sequences to each other suggests this family of auxin transporters arose from a gene duplication event (Peret, Swarup *et al*. 2012, Swarup and Peret 2012).

AUX1/LAX carriers are thought to be particularly important in the local redistribution of auxin, rather than transport of auxin over long distances throughout the plant (Parry, Marchant *et al*. 2001). AUX1 localises to the plasma membrane of certain cell types, including asymmetric localisation to the upper membrane of protophloem cells in the root - where AUX1 is thought to play a role in auxin unloading from the phloem (Swarup, Friml *et al*. 2001).

AUX1 is also expressed in columella cells and loss-of-function mutants are agravitropic with defects in auxin flux from the site of gravity perception to the site of gravity response: thus evidence supports a role for AUX1 in gravitropism (Maher and Martindale 1980, Swarup, Friml *et al*. 2001, Swarup, Kramer *et al*. 2005).

LAX3 has been shown to play a role in lateral root emergence, with *lax3* mutants displaying a reduced number of lateral roots. It is hypothesised that LAX3 is instrumental in creating an auxin accumulation in the emerging lateral root thus promoting the expression of cell wall remodelling enzymes to facilitate the emergence of the lateral root through the parent root tissues (Cosgrove 2000, Cosgrove 2005, Swarup, Benkova *et al*. 2008). This is supported by the observation that *lax3* mutants have phenotypically normal (and in fact increased numbers of) lateral root initiation sites, but are deficient in emerged lateral roots (Lucas, Godin *et al*. 2008, Lucas, Guedon *et al*. 2008).

In addition to a role in transporting nitrate, the nitrate transporter-receptor NRT1.1 has also been shown to transport auxin, lowering auxin accumulation in lateral roots at low nitrate and thus providing an intriguing potential mechanism of linking nutrient sensing with root phenotypical changes in facilitating nutrient foraging (Krouk, Lacombe *et al*. 2010).

1.3.2.2 PIN and PGP/MDR/ABCB auxin efflux carriers

Once inside the cell, the cytosolic pH (pH 7) causes IAAH to deprotonate so that it can no longer move by lipophilic diffusion. Therefore, auxin efflux carriers are required to direct the IAA out of the cell. The PIN family efflux carriers and a subset of ABCB carriers coordinate auxin export from the cell (Figure 4).

The PIN family transporters are the main contributors to polar auxin transport within plants, with *Arabidopsis* PIN homologs distributed throughout the plant kingdom (Paponov, Teale *et al*. 2005, Wang, Li *et al*. 2011, Carraro,

Tisdale-Orr *et al*. 2012, Pattison and Catala 2012). The PIN family is comprised of eight genes in *Arabidopsis*, five of which encode full-length transporters with a long hydrophilic central loop (canonical PINs) and are directly involved in mediating auxin efflux from the cell. The function of the non-canonical PINs, PIN5, PIN6 and PIN8, is less clear, but the localisation of these PINs in the ER suggests a role in intracellular auxin movement and homeostasis (Mravec, Skupa *et al*. 2009, Dal Bosco, Dovzhenko *et al*. 2012, Nisar, Cuttriss *et al*. 2014). PIN proteins are subject to many layers of regulation, including transcriptional regulation (which is itself regulated by auxin), protein stability and transport activity regulation. Directionality of intercellular auxin flow due to carrier proteins arises from specific expression and dynamic positioning (endocytic recycling and polarised targeting) of the proteins in the plasma membrane, leading to asymmetric plasma membrane localisation and thus providing a mechanism for the directional canalisation and concentration of auxin into precise regions, tissues, or even individual cells (de Reuille, Bohn-Courseau *et al*. 2006, Grieneisen, Xu *et al*. 2007, Krecek, Skupa *et al*. 2009). PINs have been demonstrated to act in various developmental roles including embryogenesis, organ formation, patterning of root meristems, vascular patterning and tropic responses (Friml, Wisniewska *et al*. 2002, Benkova, Michniewicz *et al*. 2003, Reinhardt 2003, Reinhardt 2005, Tanaka, Dhonukshe *et al*. 2006).

PIN1 is localised to the lower (rootward facing) plasma membrane of vascular cells, promoting the polar transport of auxin towards the root tip (Galweiler, Guan *et al*. 1998). Contrastingly, PIN2 is located at the upper (shootward facing) plasma membrane of lateral root cap and epidermal cells, but basally localised in root cortex cells, creating an auxin flux loop (Blilou, Xu *et al*. 2005, Wisniewska, Xu *et al*. 2006, Rahman, Takahashi *et al*. 2010). The polar localisation of PIN1 is known to be dependent on the serine-threonine kinase PINOID (PID) and the antagonistic protein phosphatase 2A (PP2A) (Friml, Yang *et al*. 2004, Michniewicz, Zago *et al*. 2007): null mutations of PIN1 and PID both give rise to shoots with defects in inflorescence development, giving a pin-like inflorescence phenotype and highlighting the importance of polar auxin transport in organ formation

(Okada, Ueda *et al*. 1991, Bennett, Alvarez *et al*. 1995, Galweiler, Guan *et al*. 1998).

Several distinct *in vivo* examples highlight the dynamic nature of PIN localisation in development, growth and tropic responses. In the early *Arabidopsis* embryo, PIN7 is localised to the upper side of suspensor cells, promoting auxin accumulation into the young embryo. However, as the embryo matures, PIN7 is redistributed along with PIN1 to the lower side of suspensor cells, redistributing auxin to the root pole and triggering the specification of a new root meristem (Friml, Vieten *et al*. 2003, Friml, Yang *et al*. 2004, Michniewicz, Zago *et al*. 2007). An even more rapid redistribution of PINs occurs during gravitropism: within minutes of the application of a gravistimulus, PIN3 switches from a uniform distribution in the plasma membrane of root tip cells to a concentrated distribution along the lower (most proximal to the gravistimulus) side of the cells. This serves to redirect auxin flow to the lower side of the root where, in concert with AUX1 and PIN2, cells in the elongation zone are inhibited by the accumulation of auxin whilst cells on the upper side of the root continue to elongate, leading to root bending towards the gravity vector (Swarup, Friml *et al*. 2001, Friml, Wisniewska *et al*. 2002, Abas, Benjamins *et al*. 2006, Harrison and Masson 2008).

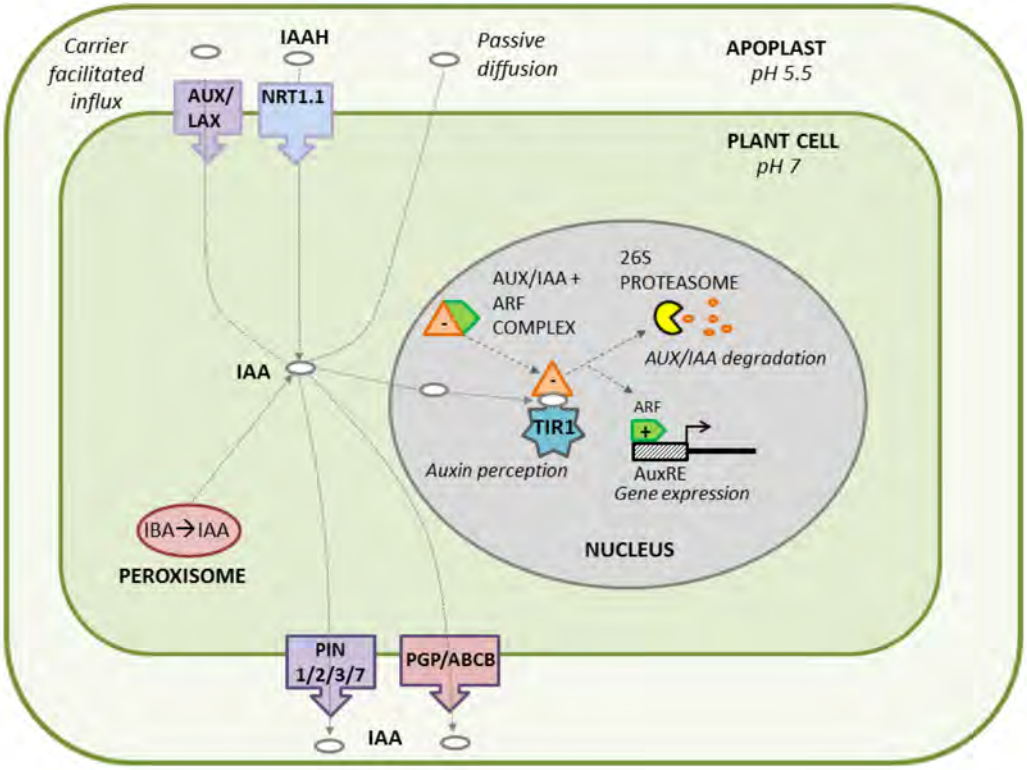

Figure 4. Simplified schematic of the intracellular pathway of auxin (IAA) and events required for auxin perception. Protonated IAA (IAAH) crosses the plasma membrane and enters the cell from the apoplast, either by passive diffusion or carrier-mediated influx (AUX/LAX and NRT1.1 influx carriers shown.) Once inside the cell, a change in pH causes IAA to become deprotonated. IAA can also be released into the cytosol via peroxisomal conversion of the storage intermediate IBA into IAA. Auxin is transported out of the cell via efflux carriers (PIN and PGP/ABCB family efflux carriers shown). Some auxin carriers, for example AUX1 and PIN2, show strong polar localisation depending on cell type and developmental context, thus providing a way to specifically direct the flow of auxin.

Once inside the cell, auxin is perceived by nuclear receptors such as TIR1, where auxin acts to facilitate the interaction of the ARF-repressive AUX/IAA proteins with ubiquitin ligase component of TIR1, thereby targeting AUX/IAA proteins for destruction by the 26S proteasome. Freed from repression, ARFs can then bind to AuxRE sequences in early auxin-response genes, regulating their expression and initiating various downstream auxin responses.

More recently, a class of intracellular PIN-like family of auxin transport proteins has been discovered using an *in silico* approach. PIN-LIKES (PILS) regulate auxin accumulation in the ER and thus may regulate intracellular auxin homeostasis and metabolism and modulate cellular sensitivity to auxin (Barbez, Kubes *et al*. 2012).

PGP/ABCB transporters also play a role in regulating auxin transport. ABCB transporters are large membrane proteins possessing typically six transmembrane helices and an ATP binding domain. Unlike PINs such as PIN1

and 2, ABCB transporters do not appear to be strongly polarised (Spalding 2013) and their exact role is more controversial, though it is possible that ABCBs use the energy released by ATP hydrolysis to actively pump auxin from the cell against the electrochemical gradient, working both synergistically and antagonistically with the (likely) passive PIN proteins in various developmental processes (Blakeslee, Bandyopadhyay *et al*. 2007, Mravec, Kubes *et al*. 2008). Originally PGP/ABCB transporters were assumed to be auxin efflux carriers: however, newer evidence suggests a dual influx/efflux function in PGP4; whether this dual transport function is a shared trait with other transporters of this family remains to be investigated (Terasaka, Blakeslee *et al*. 2005, Cho, Lee *et al*. 2007, Kubes, Yang *et al*. 2012).

1.3.2.3 IBA transport

Like IAA, there is evidence IBA undergoes long-distance transport throughout plants (Ludwig-Muller, Raisig *et al*. 1995) including both basipetal and acropetal transport in roots (Rashotte, Poupart *et al*. 2003). However, IBA does not appear to be a substrate for the IAA transporters AUX1 or PIN/ABCB family transporters (Poupart and Waddell 2000, Zolman, Yoder *et al*. 2000, Rashotte, Poupart *et al*. 2003, Yang, Hammes *et al*. 2006, Strader and Bartel 2009, Ruzicka, Strader *et al*. 2010).

Two members of the PLEIOTROPIC DRUG RESISTANCE (PDR) subclade of the ABCG family of ABC transporters, PDR8/ABCG36 (also known as PEN3) and PDR9/ABCG37, appear likely candidates for IBA efflux transport based on mutant sensitivities to IBA. The loss-of-function mutants *pdr8/abcg36* and *pdr9/abcg37* both exhibit sensitivity to IBA but not IAA (Strader, Monroe-Augustus *et al*. 2008, Strader and Bartel 2009, Ruzicka, Strader *et al*. 2010) and hyperaccumulate labelled IBA, but not IAA, in their root tips during transport assays (Strader, Monroe-Augustus *et al*. 2008, Strader and Bartel 2009). Furthermore, *pdr8/abcg36* and *pdr9/abcg37* display phenotypes suggestive of high levels of auxin accumulation in certain cell

types (such as increased elongation of root hairs), suggesting that cellular IAA levels increase if IBA efflux is decreased (Strader and Bartel 2009, Ruzicka, Strader *et al*. 2010). PDR8/ABCG36 and PDR9/ABCG37 both localise to the outward face of cells in the root epidermis, thus potentially mediating IBA efflux into the rhizosphere (Strader and Bartel 2009, Langowski, Ruzicka *et al*. 2010, Ruzicka, Strader *et al*. 2010). The purpose of this efflux is unclear, but it is possible it aids in auxin homeostasis and/or communication with biotic elements of the rhizosphere (Strader and Bartel 2011).

The mechanism of IBA influx is less clear, though RIB1 has been suggested as a potential influx carrier due to *rib1* mutants displaying resistance and altered transport for IBA, but not IAA (Poupart and Waddell 2000). On the intracellular level, since IBA-IAA conversion is peroxisomal, uptake of IBA into the peroxisome is also necessary: this is thought to be mediated by the protein PXA1 (Zolman, Yoder *et al*. 2000, Zolman, Silva *et al*. 2001, Strader and Bartel 2009).

1.3.3 Auxin signalling

The auxin signalling pathway is (in the absence of auxin) under repression by members of the AUX/IAA family of repressor proteins, which bind to the transcriptional regulators AUXIN RESPONSE FACTORs (ARFs) preventing their action; AUX/IAA proteins also interact with the co-repressor TOPLESS (TPL) which inhibits transcription of auxin-responsive genes through alteration of chromatin structure (Long, Ohno *et al*. 2006, Szemenyei, Hannon *et al*. 2008, Wang, Kim *et al*. 2013).

There are six members of the TRANSPORT INHIBITOR RESPONSE 1/AUXIN SIGNALING F-BOX (TIR/AFB) family of auxin receptor proteins in *Arabidopsis*. TIR1 and the related AFB1-5 proteins represent the F-box component of a SKP1-Cullin-F-Box (SCF) E3 ubiquitin ligase complex. The binding of auxin by TIR1/AFB family auxin-receptor protein complexes promotes the interaction of AUX/IAA proteins with the ubiquitin ligase

component of the receptor complex (Xuan, Audenaert *et al*. 2015), targeting AUX/IAA proteins for destruction by the 26S proteasome and thus de-repressing the auxin signal pathway. This destruction of AUX/IAA proteins releases the corepressor TOPLESS and frees the ARF transcriptional regulators from repression, allowing them to drive expression of early auxin-responsive genes containing Auxin Response Elements (AuxREs), which are involved in controlling the organogenesis cell division programme (Fukaki, Tameda *et al*. 2002, Dharmasiri, Dharmasiri *et al*. 2005, Kepinski and Leyser 2005, Fukaki, Okushima *et al*. 2007). The ARFs themselves do not require auxin for targeting or binding to AuxREs, thus the primary purpose of auxin in the auxin signalling pathway appears limited to promoting the release and degradation of repressors (Ulmasov, Hagen *et al*. 1999, Guilfoyle and Hagen 2001, Tiwari, Hagen *et al*. 2003).

Though TIR1 and all AFB proteins have been shown to bind auxin, the properties of the different receptors are not equal. TIR1 and AFB2 interact much more strongly with AUX/IAA proteins than AFB1 and AFB3 (Parry, Calderon-Villalobos *et al*. 2009, Greenham, Santner *et al*. 2011) and in contrast to the other members of the family, AFB4 negatively regulates auxin signalling (Greenham, Santner *et al*. 2011).

The ARF and AUX/IAA gene families respectively contain 23 and 29 members in *Arabidopsis.* The expression patterns of different *AUX/IAA* and *ARF* proteins varies spatially and developmentally, thus additional control in spatial and developmental contexts can be achieved through differing AUX/IAA-ARF pairs, and different combinations of these partially account for the diversity in phenotypic responses generated in response to a generic auxin signal (Okushima, Overvoorde *et al*. 2005, Overvoorde, Okushima *et al*. 2005, Dreher, Brown *et al*. 2006).

AUX/IAA proteins are usually comprised of four domains: domain I is a repressive domain which binds TOPLESS (Tiwari, Hagen *et al*. 2004) whilst domain II contains a sequence recognised by the TIR1 F-protein of the SCF E3 ligase complex; this domain is considered a degron since it promotes proteolysis (Dreher, Brown *et al*. 2006). Domains III and IV contain sequences

similar to those found in the C-terminal portions of ARF proteins, and mediate homo and hetero-dimerisation between AUX/IAAs and ARFs (Kim, Harter *et al*. 1997, Ulmasov, Hagen *et al*. 1997, Ulmasov, Murfett *et al*. 1997, Ouellet, Overvoorde *et al*. 2001). Different AUX/IAA proteins display differing half-lives both in terms of basal degradation and auxin-accelerated degradation, with some 'non-canonical' AUX/IAA proteins such as IAA20 (which lack domain II) appearing to be largely insensitive to auxin-mediated degradation (Dreher, Brown *et al*. 2006). Interestingly, AUX/IAA proteins themselves appear to be necessary for perception of auxin by TIR1, with auxin affinity being largely determined by the characteristics of the bound AUX/IAA protein (Villalobos, Lee *et al*. 2012).

ARF proteins are modular transcription factors, usually consisting of three domains: an N-terminal DNA Binding Domain (DBD), a central Activation Domain (AD) and a C-terminal Dimerization Domain (CTD), which (with the exception of ARF3, ARF13 and ARF17) contains similar amino acid sequences to domains III and IV of AUX/IAA proteins (Guilfoyle and Hagen 2001, Tiwari, Hagen *et al*. 2003, Korasick, Westfall *et al*. 2014). ARFs can be either transcriptional activators (ARF5–8 and ARF19) or repressors (Ulmasov, Hagen *et al*. 1999, Ulmasov, Hagen *et al*. 1999, Tiwari, Hagen *et al*. 2003, Yamasaki, Kigawa *et al*. 2004), and increasingly evidence shows ARFs can be both activators and repressors depending on the cell type and environment (Ulmasov, Hagen *et al*. 1999, Zouine, Fu *et al*. 2014). Furthermore, the same gene can be a target for both activating and repressing ARFs: for example the auxin-responsive gene GH3.6 is a target for at least three activator ARFs and one repressor ARF respectively (Okushima, Overvoorde *et al*. 2005, Wilmoth, Wang *et al*. 2005). Higher order ARF mutants display much more severe phenotypes than single mutants in *Arabidopsis*, suggesting a degree of redundancy in ARFs (Remington, Vision *et al*. 2004, Guilfoyle and Hagen 2007).

Another protein, AUXIN BINDING PROTEIN 1 (ABP1) has previously been suggested to function as an auxin receptor with a role in various cellular and developmental contexts (Jones 1994, Chen, Ullah *et al*. 2001, Woo, Marshall *et al*. 2002, Robert, Kleine-Vehn *et al*. 2010, Xu, Wen *et*

al. 2010, Chen, Naramoto *et al*. 2012, Chen, Grandont *et al*. 2014, Xu, Dai *et al*. 2014). However more recently, examination of null alleles generated through CRISPR/CAS9 approaches has shed doubt on the importance of ABP1 in auxin signalling and developmental processes (Enders, Oh *et al*. 2015, Gao, Zhang *et al*. 2015). Furthermore, genome sequencing of the *Arabidopsis abp1-5* mutant (generated through TILLING) shows that in addition to the TILLING-induced lesion, the *abp1-5* mutant contains another ~8000 single nucleotide polymorphisms, at least one of which could be responsible for a long hypocotyl phenotype previously attributed to ABP1 knockout (Enders, Oh *et al*. 2015). Thus, the role of ABP1, if any, in auxin perception and auxin-driven developmental processes remains uncertain.

Predictably, since the auxin signalling pathway is so crucial for lateral root formation, several mutants for the various modules of this pathway display altered root phenotypes. For example, *tir1 afb2 afb3* mutants have defects in pericycle cell development and abnormal primordia (Dubrovsky, Napsucialy-Mendivil *et al*. 2011) and studies have isolated various mutants for the AUX/IAA and ARF modules of the auxin response pathway - such as the solitary root (*slr*) mutant, which has a complete absence of lateral roots due to a gain-of-function mutation that increases the repressive activity of IAA14 (Fukaki, Tameda *et al*. 2002). In addition to *slr*, other gain-of-function IAA mutants also exhibit lateral root phenotypes: the *shy2/iaa3* and *iaa28 Arabidopsis* mutants both have reduced lateral root numbers (Rogg, Lasswell *et al*. 2001, Tian, Nagpal *et al*. 2003). Conversely, the *axr2-1/iaa7* mutant demonstrates an increased number of lateral roots, suggesting that different members of the AUX/IAA family may have varying responses to auxin (Nagpal, Walker *et al*. 2000).

Lateral root organogenesis is known to be under the control of at least two separate auxin signalling modules: firstly a SLR/IAA14-ARF7 and ARF19 module, which controls the initial activation of cell cycle progression and asymmetric pericycle cell division (Fukaki, Tameda *et al*. 2002, Vanneste, De Rybel *et al*. 2005, Fukaki and Tasaka 2009). The BODENLOS/IAA12-MONOPTEROS/ARF5 module subsequently acts downstream of cell cycle and division control to regulate the cellular organisation and patterning of the

new lateral root (De Smet, Lau *et al*. 2010). SOLITARY ROOT (SLR)/IAA14 paired with ARF7-ARF19 directs transcription of the early auxin response LATERAL ORGAN BOUNDARIES/ASYMMETRIC LEAVES LIKE (LBD/ASL) genes, which encode transcription factors for a set of genes controlling cell proliferation and patterning. In turn, these genes direct the initial asymmetric pericycle cell divisions during lateral root formation (Okushima, Fukaki *et al*. 2007). Correspondingly, loss-of-function *monopteros (mp)/arf5* and *bodenlos* (*bdl)/iaa12* mutants display irregularly positioned lateral roots (De Smet, Lau *et al*. 2010). The ARF protein MONOPTEROS was also demonstrated to control both its own expression and the expression of its repressor, the AUX/IAA protein BODENLOS, subject to a threshold level of auxin, suggesting that the ARF-AUX/IAA auxin response may be self-regulating (Lau, De Smet *et al*. 2011).

There are two main kinds of auxin reporters currently available. The earliest of these are the DIRECT REPEAT5 (DR5)-driven reporters. DR5 is a synthetic auxin-responsive promoter element created by site-directed mutagenesis of the native Auxin Response Element (AuxRE) found in the native soybean GH3 promoter, containing 11 bp tandem direct repeats featuring the auxin-responsive TGTCTC sequence (Ulmasov, Hagen *et al*. 1997) (Figure 5). This promoter has been used to drive expression of various reporters such as β-glucuronidase (GUS), luciferase, or fluorescent protein reporters such as green or yellow fluorescent protein (GFP/YFP) in order to visualise auxin responsiveness and distribution in various tissues.

DR5-driven reporters are relatively slow, since they rely on changes in gene expression to produce a 'readout' (~120 minutes post-auxin (1 μM NAA) application required for a change in DR5::VENUS fluorescence: (Brunoud, Wells *et al*. 2012). The main area of delay in producing a responsive readout is thought to occur during the post-transcriptional processing stage (Brunoud, Wells *et al*. 2012). Furthermore, the rate of degradation of the reporter should be considered: in particular many fluorescent proteins without degrons specifically attached to them have half-lives of 24 h, and thus apparent increases in fluorescence could in fact be merely representative of accumulation of the reporter (Li, Zhao *et al*. 1998). In addition, the AuxRE

sequence used in the DR5 promoter, TGTCTC, was recently found to not be a high-affinity binding site for ARFs and a different sequence, TGTCGG, was more optimal (Boer, Freire-Rios *et al*. 2014). Thus, a new reporter has been created, DR5v2, where the nine original tandem AuxRE repeats have been replaced with repeats featuring the new sequence. *DR5v2* has a broader expression range than the original DR5 and may be more sensitive to lower concentrations of auxin; however, neither DR5 and DR5v2 display linear responses to auxin and thus cannot be used to directly infer auxin levels (Liao, Smet *et al*. 2015).

More recently, a new independent auxin reporter, DII:VENUS, has also been developed (Figure 5). DII:VENUS is a nuclear-localised sensor measuring auxin response and distribution. It is based on the protein sequence of the AUX/IAA interaction domain (DII) of IAA28, fused to a fast-maturing YFP (VENUS), expressed under a constitutive promoter (35S). Since it is based on an AUX/IAA sequence, the sensor interacts with the auxin receptors TIR1, AFB1 and AFB5 and thus is degraded in response to auxin. For this reason, DII:VENUS can be considered a 'negative' reporter of auxin abundance, and shows an inverted auxin distribution map of the root as a whole. Furthermore, since the response of this reporter is based on protein degradation rather than gene expression, DII:VENUS is quick to respond to changes in auxin status, with exogenous treatment with auxin found to elicit a change in fluorescence within minutes of application; and also responding to native auxin distribution changes during the process of gravitropism (Brunoud, Wells *et al*. 2012).

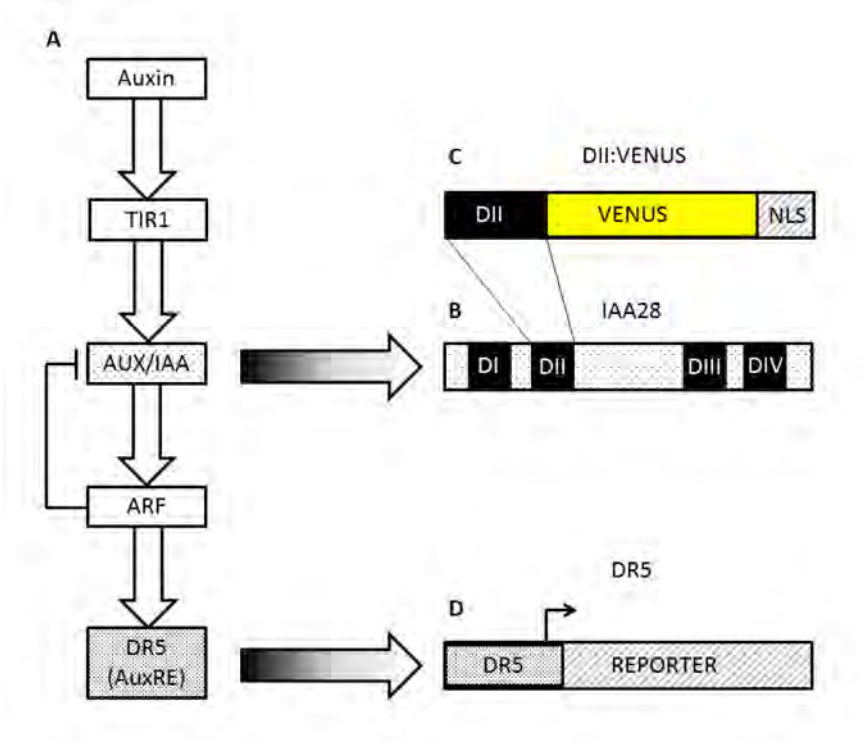

Figure 5. Comparison of DII and DR5 based reporters. (A) Simplified schematic of components involved in auxin perception. **(B)** The AUX/IAA protein IAA28 has four domains (DI-DIV). DII is recognised as a degron by the E3 ubiquitin ligase complex of TIR1 and promotes the destruction of the AUX/IAA protein by the 26S proteasome. The DII sequence from IAA28 forms the basis of the DII:VENUS reporter. **(C)** DII:VENUS is comprised of IAA28 DII fused to a fast-folding yellow fluorescent protein (VENUS) with a nuclear localisation sequence (NLS). When the DII sequence interacts with the $TIR1^{SCF}$ complex, the entire protein is targeted for destruction; thus DII:VENUS is a 'negative' spatiotemporal reporter for the presence of auxin. **(D)** DR5-driven reporters are promoter fusions of the synthetic promoter DR5, which contains auxin responsive elements (AuxREs) representing the binding site for ARFS. DR5-driven expression is higher in the presence of auxin, thus unlike DII:VENUS, DR5-reporters are 'positive' reporters for the presence of auxin. DR5 promoters have been used to drive a variety of reporters including GUS, luciferase and fluorescent proteins such as VENUS YFP.

Since degradation of the DII:VENUS sensor is necessary to produce a 'readout', the distribution and expression of auxin receptors, and/or the degradation of the AUX/IAA-based fusion protein, can affect the accuracy of auxin status detection by this tool. The use of a 35S promoter can also lead to issues interpreting the data in certain tissue types (higher 35S promoter activity in the epidermis, cortex, elongation zone and in external root cap cells leads to an underestimation of auxin abundance in these tissues (Brunoud, Wells *et al*. 2012); and conversely, 35S-driven *DII:VENUS* does not work well in some tissues, such as embryonic tissues (Volker, Stierhof *et al*. 2001).

In order to overcome the limitations of 35S promoters in embryonic tissue and the challenges verification/normalisation of signal whilst using

fluorescent reporters such as DII:VENUS, a double reporter R2D2 was also created. R2D2 combines an auxin-responsive yellow fluorescent reporter (DII:VENUS) with a second, non-auxin responsive nuclear-localised fluorescent red reporter (mDII:ntdTomato). Both reporters are driven by the RPS5A promoter which is active in dividing cells including embryo and meristematic tissues (Weijers, Franke-van Dijk *et al*. 2001, Liao, Smet *et al*. 2015). Crucially, because both reporters are combined within the same line, comparison of fluorescence ratios and verification of a genuine auxin-driven response is more reliable than comparison of separate reporter lines.

1.4 Cytokinin signalling

In addition to auxin, various other hormones are known to influence root development. Cytokinin interacts both antagonistically and synergistically to auxin in many growth and developmental processes, including root patterning and apical meristem regulation. Cytokinin is inhibitory to root growth: plants where endogenous cytokinin is lowered display elongated primary roots. Cytokinin also regulates lateral root organogenesis in an inhibitory manner, with perturbations in cytokinin levels and/or metabolism shown to interfere with LR density and development (Sponsel, Schmidt *et al*. 1997, Werner, Motyka *et al*. 2003, Bao, Shen *et al*. 2004, De Smet, Zhang *et al*. 2006, Li, Mo *et al*. 2006, Mouchel, Osmont *et al*. 2006, Laplaze, Benkova *et al*. 2007).

The cytokinin signalling pathway has similarities to the prokaryotic two-component signal (TCS) transduction pathways, and is comprised of sensory 'hybrid' kinases, histidine phosphotransfer proteins and downstream response regulators (Figure 6). Cytokinin is perceived by the receptor histidine-kinases ARABIDOPSIS HISTIDINE KINASE2 and 3 (AHK2 and AHK3) and CYTOKININ RESPONSE 1/WOODENLEG/AHK4 (CRE1/WOL/AHK4). These kinases are comprised of an extracellular cytokinin-binding domain (the cyclases/histidine kinases associated sensory

extracellular or CHASE domain), a histidine kinase domain, and a receiver domain.

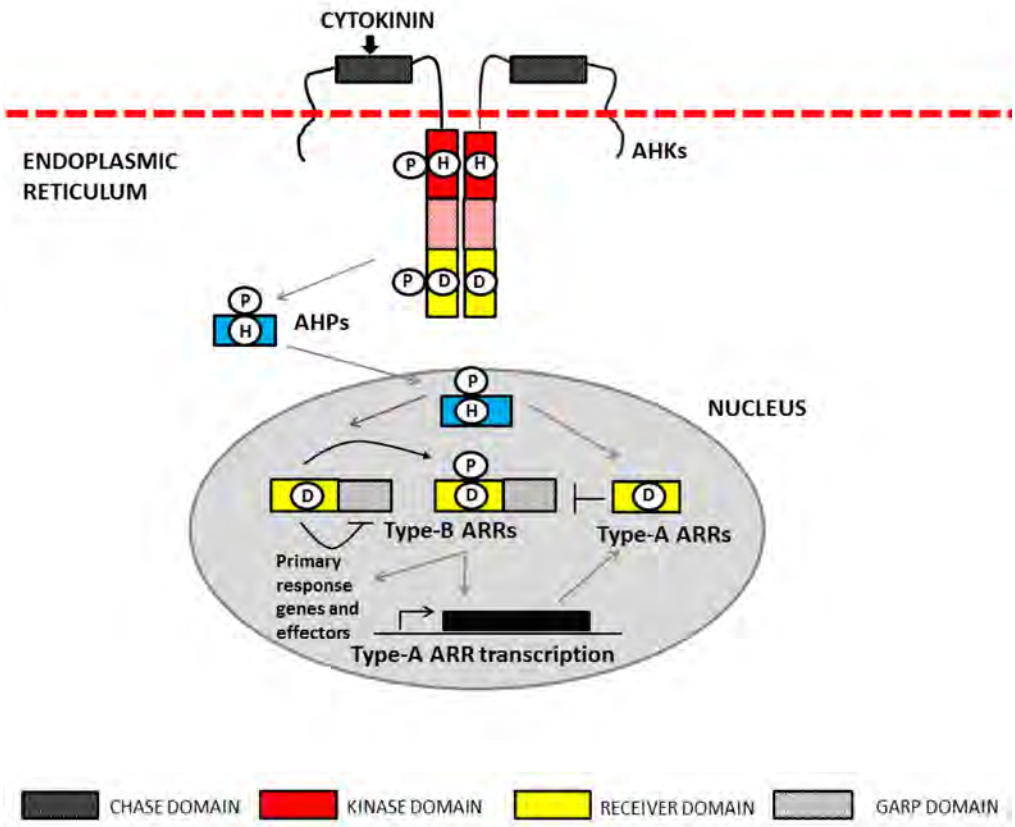

Figure 6. Simplified schematic of the two-component phosphorelay system in cytokinin signalling. Cytokinin is perceived by the CHASE domain of the AHK cytokinin receptor kinases. The kinase auto-phosphorylates a conserved His (H) residue within the kinase domain; the phosphoryl group from this is subsequently transferred to a conserved Asp (D) within the receiver domain. The phosphoryl group is then relayed to a His residue within an AHP protein; this AHP protein translocates into the nucleus before transferring the phosphoryl group to an Asp residue within an ARR.

Type-B ARRs contain a DNA-binding GARP domain which is negatively regulated by the ARR's receiver domain. Phosphorylation of the receiver domain releases the GARP domain from repression, allowing the type-B ARR to induce the expression of type-A ARRs and other cytokinin primary response genes and effectors. Type A-ARRs negatively regulate both cytokinin responses and their own expression, forming a feedback loop.

Following perception of cytokinin, phosphate is transferred in a relay system through the signalling components in the sequence of His to Asp to His to Asp (Inoue, Higuchi *et al*. 2001, Higuchi, Pischke *et al*. 2004, Nishimura, Ohashi *et al*. 2004, Riefler, Novak *et al*. 2006). Cytokinin receptors are primarily localised to the ER with the CHASE binding domain situated within

the lumen of the ER, suggesting this is the principal site of cytokinin binding (Caesar, Thamm *et al*. 2011, Lomin, Yonekura-Sakakibara *et al*. 2011, Wulfetange, Lomin *et al*. 2011).

Phosphotransfer from the receptor kinases to the response regulators is performed by the ARABIDOPSIS HISTIDINE-PHOSPHOTRANSFER PROTEINS (AHPs). There are five functional members of the AHP protein family in *Arabidopsis*, with a sixth member, AHP6, missing the His target of phosphorylation (Suzuki, Imamura *et al*. 1998, Tanaka, Suzuki *et al*. 2004, Hutchison, Li *et al*. 2006, Hutchison and Kieber 2007). AHP1–5 are partially redundant positive modules of cytokinin signalling, whilst AHP6 is a negative cytokinin response regulator which inhibits the phosphotransfer relay (Hutchison, Li *et al*. 2006, Mahonen, Bishopp *et al*. 2006, Hutchison and Kieber 2007). Independently from phosphorylation status, AHPs are dynamically transported in and out of the nucleus, enabling them to transfer phosphates between the AHK receptors and the response regulators (Punwani, Hutchison *et al*. 2010, Punwani and Kieber 2010).

There are 23 members of the ARABIDOPSIS RESPONSE REGULATOR (ARR) family in *Arabidopsis*. These can be divided into either type-A or type-B ARRs based on their sequences, structures and transcriptional responses to cytokinin (D'Agostino, Deruere *et al*. 2000, Mason, Li *et al*. 2004). Both type A and Type B ARRs are comprised of an N-terminal receiver domain containing an Asp residue which is the target for phosphotransfer, but type-B ARRs also have a DNA-binding Myb (GARP) domain. Type A-ARRs are rapidly induced by cytokinin and act as negative regulators of cytokinin signalling, whilst type-B ARRs act as positive regulators in cytokinin signalling (Brandstatter and Kieber 1998, D'Agostino, Deruere *et al*. 2000, Jain, Tyagi *et al*. 2006, Argyros, Mathews *et al*. 2008, Tsai, Weir *et al*. 2012). A synthetic cytokinin reporter, TCS::GFP, is available. It was created by the fusion of a typical type-B ARR binding site with a GFP reporter (Muller and Sheen 2008); this line was later refined to give the more recent TCSnew reporter, which is based on a DNA sequence more optimised for ARR binding (Zurcher, Tavor-Deslex *et al*. 2013).

Crosstalk between auxin and cytokinin is important in regulating many processes in plants, with auxin and cytokinin having both antagonistic and synergistic roles in different contexts. For example, in the root apical meristem, cytokinin acts through the AHK3 receptor and downstream transcription factor ARR1 to promote expression of the auxin-signalling repressor *SHY2/IAA14*, which in turn downregulates expression of the auxin transporters *PIN1*, *PIN3*, and *PIN7* modulating auxin flow to the meristem; thus auxin and cytokinin have antagonistic roles in the control of whether meristematic cells divide or differentiate (Dello Ioio, Nakamura *et al*. 2008). Correspondingly, exogenous application of auxin or cytokinin respectively increases or decreases root apical meristem size (Beemster and Baskin 2000, Blilou, Xu *et al*. 2005, Dello Ioio, Linhares *et al*. 2007). Similarly, in the shoot apical meristem, ARF5/MONOPTEROS transcriptionally regulates the cytokinin signalling repressors ARR7 and ARR15 (Zhao, Andersen *et al*. 2010). There are many other examples of the complementary nature of auxin and cytokinin signalling in various developmental processes contained within the comprehensive recent review Schaller, Bishopp *et al.* 2015.

1.5 Gibberellic Acid (GA) signalling

Gibberellic acid (GA) is a phytohormone belonging to a large family of structurally related compounds, with GA_1 and GA_4 being the primary active forms in plants. GA is known to regulate a variety of processes including seed germination, stem elongation and flower growth. Although GA can be translocated over long distances throughout the plant, the sites of GA synthesis and perception generally overlap (Xu, Liu *et al*. 2014).

Sharing conceptual similarities with the auxin signalling pathway, in the absence of the hormone the GA signalling pathway is under repression by nuclear-located DELLA proteins (Gao, Xiao *et al*. 2011). Upon perception of GA by the soluble receptor GA-INSENSITIVE DWARF1 (GID1), a conformational change is elicited that allows the GA-GID1 complex to interact

with the N-terminus of the DELLA protein. This complex subsequently interacts with SLY1, a $SCF^{SLY1/GID2}$ E3 ubiquitin ligase F–box protein which targets the repressive DELLA for destruction via the 26S pathway (McGinnis, Thomas *et al*. 2003, Sasaki, Itoh *et al*. 2003, Murase, Hirano *et al*. 2008, Shimada, Ueguchi-Tanaka *et al*. 2008).

Gibberellic acid promotes primary root growth in *Arabidopsis*, with GA accumulating in the endodermis of the root within the elongation zone, which is also the most responsive tissue in the root (Ubeda-Tomas, Swarup *et al*. 2008, Ubeda-Tomas, Federici *et al*. 2009, Shani, Weinstain *et al*. 2013). During the auxin-cytokinin (SHY2/ARR1) antagonistic determination of root apical meristem size, GA acts antagonistically to cytokinin, repressing cytokinin's inhibitory effect on root growth (Moubayidin, Perilli *et al*. 2010).

One DELLA protein, REPRESSOR OF ga1-3 (RGA) (Silverstone, Ciampaglio *et al*. 1998) forms the basis of a reporter, RGA:GFP, that is degraded in the presence of GA, in a manner similar to that of DII:VENUS under auxin treatment (Silverstone, Jung *et al*. 2001).

1.6 Small signalling peptides and receptor kinases in plant development

Although much emphasis has traditionally been placed on the roles of lipophilic hormone pathways in plant development, it is becoming increasingly clear that alternative signalling pathways, such as peptide signalling, are also involved (Lindsey, Casson *et al*. 2002, De Smet, Vassileva *et al*. 2008, Katsir, Davies *et al*. 2011, Murphy, Smith *et al*. 2012). Many of these signalling peptides are known to be perceived through receptor-like kinases (RLKs). From the first stages of plant life, cell–cell communication mediated by small signalling peptides and receptor kinases plays a key role in development (Escobar-Restrepo, Huck *et al*. 2007, Okuda, Tsutsui *et al*. 2009, Kessler, Shimosato-Asano *et al*. 2010).

Signalling peptides are small (usually less than 20 amino acids in the mature form, and rarely more than ~120 amino acids even as a full length precursor) and often present in very low (nanomolar range) physiological concentrations: often expression is low and restricted to specific cells or developmental stages. Therefore finding them presents a challenge. Furthermore, microarrays and other tools designed for identification of associated genes have not traditionally been a particularly useful tool in peptide hormone discovery: small genes are often overlooked or not adequately represented on arrays, and are poorly predicted using gene prediction algorithms as they are difficult to distinguish from short, random ORFs (Olsen, Mundy *et al*. 2002, Lease and Walker 2006). In addition, microarrays are also blind to how post-translational modifications affect protein activity, a drawback which is particularly pertinent due to the crucial role of post-translational modification processes in the activation of many signalling peptides (Kodadek 2001). Due to the small size of peptide-encoding genes, T-DNA insertion mutants are often unavailable as tools for researching small peptides, and in any case, the high degree of redundancy in these gene families could confound any findings obtained from any loss-of-function mutants identified.

Song *et al.* (2014) suggested one way to circumvent the technical difficulties in generating informative loss-of-function phenotypes in small peptides is an antagonistic peptide approach: using CLV3 as a template, the authors suggested mutation of the sixth glycine residue in CLE peptides to a threonine residue [G6T] could induce a dominant-negative phenotype by competing with active, native peptide for receptor binding. This approach has also been used to study CLE19 (Xu, Ren *et al*. 2015)(See section 1.6.5 'Small signalling peptides in embryogenesis). However, more recently doubt has been cast on the extent to which the CLE antagonistic peptide technology can induce a dominant negative phenotype in all cases (for example: $CLE45^{G6T}$), and thus findings generated by this technology should be interpreted with caution and not universally accepted as equivalent to gene knockout - especially without knowledge of precise ligand-receptor interactions and

comparisons with confirmed gene knockout lines (Czyzewicz, Wildhagen *et al*. 2015).

While plant genomes encode >600 putative RLKs and >1000 potential secreted peptides, so far very few have been shown to form pairs (Shiu and Bleecker 2001, Torii 2004, Butenko, Vie *et al*. 2009, Butenko, Wildhagen *et al*. 2014). Fyears (Matsubayashi, Ogawa *et al*. 2002, Hirakawa, Shinohara *et al*. 2008, Ogawa, Shinohara *et al*. 2008, Stenvik, Butenko *et al*. 2008, Kinoshita, Betsuyaku *et al*. 2010, Haruta, Sabat *et al*. 2014, Tabata, Sumida *et al*. 2014). Many of these ligand-receptor pairs have been identified on the basis of corresponding expression patterns and genetic evidence from corresponding changes of phenotypes in respective ligand and/or receptor mutations. However, more recently novel assays for matching ligands and receptors - such as ectopic expression of receptor genes in individual cells for the purpose of screening potential ligands - have become increasingly popular (Butenko, Wildhagen *et al*. 2014, Tabata, Sumida *et al*. 2014). In addition, a novel comparative phosphoproteomics approach has also recently been used to identify a ligand-receptor pair for RALF and FERONIA, since the phosphorylation patterns of membrane receptor are altered upon ligand binding (Haruta, Sabat *et al*. 2014, Stes, Gevaert *et al*. 2015).

Some of these peptides, RLKs, the discovery and processing of small signalling peptides, and their roles in various aspects of plant development are introduced below.

1.6.1 The use of bioinformatics in small signalling peptide discovery

The identification of several peptide signal-encoding genes shows that *in silico* methods using bioinformatics tools can be useful for initial identification of small signalling peptides. For example, SignalP is an algorithm which can predict the presence and location of signal peptides and cleavage sites in an amino acid sequence. It has been used as a tool during the discovery and characterisation of several secreted peptides, including IDA and CEP1

(Butenko, Patterson *et al*. 2003, Ohyama, Shinohara *et al*. 2009), and was the starting point for the discovery of the RALFL family (Olsen, Mundy *et al*. 2002). Some of the results generated from an initial SignalP search of an *Arabidopsis* genome database were compared for homology to known peptides - using various types of BLAST search and CLUSTALW alignments - leading to discovery of a potential uncharacterised gene family in *Arabidopsis* (Olsen, Mundy *et al*. 2002). Similarly, computer programs (PHDsec and SOPMA) were used to compare the secondary structure of the identified peptides. Some members of this family had high sequence similarity to RALF peptides, leading to them being designated the RALF-LIKE (RALFL) family (Olsen, Mundy *et al*. 2002, Haruta and Constabel 2003). *Arabidopsis* has been shown to have around 40 RALFL genes, which are expressed throughout the plant, and which impact on various developmental processes, including seedling development, stem development, and root development (Srivastava, Liu *et al*. 2009, Bedinger, Pearce *et al*. 2010).

CEP1 was identified through an *in silico* approach by analysis of an *Arabidopsis* genome database, by searching for paralogues to the peptide CLE44 (Ohyama, Shinohara *et al*. 2009). The reasoning behind this approach was that signalling peptides, such as PSK, PSY1 and CLE, are encoded by several paralogous genes, and have many specific features which can be programmed in to computational searches to maximise the quality of the final candidate pool, such as size limitations, presence of an N-terminal signal sequence, and cysteine paucity. As proof of concept, the candidate pool identified by this search consisted of approximately 40% of peptides with known or predicted function, such as PSK and PSY precursors, IDA/IDA-LIKE (IDL), and several CLE family peptides. Identification of one uncharacterized gene family led to identification of a 15 amino acid hydroxyprolinated peptide, namely CEP1 (Ohyama, Shinohara *et al*. 2009).

Another *in silico* approach was then adopted using similar search parameters as those used earlier to identify CEP1, but including additional search criteria to identify peptides with an Asp-Tyr tyrosine sulfation motif. From this search, a candidate polypeptide family, RGF, was found with features matching all the search criteria. Mass spectrometry of peptides derived

from plants overexpressing one member identified a 13 amino acid tyrosine-sulphated peptide, derived from the C-terminal domain of the full length cDNA-encoded protein. In parallel, homology searches using the C-terminal sequence of CLE18 also revealed 10 members of the same RGF/GLV/CLEL family (Meng, Buchanan *et al*. 2012). It has been suggested that the active peptide sequence of RGF1 is derived from the CLE motif of its precursor, but the RGF1/CLE18 precursor in fact contains not one, but two functional motifs within its sequence: the RGF1/CLE18 13-amino acid CLE motif, spanning residues 35–48 (not the extreme C-terminus, as would be expected for a CLE motif), and also a second, previously undiscovered motif spanning residues 75–87 designated the 'CLEL' motif because of the similarity to the classical CLE motif (Matsuzaki, Ogawa-Ohnishi *et al*. 2010, Meng, Buchanan *et al*. 2012). Homology searches revealed that similar motifs were found in nine other proteins besides the RGF1/CLE18 precursor.

1.6.2 Peptide processing and maturation

The secreted signalling peptides discovered in plants thus far can be considered to fall into two broad groups: small post-translationally modified peptides and cysteine rich peptides. The small post-translationally modified peptides are a group of secreted signalling peptides characterised by small (<20 amino acid) mature peptide size, which are cleaved from a longer translated precursor peptide; a general paucity of cysteine residues, and are subject to one or more post-translational modification. Several small post-translationally modified peptides have been identified, including CLV3/CLE/TDIF, PSK, PSY1, CEP1, IDA and RGF/CLEL/GLV. The cysteine-rich peptides have several uniting features despite a generally high amino acid sequence divergence between individual peptides: though often larger than small post-translationally modified peptides, they are still small (<160 amino acids), they are cationic, and they have a conserved N-terminal signal peptide. In contrast to small post-translationally modified peptides, their C-terminal domain is cysteine-rich, typically containing 4–16 cysteine residues. The cysteine-rich peptide family

of peptide hormones includes the RALF/RALFL, EPF1, EPF2, TPD1, STOMAGEN/EPFL9 and ENOD peptides.

Many small signalling peptide genes encode a protein that is longer than the mature, active form of the peptide. Several of them are expressed as ~100 amino acid prepropeptides, which require proteolytic processing by endopeptidases and cleavage of a signal peptide by signal peptidase, to produce a functional peptide. The small post-translationally modified peptide and the cysteine-rich peptide groups require different types of processing: both usually undergo removal of signal sequences (by signal peptidase) and removal of extra mRNA-encoded precursor sequence (some cysteine rich peptides do not require this step); before processing, the small post-translationally modified peptides undergo enzyme-catalysed modifications to selected residues of the mature-length peptide.

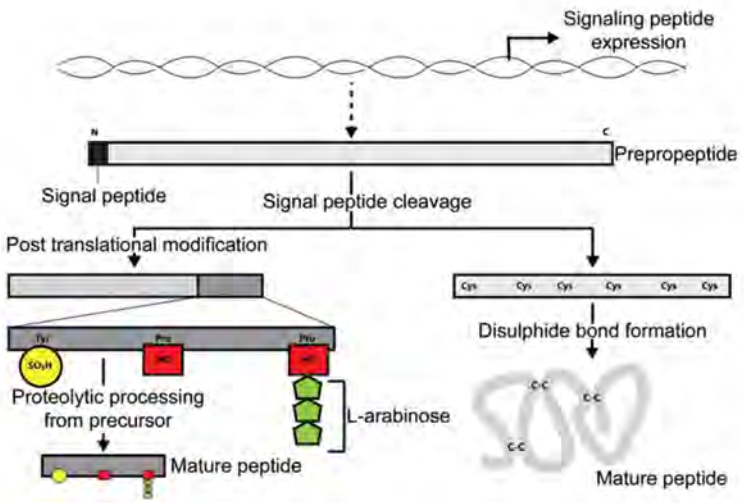

Figure 7. Posttranslational modifications and processing of small signalling peptides. Following entry of the full-length mRNA-encoded prepropeptide to the secretory pathway, the N-terminal hydrophobic signal peptide is cleaved by a signal peptidase. Small posttranslationally modified peptides and Cys-rich peptides then follow different pathways to produce an active peptide. All small posttranslationally modified peptides discovered in plants thus far undergo one or more of three types of post translational modification: Tyr sulfation (yellow), Pro hydroxylation (red), and/or Hyp arabinosylation (green). Proteolytic cleavage of the modified peptide from the precursor sequence completes activation. Cys-rich peptides often do not require proteolytic processing from a precursor, though there are exceptions. Activation of Cys-rich peptides is completed upon formation of disulphide bonds between the conserved Cys residues of the peptide, thus bringing the peptide to an active conformation.

Schematic is based on Matsubayashi *et al*. (2012) with permission.

Following processing, cysteine rich peptides are predicted to form disulphide bonds between the conserved cysteine residues in their sequence to give the peptides their final active conformation – disulphide bonds have been shown to be absolutely required for function in the RALF peptides (Pearce, Moura *et al*. 2001).

1.6.3 Small signalling peptide post-translational modifications

So far, three types of post-translational modification have been observed to occur to signalling peptides in plants: tyrosine sulfation, proline hydroxylation and hydroxyproline arabinosylation. Peptide modification is known to be important for optimal bioactivity of peptides (hydroxyprolination of proline 7 in the PIP motif of IDA peptides increases activity 30-fold compared to the unmodified sequence: (Shinohara and Matsubayashi 2013, Butenko, Wildhagen *et al*. 2014). Potential explanations for the observed increase in bioactivity of posttranslationally modified signalling peptides are i) a role in conformational stability of the peptide; ii) increasing receptor-ligand interaction, other protein-protein interaction, and/or general reactivity of the peptide, for example by providing projecting chemical group side-chains (Ohyama, Shinohara *et al*. 2009, Shinohara and Matsubayashi 2013); iii) a means of generating diversity in peptides with limited residue numbers, without greatly adding to molecular weight (which is a particularly important consideration for peptides undergoing secretion and/or those potentially required to travel symplastically) (Matsubayashi 2014).

Tyrosine sulfation is thought to be catalysed by the plant-specific TPST, a 62 kDa type I transmembrane protein localised in the Golgi (Komori, Amano *et al*. 2009). The peptide substrate is known to require, as a minimum, an aspartic acid residue N-terminally adjacent to a tyrosine residue (an Asp-Tyr motif). Proline hydroxylation is catalysed by PROLYL-4-HYDROXYLASE (P4H) a type II Golgi and ER-membrane protein of the 2-OXOGLUTARATE-DEPENDANT DIOXYGENASE family (Myllyharju

2003). Thirteen genes for P4H have been identified in *Arabidopsis*, but no substrate consensus sequence for proline hydroxylation has yet been established (Matsubayashi 2012). Following the conversion of proline to hydroxyproline, a subset of peptides undergoes a further post-translational modification, hydroxyproline arabinosylation. In a post-translational modification unique to plants, an O-linked L-arabinose chain (triarabinoside) is added to certain hydroxyproline residues within the peptide. The linkage of the triarabinoside chain to the hydroxyproline residue and the linkage of the sugar moieties within the chain to each other are separate reactions and hypothesised to occur in two steps mediated by different enzymes. A hydroxyproline arabinosyltransferase catalyses a β-linkage between the hydroxyl group of Hyp to an arabinose, and an arabinosyltransferase mediates the β1,2-linkage between the arabinose residues themselves (Matsubayashi 2012). Hydroxyproline arabinosylation has been directly shown through NMR to increase conformational stability in CLV3, and increase interaction with the CLV1 receptor (Ogawa, Shinohara *et al*. 2008, Ohyama, Ogawa *et al*. 2008, Shinohara and Matsubayashi 2013, Ogawa-Ohnishi and Matsubayashi 2015).

An *Arabidopsis* TPST mutant, *tpst-2*, was recently found to be hypersensitive to copper during root elongation and showed increased ethylene production (Wu, Kamiya *et al*. 2015). However, exogenous application of PSK inhibited the excess ethylene production in the *tpst-2* mutant, suggesting that PSK may play a role in ethylene regulation, and that correct sulphation of PSK is essential to this role. Correspondingly, another knockout mutant for TPST, *tpst-1*, displayed a reduced seed production and slowed pollen tube growth phenotype which could also be rescued via exogenously applied PSK (Stuhrwohldt, Dahlke *et al*. 2015).

Three hydroxyproline arabinosyltransferase (HPAT) enzymes involved in the posttranslational modification of small peptides including PSY1 and CLE have recently been identified in *Arabidopsis* through affinity purification using PSY1 as an affinity probe (Ogawa-Ohnishi and Matsubayashi 2015). As Golgi-localised transmembrane proteins with similarities to glycosyltransferase GT8 family proteins, HPATs are the initial enzyme in the Hyp-O-oligoarabinoside biosynthetic pathway and as such are required for elongation

of hydroxyproline-linked arabinose chains. Since HPATs are also involved in modifying a range of proteins including extensions, loss-of-function HPAT mutants exhibit a variety of pleiotropic phenotypes including defects in cell wall thickening and pollen tube growth. Thus, a mass spectrometry approach was used to examine CLE2 peptide arabinosylation in HPAT mutant backgrounds. Loss-of-function mutants for HPAT3 were impaired in CLE2 hydroxyproline arabinosylation, whilst levels were normal in *hpat1/hpat2* double mutants. This suggests HPATs are substrate-specific, and using a similar MS-based approach in HPAT mutants could help elucidate other peptide targets of specific HPATs.

In addition to the three HPATs discovered in *Arabidopsis,* several arabinosyltransferases have been recently found in tomato (*Solanum lycopersicum*): four HPAT homologs (including a hydroxyproline-O-transferase similar to HPAT3, FASCIATED INFLORESCENCE [FIN]) and two further arabinosyltransferases homologous to the *Arabidopsis* GT77-family arabinosyltransferases: XYLOGLUCANASE 113 (XEG113) (also known in tomato FASCIATED AND BRANCHED 2 [FAB2]); and REDUCED RESIDUAL ARABINOSE 3 (RRA3), which act downstream of the HPAT FIN to continue extension of arabinoside chains to the full three residues (Xu, Liberatore *et al*. 2015). FIN, XEG113/FAB2 and RRA3 are required for full hydroxyproline arabinosylation of the tomato homolog of CLV3 (SlCLV3); mutants defective in the aforementioned arabinosyltransferases displayed enlarged shoot meristems and fasciated flowers, consistent with the role of active (modified) CLV3 in restricting meristem size through its cognate receptor CLV1 (FAB in tomato). The mutant phenotype could be rescued by exogenous application of triarabinosylated SlCLV3, further demonstrating the importance of posttranslational modifications in the native function and bioactivity of this family of peptides. As with HPATs in *Arabidopsis*, the other arabinosyltransferases demonstrate substrate specificity with different families of arabinosyltransferases targeting different proteins and peptides depending on species and developmental context; this includes pollen tube growth (Ogawa-Ohnishi and Matsubayashi

2015), root hair elongation (Velasquez, Ricardi *et al*. 2011) and rhizobia-induced root nodulation (Schnabel, Kassaw *et al*. 2011).

CLE family proteins are united by their possession of a conserved 12–14 amino acid CLE motif, usually positioned at the extreme C-terminus of the full length translated precursor protein, and a hydrophobic N-terminal signal sequence (Cock and McCormick 2001, DeYoung and Clark 2001). Cleavage of the CLE motif from the precursor is necessary for production of the mature 12–13 amino acid peptide, which may then undergo further post translational modification (Fiers, Golemiec *et al*. 2005, Fiers, Golemiec *et al*. 2006, Ito, Nakanomyo *et al*. 2006, Ni and Clark 2006, Ohyama, Shinohara *et al*. 2009, Katsir, Davies *et al*. 2011). The CLE motif contains four conserved prolines at positions 4, 7 and 9: Pro4 and Pro7 appear to have particular significance as the targets for posttranslational modification (hydroxylation: Pro4/Pro7; arabinosylation: Pro7). The *CLV3* gene is expressed as a protein of 96 amino acids, including an 18 hydrophobic N-terminal amino acid signal peptide to direct the peptide for secretion, a typical feature of CLE peptides. Several CLE domain proteins have been shown to fully or partially complement *clv3* mutants, demonstrating a degree of functional homology within the CLE family (Ni and Clark 2006, Ohyama, Ogawa *et al*. 2008, Ohyama, Shinohara *et al*. 2009). Cleavage rom the precursor occurs at the arginine at the beginning of the CLE motif (Arginine 70); this residue is conserved absolutely through all CLE proteins including CLV3 (Oelkers, Goffard *et al*. 2008). Following processing, CLV3 undergoes further post-translational modification. The amino acid sequence of CLV3 contains three prolines; the first two N-terminal prolines are hydroxylated; one hydroxyproline (Hyp7) then undergoes hydroxyproline arabinosylation. The arabinosylation of Hyp7 has been shown to enhance binding of CLV3 to the receptor CLV1 (Kondo, Sawa *et al*. 2006). TDIF (CLE41/CLE42) is cleaved from a 99–112 amino acid protein precursor before proteolytic processing to separate the 12 amino acid CLE domain from the precursor (Ito, Nakanomyo *et al*. 2006).

Similar mechanisms may also apply to the RGF/CLEL/GLV family peptides with regards to the processing of the CLE motif from the precursor, which then requires tyrosine sulfation for optimum bioactivity, possibly by

enhancing receptor binding (Matsubayashi 2011). In agreement with the findings for the RGF1 peptide, tyrosine sulfation of synthetic peptides representing the proposed active form of GLV1/RGF6 and GLV3/RGF4, increased their bioactivity by 2–3 orders of magnitude. Acidic residues surrounding the tyrosine residues appear essential for optimal sulfation activity, probably by a new class of tyrosylprotein sulfotransferases unique to plants (Hanai, Nakayama *et al*. 2000). However, though it seems likely, it is unknown if CLEL motifs are extracted from the full length precursor in the same way as the CLE motifs; and in contrast to the CLE motif peptides derived from RGF/CLEL/CLV precursor sequences, CLEL-motif peptides do not appear to require any post translational modification, such as tyrosine sulfation, for optimal activity (Meng, Buchanan *et al*. 2012). This opens the possibility that other precursors might also give rise to multiple functional peptides which are subject to distinct proteolytic processing and post-translational modifications.

1.6.4 Small signalling peptides in anther and pollen development

The tapetal layer of the anther is important to pollen development as it provides essential lipids and small molecules that aid in pollen formation and pollen wall generation (Goldberg, Beals *et al*. 1993, Ge, Chang *et al*. 2010, Zhou, Wei *et al*. 2010). A small signal peptide, TAPETAL POLLEN DEVELOPMENT (TPD) is thought to act between the microsporocyte and the tapetal development stage in pollen to initiate tapetal cell differentiation (Ge, Chang *et al*. 2010). Recently, a number of CLE peptides have also been shown to be expressed during anther development. *CLE1*, *CLE11*, *CLE12*, and *CLE13* are all found within the pollen granules during various stages of pollen development, while *CLE25* is expressed only during early anther development (Jun, Fiume *et al*. 2010). However, their role in anther and pollen development remains to be demonstrated.

EXCESS MICROSPOROCYTES1 (EMS1)/EXTRA SPOROGENOUS CELLS encodes an LRR-RLK that is localized to the cell surface and is needed for tapetal cell development (Ge, Chang *et al*. 2010). The *ems1* mutant displays a lack of tapetal cell development and an excess production of microsporocytes (Albrecht, Russinova *et al*. 2005). The nearly identical phenotypic similarities between *tpd1*, *ems1*, and double *tpd1 ems1* mutants suggest a strong link between receptor and putative ligand during tapetal cell and pollen development (Yang, Xiea *et al*. 2003, Albrecht, Russinova *et al*. 2005).

SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE1 (SERK1) and SERK2 are also proposed receptors for the TPD1 ligand, as *serk1 serk2* also results in a lack of tapetal layer, with extra meiotic cells, but biochemical evidence is lacking at present (Albrecht, Russinova *et al*. 2005, Ge, Chang *et al*. 2010).

The BARELY ANY MERISTEM1 (BAM1) and BAM2 receptors have also been shown to play a role in anther and pollen development (Hord, Chen *et al*. 2006, Deyoung and Clark 2008). Since the BAM receptors have been shown to directly bind with CLE family peptides, it is possible that the BAM1 and BAM2 perceive CLE1, CLE11, CLE12, CLE13, and/or CLE25 anther and pollen expressed peptides (Hord, Chen *et al*. 2006, Jun, Fiume *et al*. 2010, Shinohara, Moriyama *et al*. 2012).

Recently, signalling through PSK RECEPTOR 1 and 2 (PSKR1/2) and PSK2 was shown to regulate pollen tube growth and guidance in *Arabidopsis.* The double knockout mutant *pskr1-3 pskr2-1* exhibited reduced seed production and slowed pollen tube growth which could be rescued via exogenous application of PSK (Stuhrwohldt, Dahlke *et al*. 2015). It has been proposed that interactions between *PSK* expressed in the female sporophytic tissue and/or female gametophyte and *PSKR1* and *2* expressed in the pollen tube are required for correct guidance of the pollen tube from the transmitting tract to the embryo sac.

1.6.5 Small signalling peptides in embryogenesis

A defining feature of higher plants is double fertilization: the egg is fertilized by two sperm cells which are transported to the ovary and released by the pollen tube: One sperm cell fertilizes the egg, which will develop into the embryo, and the other leads to endosperm formation, which are cells that regulate and nourish the embryo during development. (De Smet, Lau *et al*. 2010, Lau, Slane *et al*. 2012). Several *CLE* peptides recently have been shown to be expressed during embryogenesis; specifically, CLE8 has been directly linked to embryo and endosperm proliferation and differentiation (Jun, Fiume *et al*. 2010, Fiume, Monfared *et al*. 2011, Wang, Zhang *et al*. 2011, Fiume and Fletcher 2012). CLE8 has been shown to act upstream of WUSCHEL-LIKE HOMEOBOX8 (WOX8), evidenced by severely reduced *WOX8* expression in *cle8* mutants. Conversely, overexpression of *CLE8* shows a marked increase in *WOX8* expression, suggesting that these two proteins act synergistically in suspensor and endosperm development (Fiume and Fletcher 2012).

Little is known about the targets of the CLE8 signalling peptide involved in embryonic development. Several RLKs have already been identified as integral to normal embryo and seed development (Luo, Dennis *et al*. 2005, Nodine, Bryan *et al*. 2011, Fiume and Fletcher 2012, Shinohara, Moriyama *et al*. 2012). As there is often a strong similarity between binding affinities within CLE family peptides, it is likely that the target for CLE8 signalling is an LRR-RLK.

In addition to *CLE8*, *CLE19* is also expressed in the embryo and evidence suggests it may regulate cotyledon establishment and endosperm development in both *Arabidopsis* and *Brassica napus* (Fiers, Hause *et al*. 2004). Plants expressing a *CLE19 G6T* construct under the native CLE19 regulatory element led to seed abortion and defective cotyledon establishment and endosperm development (Xu, Ren *et al*. 2015), though due to the concerns over antagonistic peptide approaches in CLE peptide characterisation, further characterisation of the role of CLE19 may be required (see section 1.6 ‘Small signalling peptides and receptor kinases in plant development).

1.6.6 Small signalling peptides in the Shoot Apical Meristem (SAM)

The leaves, stems, and floral parts of plants develop from the SAM, a conserved stem cell niche that resides at the top of the apical-basal axis of the plant. It is a tightly organized and regulated structure separated in three zones: the peripheral zone (PZ), the central zone, and the rib zone. The central zone consists of a highly conserved stem cell population, residing directly above the organizing centre (OC), whose daughter cells are displaced into the PZ. It is the cells located in the PZ that generate the aerial tissues and organs of the plant (Fletcher and Meyerowitz 2000, Stahl and Simon 2005).

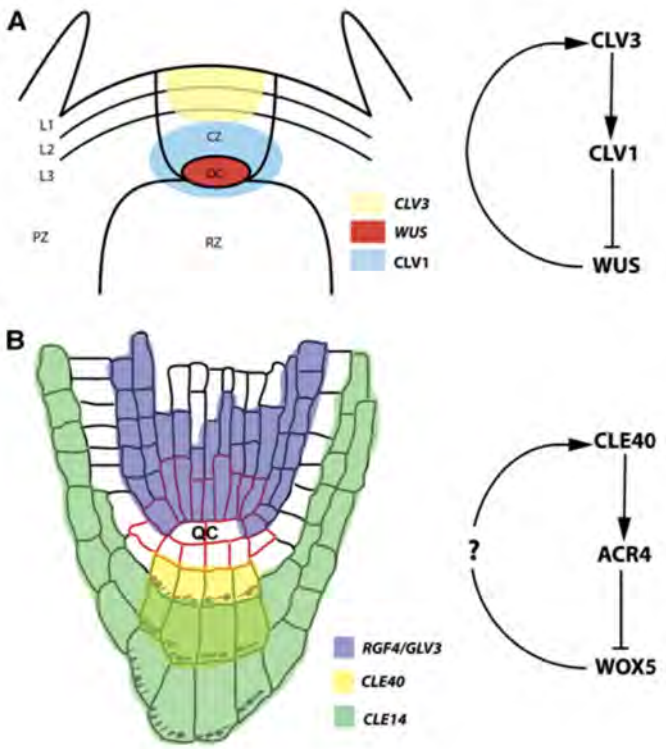

Figure 8. CLV3 and CLE40 regulate the stem cell niche in the SAM and RAM. (A) *CLV3* (yellow) is expressed in the central zone (CZ) of the SAM and inhibits expression of *WUS* (red) in the OC. WUS itself promotes *CLV3* expression. The CLV1 receptor is expressed below layers one and two of the SAM (L1 and L2). On the right is the proposed CLV3-CLV1-WUS signalling module. PZ, peripheral zone; RZ, rib zone. **(B)** Several peptides, such as *CLE14*, *CLE40*, and *RGF4/GLV3*, are expressed in the RAM in overlapping domains to control cell identity and meristematic activity. On the right is the proposed CLE40-ACR4-WOX5 signaling module. At present, there is no evidence for WOX5 controlling *CLE40* expression (?). Red outline, stem cells and QC; purple, starch granules.

In shoot development, the peptide ligand CLAVATA3/ESR (CLV3) is a major regulator of the stem cell niche (Fletcher, Brand *et al*. 1999, Kondo, Sawa *et al*. 2006) (Figure 8). CLV3 is shown to be involved in a negative feedback signalling pathway with the stem cell promoting homeodomain transcription factor protein WUSCHEL (WUS) (Mayer, Schoof *et al*. 1998, Fletcher, Brand *et al*. 1999, Schoof, Lenhard *et al*. 2000, Nimchuk, Tarr *et al*. 2011). *CLV3* is expressed in stem cells at the meristem apex, and the CLV3 peptide is thought to diffuse down toward the OC to restrict the expression of the transcription factor *WUS*, thereby regulating and inhibiting the expansion of stem cells in the SAM (Kinoshita, Betsuyaku *et al*. 2010, Nimchuk, Tarr *et al*. 2011, Yadav, Perales *et al*. 2011). Recently a CLV3-like peptide, OsCLE48, was also found to be required for correct shoot apical meristem development in rice (Guo, Zhang *et al*. 2015). *OsCLE48* when overexpressed in *Arabidopsis* inhibits shoot meristem development, whilst expression under the native Arabidopsis *CLV3* promoter rescues *clv3-2* knockout mutant phenotypes; however, unlike CLV3, no root apical meristem defects were observed when *OsCLE48* was overexpressed in *Arabidopsis* (See section 1.6.7 'Small signalling peptides in primary root development'). Tellingly, OsCLE48 was found to be an auxin-responsive gene, suggesting the possibility that it and other CLE peptides may exert their influence through modulation of auxin transport or signalling.

In addition to CLV3, there are over 30 CLE-related peptides containing the CLE motif, of which, CLE16, CLE17, and CLE27 have also been identified in and around the SAM (Lenhard and Laux 2003, Sharma, Ramirez *et al*. 2003, Fiers, Golemiec *et al*. 2005, Jun, Fiume *et al*. 2010). CLV3 has been proposed to interact with four major membrane-associated protein receptors: CLV1, CLV2-CORYNE (CRN), TOAD2/RPK2 (Clark, Williams *et al*. 1997, Jeong, Trotochaud *et al*. 1999, Muller, Bleckmann *et al*. 2008), and, more recently, FLS2 (Lee, Chah *et al*. 2011).

The *CLV3* and the LRR-RLK *CLV1* expression domains significantly overlap and competitive binding assays previously suggested CLV3 directly binds to the CLV1 protein receptor (Fletcher and Meyerowitz 2000, Ogawa,

Shinohara *et al*. 2008, Bleckmann, Weidtkamp-Peters *et al*. 2010, Nimchuk, Tarr *et al*. 2011). A recent photoaffinity approach has decisively confirmed CLV3 does indeed bind directly to CLV1 and additionally, two other receptor kinases, BAM1 and BAM2; (Shinohara and Matsubayashi 2015). In support of this finding, *clv1 bam1* double mutants exhibited accumulation of stem cells in the RAM and were insensitive to exogenous application of CLV3 peptide, consistent with the role of CLV3 in restricting stem cell proliferation in the SAM through regulation of *WUS* expression.

BAM1–BAM3 genes encode RLKs with homology to CLV1 (DeYoung, Bickle *et al*. 2006). Single *bam1*, *bam2*, and *bam3* mutants do not produce obvious phenotypes, but double and triple mutants produce striking arrested development of meristematic tissues, the opposite phenotype to *clv1*. BAM1 has also been shown to bind with a high degree of affinity to numerous CLE peptides (*CLE8* to *CLE14*), which are expressed during organ-specific developmental processes (Jun, Fiume *et al*. 2010, Shinohara, Moriyama *et al*. 2012). Since *CLV1* and *BAM* expression profiles do not overlap, it has been suggested that BAMs, located in the periphery of the meristem, may sequester CLE (or similar) peptides from entering the meristem, thereby influencing *WUS* expression (Deyoung and Clark 2008, Butenko, Vie *et al*. 2009, Wang and Fiers 2010).

CLV2 has been shown to associate with the CRN protein to form a receptor-like protein complex, and CRN requires CLV2 to localize to the plasma membrane (Bleckmann, Weidtkamp-Peters *et al*. 2010). The CLV2-CRN complex has been proposed to act in the CLV3 signalling pathway (Nimchuk, Tarr *et al*. 2011). No direct binding of CLV3 to CLV2/CRN or TOAD2/RPK2 was observed via this photoaffinity labelling approach (Shinohara and Matsubayashi 2015), however, this does not rule out the possibility that CLV2/CRN and TOAD2/RPK2 may function as co-receptors. For example, single *rpk2* and *clv1 clv2 rpk2* triple mutant lines display phenotypes highly similar to *clv3* mutants, suggesting a functional role for RPK2 in CLV3 perception and repression of *WUS* expression in the meristem (Kinoshita, Betsuyaku *et al*. 2010). A CLV3/CLE, BAM1 and RPK signalling

network has also been shown to act in the root meristem (see section 1.6.7 'Small signalling peptides in primary root development'.).

CLV3 was also previously reported to interact with the FLS2 receptor (Lee, Chah *et al*. 2011, Lee, Kuroha *et al*. 2012), which is known to recognize the unrelated bacterial flagellin-derived peptide flg22 and activate plant innate immune responses (Gomez-Gomez and Boller 2000). The CLV3 interaction with FLS2 in the meristem might play a role in protection of the meristem from bacterial pathogen infection (Lee, Chah *et al*. 2011). However, as other researchers have been unable to confirm binding of CLV3 to FLS2 under a variety of conditions (Mueller, Bittel *et al*. 2012, Mueller, Chinchilla *et al*. 2012, Segonzac, Nimchuk *et al*. 2012), it appears that more definitive results are necessary to support this interaction and its importance *in vivo*. Therefore, at present, the binding of CLV3 by FLS2 and a potential role in immune responses in the meristem remains an open question.

The RLK ERECTA (ER) is generally associated with stomatal development but has been recently shown to be involved in inflorescence development (Uchida, Lee *et al*. 2012). Single *er* mutants show a strong compacted inflorescence phenotype, very similar to *epfl4 epfl6* double mutants. Knockout experiments have shown a negligible role of ERECTA-LIKE1 (ERL1) and ERL2 during inflorescence development. Transcriptional analysis has shown that EPFL4 and EPFL6 are predominantly expressed in endodermal tissue and, through co-immunoprecipitation assays, have been shown to interact directly with ER in neighbouring phloem tissue following their diffusion through cell layers (Uchida, Lee *et al*. 2012). Interestingly, both auxin-regulated genes (such as ARGOS that is required for meristematic competence of aboveground organs), and gibberellin metabolic genes, were differentially regulated in *er* and *epfl4 epfl6* (Uchida, Lee *et al*. 2012). This suggests that potential interactions between phytohormones and signalling peptides participate in non-cell-autonomous control of inflorescence development.

1.6.7 Small signalling peptides in primary root development

The underground parts of the plant, the main root, lateral roots, and root hairs, all of which play a role in water and nutrient uptake (De Smet 2012, Smith and De Smet 2012), are derived from cells originating in the root apical meristem (RAM). The RAM consists of stem cells surrounding the quiescent centre (QC), which transmits developmental cues to the neighbouring stem cells. Stem cells can generate further stem cells, in a non-differentiated state, as well as daughter cells that differentiate into respective cell layers (van den Berg, Willemsen *et al*. 1997).

Among the peptides involved in developmental processes in the RAM, the CLE and the ROOT GROWTH FACTOR/GOLVEN/CLE-LIKE (RGF/GLV/CLEL) families of peptides are the most prominent (Casamitjana-Martinez, Hofhuis *et al*. 2003, Hobe, Muller *et al*. 2003, Stahl and Simon 2009, Matsuzaki, Ogawa-Ohnishi *et al*. 2010, Meng, Buchanan *et al*. 2012, Whitford, Fernandez *et al*. 2012, Fernandez, Drozdzecki *et al*. 2015).

Communication between QC and distal columella (stem) cells in the RAM has been elucidated as a signal transduction pathway between WOX5 and CLE40. *CLE40* is expressed in the inflorescence apices, leaves, and roots of the plant but has only been observed at functionally significant levels within the root tip. The *cle40* mutant shows a significant enlarged root phenotype due to rapid and aberrant division of columella initial cells. This overproliferation of initial cells is connected to an expansion of the *WOX5* domain, suggesting that CLE40 has a role in negatively regulating *WOX5*, thus possibly controlling or inhibiting the signals transmitted from the QC to the surrounding stem cells (Hobe, Muller *et al*. 2003, Stahl and Simon 2009).

It has been suggested that CLE40 acts as a secreted peptide ligand to the RLK ARABIDOPSIS CRINKLY4 (ACR4) in the RAM (Stahl and Simon 2009). *ACR4* is expressed in the columella stem cells, in close proximity of CLE40 (De Smet, Vassileva *et al*. 2008, Stahl and Simon 2009), and *cle40* and *acr4* mutants have similar phenotypes, namely, an expansion in the *WOX5* domain and additional layers of columella stem cells (De Smet, Vassileva *et al*. 2008, Stahl and Simon 2009). As *CLE40* is expressed in differentiated

columella cells, the expression overlaps with an area of weak *ACR4* expression (Stahl and Simon 2009). This area of weak *ACR4* expression has been shown to be upregulated by CLE40 treatment. ACR4 is thought to act as a dynamic buffer when CLE40 levels are too high. Increased ACR4 levels will sequester CLE40, allowing the expression of *WOX5*. *WOX5* in turn expands and protects the quiescent centre, counteracting the effects of CLE40 (Stahl and Simon 2009). Alternatively, CLE40 could activate ACR4 signalling, but this remains to be demonstrated biochemically. Intriguingly, this CLE40-ACR4-WOX5 signalling pathway in the RAM is highly similar to the CLV3-CLV1-WUS signalling pathway in the SAM.

Additionally, CLE26 has been suggested to work in a similar way to CLE45 in vascular development (see section 1.6.9 'Small signalling peptides in vascular development'). *CLE26* is expressed at the phloem pole and exogenous application of CLE26 results in severe shortening of the primary root in *Arabidopsis*, *Brachypodium* and wheat and a decrease in lateral root number in *Arabidopsis* and wheat (Czyzewicz, Shi *et al*. 2015). However, unlike CLE45, CLE26 most likely does not bind to the receptor BAM3, since *bam3* mutants display equal sensitivity to CLE26 as wild-type controls; however, strong binding of CLE26 to BAM1 and BAM2 has been demonstrated (Guo, Han *et al*. 2010). In addition, moderate binding of CLE26 to CLV1 and CLV2 was also demonstrated. Like CLE45, CLE26 appears to affect the distribution of auxin in roots, namely decreasing it in the root apical meristem; evidence suggests this may be due to a role in post-translational regulation of PIN1 auxin transporters (Czyzewicz, Shi *et al*. 2015).

Various other CLE family peptides have been shown to be expressed in the RAM: *CLE12*, *CLE14*, *CLE16*, *CLE17*, *CLE22*, and *CLE27* (Jun, Fiume *et al*. 2010, Meng and Feldman 2010). When applied exogenously, several CLE peptides (such as CLE14, CLE19, and CLE20) can irreversibly induce differentiation of stem cells rather than division and elongation, resulting in early consumption of the RAM and causing a strong short root phenotype (Casamitjana-Martinez, Hofhuis *et al*. 2003, Fiers, Hause *et al*. 2004, Fiers, Golemiec *et al*. 2005, Jun, Fiume *et al*. 2010, Meng and Feldman 2010). Curiously, overexpression of *CLE9*, *CLE10*, *CLE11*, or *CLE13* results in

severe inhibition of primary root elongation, while *CLE18*, *CLE25*, and *CLE26* overexpression leads to enhanced root elongation (Strabala, O'Donnell *et al*. 2006). How CLE family peptides, with highly similar homologies, can have antagonistic roles in root elongation has yet to be elucidated, but it may involve competitive receptor binding, as seen for similar antagonistic roles of related signalling peptides observed during stomatal development (Kondo, Nakamura *et al*. 2008, Lee, Hnilova *et al*. 2015) (see section 1.6.10 'Small signalling peptides in stomatal development'.). The phytohormone cytokinin was shown to be involved in processing or transmitting CLE peptide signals, thus regulating the effects of CLE14 and CLE20 *in planta*; exogenous application of CLE14 and CLE20 results in a strong short root phenotype, whilst treatment with cytokinin largely rescued the reduction in primary root length (Meng and Feldman 2010).

The peptides CLE14, CLE19, and CLE20 also appear to act along the CLV2-CRN/SOL2 receptor pathway in the root tip, a pathway that is prevalent in the development of the SAM. The *clv2* and *crn* mutants show a strong short root phenotype that is highly similar to the short root phenotype observed in *cle14*, *cle19*, and *cle20*. Thus, the CLV2-CRN/SOL2 receptor complex is not only vital to SAM stem cell maintenance through CLV3 signalling, but is also inherent in regulating the RAM through CLE14, CLE19, and CLE20 signalling (Meng and Feldman 2010). Recently, a genetic screening and co-immunoprecipitation approach generated evidence supporting further parallels with the SAM CLV3/CLE-RLK-based model (see section '1.6.6 'Small signalling peptides in the Shoot Apical Meristem (SAM)' and Figure 8): BAM1 and RPK2 were shown to form heteromeric complexes which, along with CLV2-CRN/SOL2, perceive CLE peptides involved in RAM and restrict stem cell proliferation.

Two other signalling peptides expressed in roots, *PHYTOSULFOKINE* (*PSK*) and *PSY1*, are also involved in root elongation (Amano, Tsubouchi *et al*. 2007, Matsuzaki, Ogawa-Ohnishi *et al*. 2010). These peptides can restore cell elongation activity in the elongation differentiation zone of roots in *tpst* (a mutant of TYROSYL PROTEIN SULFOTRANSFERASE, an enzyme involved in post-translational tyrosine sulfation modifications known to

optimise bioactivity in some small signal peptides such as RGF) but cannot restore meristem activity to that of the wild type, as evidenced by additional QC cells and aberrant columella cells (Matsuzaki, Ogawa-Ohnishi *et al*. 2010). Triple mutants for RGF family sulphated peptides, *rgf1 rgf2 rgf3*, exhibit a similar phenotype as that of *tpst*, being a short root phenotype with a reduction in meristematic cells. Exogenous application of RGF1/GLV11/CLEL8 to *rgf1 rgf2 rgf3* rescued cell numbers to that of the wild type, suggesting a strong functional redundancy between these peptides. The overexpression of RGF1/GLV11/CLEL8 shows the opposite phenotype, with seedlings possessing enlarged meristems, due to additional columella cells (Matsuzaki, Ogawa-Ohnishi *et al*. 2010). Thus, the redundant, sulphated RGF/GLV/CLEL family of peptides is clearly inherent to the maintenance of the stem cell population within the root and appears to act on PLETHORA (PLT) transcription factors (Matsuzaki, Ogawa-Ohnishi *et al*. 2010). PLTs are expressed in response to auxin and, in concert with PIN FORMED (PIN) proteins, act on auxin signalling required for root stem cell specification (Aida, Beis *et al*. 2004, Galinha, Hofhuis *et al*. 2007).

Interestingly, RGF/GLV/CLEL peptides also have an effect on auxin-dependent gravitropic response within roots, resulting in a waving phenotype and increased RAM size, when overexpressed or exogenously applied. Specifically, RGF1/GLV11/CLEL8 interacts with the PIN2 protein, namely, accumulating the PIN2 proteins at the plasma membrane, through an unknown process, thereby affecting auxin transport (Meng, Buchanan *et al*. 2012, Whitford, Fernandez *et al*. 2012, Fernandez, Drozdzecki *et al*. 2015).

RAPID ALKALINIZATION FACTOR (RALF) peptides are cysteine-rich peptides with approximately 115 amino acids (5 kDa) in the preproprotein. First discovered in *Nicotiana tabacum*, exogenous application of RALF causes fast alkalinisation of the media when leaf cells are grown in culture (Pearce, Moura *et al*. 2001). RALF peptides are also found in *Arabidopsis*, *Medicago truncatula, Solanum spp., Nicotiana spp*. and poplar, with at least 34 RALF genes in *Arabidopsis* (Murphy and De Smet 2014). The RALF peptides reversibly inhibit *Arabidopsis* root growth and root hair initiation when seedlings are cultured in synthetic RALF-containing media (Pearce, Moura *et*

al. 2001, Bedinger, Pearce *et al*. 2010). Overexpression of *RALF23* produces slow-growing seedlings, with roots that have a reduced capacity to acidify the rhizosphere, which may have developmental implications, resulting in a semi-dwarf phenotype (Srivastava, Liu *et al*. 2009). The recent identification of FERONIA as a receptor for RALF has provided a molecular mechanism for RALF action: interaction of RALF with FERONIA phosphorylates a plasma membrane H^+-ATPase 2 and inhibits proton transport, increases apoplastic pH and thus reduces cell elongation (Haruta, Sabat *et al*. 2014). Exogenous application of RALF1 also causes a significant rise in cytoplasmic Ca^{2+} concentration and increases abundance of calcium-dependant kinase 9, suggesting a functional role in Ca^{2+} modulation and signal transduction (Haruta, Monshausen *et al*. 2008). In support of this, FERONIA loss-of-function mutants (*fer4*) do not exhibit increases in Ca^{2+} signalling and cell elongation inhibition following RALF treatment (Haruta, Sabat *et al*. 2014). However, since *fer4* is not completely insensitive to RALF application at high concentrations, it is likely that yet further RALF receptors exist, possibly with different expression patterns and affinities/binding preferences for specific RALF peptides (Haruta, Sabat *et al*. 2014, Morato do Canto, Ceciliato *et al*. 2014, Murphy and De Smet 2014).

1.6.8 Small signalling peptides in lateral root development

While the role of phytohormones during lateral root initiation has been highlighted extensively, only recently has the RGF/GLV/CLEL family of peptides been shown to significantly delay lateral root development when overexpressed (Peret, De Rybel *et al*. 2009, Meng, Buchanan *et al*. 2012). Specifically, overexpression of *CLEL6* and *CLEL7* results in inhibition of pericycle division in the second round of pericycle cell divisions, arresting the initiation of lateral roots. Recently, RGF8/GLV6/CLEL2 has also been shown to regulate early pericycle cell divisions: *RGF8/GLV6/CLEL2* is expressed in the first stages of primordium development and increasing RGF8/GLV6/CLEL2 activity through gain-of-function mutants, ectopic

expression restricted to the xylem pole pericycle, or exogenous peptide application results in supernumerary pericycle divisions with fewer emerged lateral roots (Fernandez, Drozdzecki *et al*. 2015). It is thought RGF8/GLV6/CLEL2 acts to pattern the earliest asymmetric pericycle cell divisions, since expression is induced in LRFCs prior to the first division and excess RGF8/GLV6/CLEL2 decreases asymmetry of these cell divisions.

Interestingly, analyses on *CLEL*-overexpressing plants in conjunction with auxin-containing media have shown that the CLEL peptides actually act in an auxin-independent manner (Meng, Buchanan *et al*. 2012). In addition, the *CLE20* peptide is also expressed in lateral roots, suggesting a possible dual role of the peptide in RAM maintenance and lateral root development (Meng and Feldman 2010).

The small 15–amino acid hydroxyprolinated peptide C-TERMINALLY ENCODED PEPTIDE 1 (CEP1) negatively affects primary and lateral root growth, though the mechanism by which it does so has not yet been fully elucidated. Originally discovered via an *in silico* approach, CEP1 belongs to a family of 15 related peptides in *Arabidopsis* (Delay, Imin *et al*. 2013, Roberts, Smith *et al*. 2013), with other related CEPs (including 'Group I' [canonical] and 'Group II' [non-canonical] sequences) found across a wide range of angiosperm families including *Brassicaceae*, *Fabaceae*, *Poaceae*, *Rosaceae* and *Solanaceae* and the gymnosperm family *Pinaceae* (Ogilvie, Imin *et al*. 2014). Full-length CEP peptides are 82–126 amino acids long and share a basic structure of an N-terminal secretion signal, an N-terminal region with little sequence homology between family members, and a CEP motif (usually a single domain located at the extreme C-terminus of the prepropetide: however, there are exceptions – CEP2, CEP6, CEP9 and CEP10 contain multiple CEP motifs within the prepropeptide sequence, for example). The most common group I (canonical) CEP motif across all plant families studied so far is DFRPTAPGHSPGVGH (Ogilvie, Imin *et al*. 2014); the 15 *Arabidopsis* CEPs vary from this consensus by 2–9 residues, with AtCEP1, AtCEP5, AtCEP6.2, AtCEP9.2 and AtCEP9.5 being the most similar, and AtCEP15 the most dissimilar, though it is possible AtCEP15 and AtCEP14 are in fact group II CEPs (Delay, Imin *et al*. 2013, Roberts, Smith *et al*. 2013). Group II CEPs are

similar to Group I CEP motifs, particularly in the C-terminal SPG(I/V)GH sequence; however, the N-terminal 9 amino acids are more divergent in group II CEPs than in those sequences belonging to Group I.

CEP genes are not found in bryophytes, lycophytes, algae and other plants that lack true branching roots, suggesting their evolution coincides with the advent of vascularisation in plants (Rensing, Lang *et al*. 2008, Banks, Nishiyama *et al*. 2011, Delay, Imin *et al*. 2013). Interestingly, CEP-like domain sequences have also been found in secreted peptides from Root Knot Nematodes (RKNs) (Bobay, DiGennaro *et al*. 2013). Many of the RKN CEP sequences are strikingly similar to the group I CEP sequences found in angiosperms, suggesting the possibility that RKNs may have acquired CEP genes through horizontal gene transfer, possibly to aid in parasitism (Jones, Furlanetto *et al*. 2005, Haegeman, Jones *et al*. 2011, Delay, Imin *et al*. 2013).

The 15 CEP peptides found in *Arabidopsis* exhibit distinct expression patterns throughout the plant (Roberts *et al*. 2013). The best characterised members of the *Arabidopsis* CEP family - *CEP1*, *CEP3* and *CEP5* - are all expressed in the root (amongst other tissues) and perturbation of their levels through either pharmacological or genetic means leads to specific root phenotypes, namely stunted primary roots and a reduction in the number of emerged lateral roots. *CEP1* is primarily expressed in the shoot apical meristem and lateral root primordia during development, and overexpression lines display stunted roots and shoots with reduced meristem size, though it does not seem to have any obvious effect on QC cells (Ohyama, Ogawa *et al*. 2008). *CEP5* is primarily expressed in the root phloem pole pericycle tissues (with lesser expression also found in leaf petioles, mature leaves and anthers: (Roberts, Smith *et al*. 2013)) and overexpression leads to similar root and shoot stunting as seen in the *CEP1* overexpression lines; conversely, knockdown of *CEP5* expression by RNAi results in a slight increase in plant height (see Chapter 2 and (Roberts, Smith *et al*. 2013). Constitutive overexpression lines for *CEP2*, *CEP3*, *CEP4*, *CEP6* and *CEP9* displayed varying degrees of phenotype severity in terms of root and shoot phenotypes and leaf morphology defects, suggesting that despite their sequence similarities, CEP peptides have unique, non-overlapping functions *in planta*.

This is supported by the fact that many CEP genes are arranged in close proximity to each other (*CEP3/CEP11* and *CEP6/CEP7/CEP8* are arranged in tandem on chromosomes 2 and 5 respectively) but the divergence in the N-terminal sequence of these genes probably shows they are not simply the result of recent gene duplication and that diversity in CEP domain sequences appears to be advantageous enough to be evolutionarily selected for (Delay, Imin *et al*. 2013). Furthermore, the functions, expression patterns and domain sequences of *CEP*s appear to be species-specific: whilst considered homologs, *AtCEP1* and *MtCEP1* have differing expression patterns (Imin, Mohd-Radzman *et al*. 2013); the *Poaceae* family contains unique CEPs not found in other families (*Oryza sativa*: OsCEP5 and OsCEP6 which are thought to act in panicle development) (Ogilvie, Imin *et al*. 2014) and the most popular consensus sequences for CEP domains show variation between monocots and dicots, and between angiosperms and gymnosperms (Ogilvie, Imin *et al*. 2014).

The small size of CEP-encoding genes means that T-DNA insertion knockouts for the various CEPs are not generally available; however, a knockout for one CEP, CEP3, has successfully been generated and showed increased root and shoot growth, contrasting accordingly with the stunting seen in *CEP3* and other *CEP* overexpression lines (Ohyama, Ogawa *et al*. 2008, Delay, Imin *et al*. 2013, Roberts, Smith *et al*. 2013).

The roles of certain CEP peptides beyond *Arabidopsis* have also been studied in the root-nodule forming model organism *Medicago truncatula*. *Medicago truncatula* roots develop nodules containing symbiotic nitrogen-fixing rhizobia which increase N supply to the plant and improve growth, particularly in N-poor soil. The process of nodule formation is similar in some aspects to lateral root formation, in that it requires dedifferentiation of pericycle and also root cortical cells, which then divide to form a nodule meristem (Timmers, Auriac *et al*. 1999). As in *Arabidopsis*, *MtCEP1* is expressed in roots and inhibits lateral root formation; however, it was also found to enhance nodulation, and conversely to *Arabidopsis*, no primary root stunting was observed. The nodules formed in *Medicago* following *MtCEP1* overexpression or exogenous application also developed faster and fixed more nitrogen than control nodules.

As well as a role in nodulation in leguminous species, current evidence suggests the CEP peptides represent a more general signalling mechanism of co-ordinating environmental cues and responses such as root branching: expression analyses show *CEP* gene expression is regulated by cues such as nitrogen availability, osmotic and salt status, and carbon dioxide levels (Delay, Imin *et al*. 2013, Imin, Mohd-Radzman *et al*. 2013, Roberts, Smith *et al*. 2013, Tabata, Sumida *et al*. 2014). Nitrate starvation in particular induces expression of *CEP3*, *CEP5* and *CEP9* and eight *MtCEPs* (Delay, Imin *et al*. 2013) with *CEP3* expression in particular increased 10-fold under nitrate depletion, and *MtCEP1* expression increased as much as 36-fold (Delay, Imin *et al*. 2013, Imin, Mohd-Radzman *et al*. 2013). Furthermore, this response appears unique to nitrate depletion, as similar effects were not observed for ammonium limitation. A role in nitrate sensing is also supported by the observation from *Medicago* that artificially increased MtCEP1 abundance promotes nodulation even under high nitrate; a condition which usually strongly suppresses nodule formation (Imin, Mohd-Radzman *et al*. 2013).

Recently, evidence has been presented showing that various CEPs are perceived by the receptors XYLEM INTERMIXED WITH PHLOEM 1 (XIP1)/CEPR 1 and CEPR2 (Bryan, Obaidi *et al*. 2012, Tabata, Sumida *et al*. 2014). Loss-of-function mutants for these related leucine-rich receptor kinases (LRR-RKs) are insensitive to CEP1-mediated root stunting and display elongation of lateral roots, dwarfism, pale green leaves and anthocyanin accumulation which are phenotypes associated with nitrate starvation (Tabata, Sumida *et al*. 2014). Several nitrate transporters are downregulated in these mutants, including *NRT2.1, NRT3.1* and the dual nitrate/auxin transporter *NRT1.1* (see section 1.7.1 'Nitrate'). Accordingly, *CEP1* through *CEP11* overexpression lines had elevated *NRT2.1* expression. Split root experiments in nitrate rich and nitrate depleted media respectively showed that perception of low nitrate was sufficient to induce *NRT* upregulation even in roots situated in N-rich medium, and reciprocal grafting between *xip1/cepr1* and *cepr2* loss-of-function mutants and wild-type scions/rootstocks showed that wild-type shoots were necessary to elicit NRT upregulation in response to exogenous CEP1

treatment, whilst wild-type roots were not required (Tabata, Sumida *et al*. 2014).

Thus, the current working mechanism is that following an earlier perception event (possibly by the nitrate transporters themselves due to their dual transporter/receptor 'transceptor' status), nitrate starvation is communicated through the secretion of root-derived CEP peptides, which translocate and are perceived by shoot-located receptors. This in turn leads to an upregulation of nitrate transporters and promotes other adaptive phenotypical responses to low nitrate (Tabata, Sumida *et al*. 2014). The exact signals downstream of ligand/receptor interaction, the shoot-descending signal which links CEP perception with a phenotypic response, and the pathway of peptide translocation to the shoot remains to be determined.

Receptor-like kinases are known to be important signalling modules in lateral root development, although it is not always known which ligand receptor pairs govern specific aspects of lateral root development. For example, ACR4 has been shown to be required for specifying the fate of neighbouring cells during pericycle cell division in lateral root initiation and restricting the division of neighbouring pericycle cells, thereby controlling the pattern of cell division (De Smet, Vassileva *et al*. 2008). ACR4 likely acts as signal receiver for one, or many, small signalling peptides generated in or surrounding the lateral root initiation site, such as RGF/GLV/CLEL peptides acting during lateral root initiation (Meng, Buchanan *et al*. 2012). However, the majority of known receptors for CLE peptides are LRR-RLKs (e.g., CLV1, TDR/PHLOEM INTERCALATED WITH XYLEM [PXY], and BAM), and the active domain of ACR4 has been shown to consist of extracellular crinkly repeats, resulting in a critical difference in receptor structure compared with LRR-RLKs (Gifford, Robertson *et al*. 2005), suggesting that other, yet unidentified, signalling peptides and/or receptor kinases could be involved. Recently, it has been proposed that ACR4 acts in a reciprocal manner with cytokinin in the regulation of lateral root spacing (Chang, Ramireddy *et al*. 2015).

The peptide POLARIS (PLS) is also required for root growth and vascular patterning. POLARIS is a somewhat more unusual peptide, as it does not fall exactly into either the small-posttranslationally modified peptides class or the cysteine rich peptides; instead, it is a 36-amino acid non-secreted peptide that acts intracellularly (Chilley, Casson *et al*. 2006). PLS mutants have a semidominant phenotype with short roots, enhanced ethylene response, disrupted auxin transport, reduced leaf vascularisation, altered cytoskeleton dynamics and abnormal responses to exogenous auxin and cytokinin (Lindsey, Casson *et al*. 2002). PLS is a negative regulator of ethylene responses, whilst ethylene negatively regulates auxin transport and *PIN1* and *PIN2* expression (Liu, Mehdi *et al*. 2013): correspondingly, *pls* mutants have lower free IAA levels than wild-type controls, and develop fewer lateral roots. POLARIS is thought to regulate an ethylene-auxin-cytokinin crosstalk loop required for normal root growth. Though the exact molecular mechanism by which it does so is currently unclear, modelling suggests it may act via the ETR1 ethylene receptor (Liu, Mehdi *et al*. 2010), with events downstream of ethylene perception modulating auxin transport via the PIN and AUX1 auxin transporters (Moore, Zhang *et al*. 2015).

TRACHEARY ELEMENT DIFFERENTIATION INHIBITORY FACTOR (TDIF/CLE41CLE44) plays a role in regulation of lateral root organogenesis through modulation of auxin via a TDIF-TDR-BIN2 signalling cascade (Cho *et al*. 2014). TDIF is perceived by TDIF RECEPTOR (TDR). TDR directly interacts with the GSK3-family kinase BIN2, which subsequently phosphorylates ARF7 and ARF19, triggering their release from AUX/IAA repressors and increasing their DNA-binding affinities independently from auxin perception. ARF7 and ARF19 are crucial factors in lateral root organogenesis, driving expression of *LBD16* and *LBD29* (see sections 1.3 'Auxin in lateral root development' and 1.3.3 'Auxin signalling').

1.6.9 Small signalling peptides in vascular development

The vascular system in plants consists fundamentally of two functional cell types with different functions: xylem and phloem (Sieburth and Deyholos 2006). The importance of signalling peptides (especially the CLE family) in vascular development has recently become more clear (Fiers, Ku *et al*. 2007, Elo, Immanen *et al*. 2009, Hirakawa, Kondo *et al*. 2011, Kondo, Hirakawa *et al*. 2011). TDIF (CLE41/44) is shown to promote the proliferation of procambial cells in leaf and hypocotyl vasculature development, while it also inhibits the differentiation of procambial cells into tracheary elements (Ito, Nakanomyo *et al*. 2006, Whitford, Fernandez *et al*. 2008). The receptor for TDIF has been identified as the LRR-RLK TDR/PXY (Fisher and Turner 2007, Hirakawa, Shinohara *et al*. 2008, Etchells and Turner 2010). *TDR/PXY* is primarily expressed in procambium cells, while *TDIF* is expressed in adjacent phloem cells, in nonoverlapping expression domains. CLE41 must then be secreted toward the xylem where it is received by TDR/PXY, to transmit the correct positional information (Etchells and Turner 2010, Hirakawa, Kondo *et al*. 2011). The TDIF-TDR/PXY pathway controls the WUS-related HOMEOBOX gene, WOX4, which is required for controlling the fates of the vasculature cell fates (Suer, Agusti *et al*. 2011). WOX4 is essential for procambial/cambial cell proliferation, as *wox4* mutants show restricted proliferation; however, it is not essential for the inhibition of cambial cells into xylem cells. Thus, two pathways can be distinguished, namely, a TDIF-TDR/PXY-WOX4 pathway, which promotes procambial/cambial stem cell proliferation, and TDIF-unknown factors, which inhibits the differentiation of cambial cells into xylem cells (Hirakawa, Kondo *et al*. 2010, Suer, Agusti *et al*. 2011). Both TDIF pathways, however, show crosstalk between xylem and phloem cells, as the TDIF derived from phloem is then responsible for the maintenance of the procambial/cambial cells via their differentiation and proliferation capabilities (Kondo, Hirakawa *et al*. 2011).

CLE10 plays a role in xylem formation as *CLE10* overexpression results in significant inhibition of the early stages of protoxylem vessel formation, which, in roots, appears to occur through activation of cytokinin signalling (Kondo, Hirakawa *et al*. 2011). CLE10 has the ability to inhibit protoxylem differentiation in the root, by the repression of *ARABIDOPSIS*

RESPONSE REGULATOR (ARR) genes, *ARR5* and *ARR6*, which are known to negatively regulate cytokinin signalling, allowing suppression of procambium differentiation into protoxylem vessels (Hirakawa, Kondo *et al*. 2011). CLE10 is thought to act through the CLV2 receptor: *CLE10* expression normally inhibits protoxylem formation in roots; however, in *clv2* knockout lines, protoxylem formation is no longer inhibited, suggesting a link between the CLE10 signalling peptide and the CLV2 receptor (Kondo, Hirakawa *et al*. 2011).

Compared with xylem development, comparatively little is known about the signalling pathway components involved in phloem development; however, recent evidence has shown a role for CLE45 in this process through interaction with the known phloem development regulators OCTOPUS (OPS) and BREVIS RADIX (BRX). OPS and BRX are plasma-membrane located proteins; *ops* and *brx* loss-of function mutants have defects in protophloem formation and differentiation reminiscent of *CLE45* overexpression (Rodriguez-Villalon, Gujas *et al*. 2014); conversely, gain-of-function OPS lines are resistant to CLE45. Loss-of-function mutants for BRX and OPS can be rescued by loss-of-function of the putative CLE45 receptor BAM3: thus, BRX and OPS are thought to be positive regulators of protophloem formation and differentiation which work antagonistically to the negative CLE45-BAM3 signalling module, which represses specification of sieve element precursor cells. Recently, CLE26 was also found to work in a similar way in the developing protophloem, and furthermore, according to *DR5:VENUS* expression patterns, CLE45 and CLE26 increase auxin response in and above the differentiation zone of the primary root, reducing the size of the lateral root formation zone but increasing lateral roots in the higher branching zone, suggesting impaired protophloem sieve element differentiation leads to increased auxin response and altered lateral root phenotypes in mature shootward parts of the root (Rodriguez-Villalon, Gujas *et al*. 2015).

The receptor kinase ERECTA is known to regulate inflorescence architecture and stomatal development (see sections 1.6.6 'Small signalling peptides in the Shoot Apical Meristem' and 1.6.10 'Small signalling peptides

in stomatal development'). Phloem-expressed *ER* and a paralogue, *ER-LIKE1 (ERL1)*, also play a role in vascular development from procambial cells, in conjunction with endodermis-expressed *EPFL4* and *EPFL6* (Uchida and Tasaka 2013). Interestingly, interaction of EPFL4/6 with ER appears to promote stem elongation, whilst EPFL4/6-ER/ERL interaction favours vascular development: the control of two different developmental fates regulated by the same family of peptides is also seen in EPF/EPFL peptides in stomatal development (see section 1.6.10 'Small signalling peptides in stomatal development').

1.6.10 Small signalling peptides in stomatal development

Stomata are the organs responsible for the vital gas exchange required to perform respiration and are composed of two guard cells. Stomata in *Arabidopsis* are usually separated by at least one nonstomatal cell to optimize spatial arrangement between the photosynthetic epidermal surface area and the gas exchange organs. Various members of the EPF family of Cysteine-rich small peptides, specifically, EPIDERMAL PATTERNING FACTOR 1 (EPF1), EPF2, EPF-LIKE6 (EPFL6)/CHALLAH (CHAL), and EPFL9/STOMAGEN, are involved in regulating stomatal density and positioning (Hara, Kajita *et al*. 2007, Abrash and Bergmann 2010, Kondo, Kajita *et al*. 2010, Rowe and Bergmann 2010, Sugano, Shimada *et al*. 2010, Shimada, Sugano *et al*. 2011).

The overexpression of *EPF1* or *EPF2* results in reduced stomatal density, but loss-of-function mutants indicated they have differing actions (Hunt and Gray 2009, Kondo, Kajita *et al*. 2010). EPF2 may act early in development to control differentiation in meristemoids, controlling asymmetric cell division and proliferation rates, while EPF1 may affect the directionality and spacing of the developing stomata (Hunt and Gray 2009, Rowe and Bergmann 2010, Shimada, Sugano *et al*. 2011, Lee, Kuroha *et al*. 2012). This theory is strengthened by the expression patterns, with *EPF2* expressed in

mother meristemoid cells and early meristemoids, while *EPF1* is expressed in GMCs and young guard cells, showing a clear temporal-spatial division between the two peptides (Shimada, Sugano *et al*. 2011).

EPFL9/STOMAGEN is produced in the mesophyll cells of the plant and positively regulates stomatal development in the epidermis, in a role opposite to EPF1 and EPF2, despite all three peptides adopting similar structures (Hunt, Bailey *et al*. 2010, Sugano, Shimada *et al*. 2010). Overexpression of *EPFL9/STOMAGEN* greatly increases stomatal density to a degree where guard cells are often surrounded by other stomatal precursor cells (Hunt, Bailey *et al*. 2010, Sugano, Shimada *et al*. 2010). Conversely, knockout mutants show a significant decrease in stomatal density (Hunt, Bailey *et al*. 2010, Sugano, Shimada *et al*. 2010, Shimada, Sugano *et al*. 2011).

Several receptor-like proteins and RLKs, such as TOO MANY MOUTHS (TMM), ER, and ERL are involved in stomatal development (Yang and Sack 1995, Shpak, McAbee *et al*. 2005). EPF1 and EPF2 bind to ER and ERL1 receptors whilst EPF2 and EPFL9/STOMAGEN both directly bind to ER and its co-receptor TMM (Lee, Kuroha *et al*. 2012, Lee, Hnilova *et al*. 2015). This competitive binding of EPF2 and EPFL9/STOMAGEN to the same receptors provides an intriguing mechanism explaining their antagonistic functions in stomatal development; EPF2 binding was observed to elicit rapid phosphorylation of downstream signalling components, whilst EPFL9/STOMAGEN binding did not. Interestingly, the expression pattern of *EPF2* and *EPFL9/STOMAGEN* differs (*EPF2* is expressed in a subset of protodermal cells whilst *EPFL9/STOMAGEN* is secreted from underlying internal tissues) suggesting a further spatial focusing dimension to their antagonism, i.e. protodermal cell fate is influenced by the relative concentrations of the respective peptides on either side.

It is possible CHALf peptides also act through the TMM and ER receptors (Abrash, Davies *et al*. 2011). In a *tmm* background, *CHALf* overexpression leads to a striking reduction in stomatal density, suggesting that TMM binds CHALf to buffer signalling and block transmission into stomatal

lineage cells; *CHALf* overexpression phenotypes are also mediated by ER family mutations (Abrash, Davies *et al*. 2011).

BAM receptors have also been identified in aerial organs during stomatal development and bind with high affinity to peptides like CLE9. As *CLE9* is expressed during stomatal development, it is likely that BAMs are functionally involved in stomatal development (Jun, Fiume *et al*. 2010, Shinohara, Moriyama *et al*. 2012).

1.6.11 Small signalling peptides in abscission

Abscission, whereby entire organs are shed from the plant, involves the organized and programmed cell separation of the middle lamella in plant cells. The middle lamella is the area of the cell wall that is shared between two neighbouring cells, solidifying and holding the cells together. Abscission during development is essential for optimal plant growth when specific cells and organs are no longer necessary for functional processes (Stenvik, Butenko *et al*. 2006, Stenvik, Tandstad *et al*. 2008).

The small signalling peptide IDA has been shown to stimulate abscission through promotion of cell separation or through inhibiting cellular repair processes (Stenvik, Butenko *et al*. 2006, Stenvik, Tandstad *et al*. 2008). INFLORESENCE DEFICIENT IN ABCISSION (IDA) and the IDA-LIKE (IDL) subfamily regulate *Arabidopsis* developmental processes; with *IDA* predominantly expressed during floral abscission. The *ida* knockout mutants lack the ability to abscise floral organs, with plants fully retaining the petals, sepals, and filaments indefinitely. Furthermore, overexpression of *IDA* and *IDL* shows premature floral abscission, with overproliferation of abscission zone cells; however, it varies in intensity between the members of the family. IDA and IDA-like peptides may play a general role in abscission beyond floral organ abscission, since they are variously expressed at sites where cell separation occurs, such as vascular and stomatal development, root cap sloughing, lateral root emergence and seed shedding (Stenvik, Tandstad *et al*.

2008, Kumpf, Shi *et al*. 2013). Some members of the IDA/IDL family were also recently shown to be induced by different biotic and abiotic stresses, particularly salt stress but also UV, cold, and inoculation with *Pseudomonas syringae*, possibly suggesting a role for IDA/IDLs beyond normal developmental and abscission processes (Vie, Najafi *et al*. 2015).

PAMP-INDUCED SECRETED PEPTIDE (PIP) and PIP-LIKE (PIPL) peptides, which are involved in amplifying immune responses triggered by the bacterial flagellin epitope flg22 through RECEPTOR LIKE KINASE 7 (RLK7), contain a 13-amino acid C-terminal motif named the PIP motif (Hou, Wang *et al*. 2014). IDA and IDL proteins have an extended PIP motif (EPIP), which has been shown as the active part of the peptide used in abscission. Similar motifs are also found in the CEP peptides CEP14 and CEP15 (Vie, Najafi *et al*. 2015) (see section 1.6.8 'Small signalling peptides in lateral root development'). *Arabidopsis* plants subjected to synthetic IDA EPIP peptides showed phenotypes similar to *IDA* overexpression lines, with abscission occurring prematurely, whereas application of IDA EPIP to *ida* knockout lines rescued the phenotype (Stenvik, Butenko *et al*. 2006, Stenvik, Tandstad *et al*. 2008). Due to the activity of the EPIP motif, it is further hypothesized that IDA and IDL peptides are cleaved, through a similar process as CLV3, where the active EPIP site becomes available to downstream effectors, thereby initiating floral abscission (Stenvik, Butenko *et al*. 2006).

IDA and IDL peptides act as the peptide ligand to the LRR-RLK HAESA (HAE) and to a family of HAESA-LIKE (HSL) receptor kinases, which have been shown to be expressed in floral abscission zones. The highly similar expression patterns of *HAE* and *HSL2*, along with the fact that *hae hsl2* double mutants completely lack floral abscission, suggest a functional redundancy within this family of receptors. IDA and HAE-HSL2 may act along the same pathway or may in fact be ligand-receptor pairs (Stenvik, Butenko *et al*. 2006). IDA possibly regulates floral organ abscission by acting as a ligand in HAE-HSL2–mediated suppression of BP/KNAT1, which are repressors of the abscission-promoting transcription factors KNAT2 and KNAT6 (Shi, Stenvik *et al*. 2011).

1.7 Root architecture responses to soil nutrient status

The soil environment is not homogenous in terms of nutrient distribution. Therefore, plants must adapt their root architecture to exploit soil resources efficiently. The ability to sense soil nutrient status and to respond via post-embryonic growth and rearrangement of organs in this adaptive way are complex and tightly regulated processes, the complete mechanisms of which are still not fully understood. The interactions of plants with selected nutrients (nitrate and phosphorous) at the root interface are detailed below. For root responses to other nutrients, such as potassium, calcium, sulphur and iron, the reader is directed to the recent reviews (Giehl, Gruber *et al*. 2014, Kong, Zhang *et al*. 2014, Tian, De Smet *et al*. 2014, Brumbarova, Bauer *et al*. 2015, Satbhai, Ristova *et al*. 2015).

1.7.1 Nitrate

Nitrate (NO_3^-) is the primary source of soil-derived nitrogen for plants, but the distribution of nitrate in the soil solution can vary dramatically: up to four orders of magnitude (Crawford and Glass 1998). Therefore, plants need to proactively adapt to this variability in soil nitrate status through modulation of root architecture. In addition, the demand for nitrogen by the plant itself is also not always uniform, fluctuating according to carbon metabolism and growth rate: thus a multitude of short and long-range signalling mechanisms are utilised to communicate the nitrogen status of the plant and coordinate responses. The importance of nitrate status to a plant is tellingly illustrated by the fact that over 1000 genes in *Arabidopsis* are known to be regulated by nitrate status (Wang, Okamoto *et al*. 2003). Soil nitrate is prone to leaching and tends to be more concentrated in deeper soil layers (Tinker and Nye 2000, Giehl, Gruber *et al*. 2014).

Low nitrate availability is thought to stimulate the elongation of the primary root to exploit the reserves of nitrate found in deeper soils (though this idea is controversial, as there is evidence water supply is the main influence on primary root growth, independent from nitrate concentration: (Chapman, Whalley *et al*. 2011)). Low nitrate also stimulates the elongation of lateral roots, though the density of lateral roots is unaffected (Linkohr, Williamson *et al*. 2002, Lopez-Bucio, Cruz-Ramirez *et al*. 2003, Gruber, Giehl *et al*. 2013). In addition, the distribution of nitrate in the soil is relevant in terms of lateral root phenotypes, as localised supply of nitrate encourages lateral root elongation of those roots growing into nitrate-rich patches, but does not promote lateral root elongation systemically throughout the entire root system - instead repressing the elongation of lateral roots outside nitrate-rich zones (Zhang and Forde 1998, Linkohr, Williamson *et al*. 2002). Conversely, globally high nitrate also represses lateral root elongation: however, this is thought to be due to nitrogen itself acting as a signal molecule, rather than due to any nutritional effect, as similar effects on lateral root elongation were also observed in response to ammonium (NH_4^+) - another nitrogen source available to plants from soil which, unlike nitrate, increases lateral root density (Zhang and Forde 1998, Zhang, Jennings *et al*. 1999, Zhang and Forde 2000, Lima, Kojima *et al*. 2010).

The uptake of nitrate from the soil requires membrane bound transporters. In plants, nitrate uptake from the soil is mainly mediated by proteins of the Nitrate Transporter1/Peptide Transporter (NRT1/PTR) family and the NRT2 family. Uptake of nitrate from the soil can be either through high-affinity transport systems (HATS) or low affinity transport systems (LATS), the energy requirement for both of which is thought to be derived from proton gradient coupling (Meharg and Blatt 1995, Wang and Crawford 1996, Crawford and Glass 1998).

The NRT1/PTR family comprises of a large number of transport proteins conserved across bacteria, fungi, plants and animals. In *Arabidopsis,* there are 53 known members (Wang, Hsu *et al*. 2012); members of the NRT1/PTR family are generally considered low-affinity nitrate transporters and have diverse substrate specificity (Wang, Liu *et al*. 1998, Liu, Huang *et al*.

1999, Morere-Le Paven, Viau *et al*. 2011). NRT1.1 has also been shown to transport auxin (particularly at low (<0.2 mM) nitrate concentrations) and lead to auxin accumulation in lateral root tips, thus potentially linking environmental cues with hormonal and phenotypical responses (Krouk, Lacombe *et al*. 2010) (see section 1.6.8 'Small signalling peptides in lateral root development').

NRT2 family proteins are, like most NRT1/PTR proteins, proton-coupled transporters but unlike NRT1/PTR family proteins, specifically transport nitrate and are considered high-affinity nitrate transporters (Wang, Hsu *et al*. 2012, Krapp, David *et al*. 2014). There are seven known members of this family in *Arabidopsis* (Cerezo, Tillard *et al*. 2001, Orsel, Krapp *et al*. 2002, Chopin, Orsel *et al*. 2007, Li, Wang *et al*. 2007, Kiba, Feria-Bourrellier *et al*. 2012). The nitrate transport function of most NRT2 members is dependent on the protein NAR2/NRT3.1 which associates with several NRT2 proteins (Okamoto, Kumar *et al*. 2006, Orsel, Chopin *et al*. 2006, Kotur, Mackenzie *et al*. 2012). The NRT2.1-NAR2.1 high affinity uptake complex is known to play a dual role in regulating lateral root development, positively regulating lateral root initiation in low nitrate conditions but conversely function as repressors when nitrate is low but sucrose is high, suggesting this regulation occurs independently from nitrate uptake, and demonstrating the balance between carbon and nitrogen ratios in regulating nitrate responses (Little, Rao *et al*. 2005, Remans, Nacry *et al*. 2006).

In addition to the NRT1/PRT and NRT2 nitrate transporter families, there are two additional families known to be involved in the transport of nitrate within the plant: chloride channels (CLC) and Slow Anion Channel-associated 1 (SLAC1) or SLAC1-associated homologues (SLAH1–4). In *Arabidopsis* there are seven known members of the CLC family and five members of the SLAC1/SLAH family, though neither of these families have been demonstrated to be involved in uptake of nitrate from the external soil solution (Krapp, David *et al*. 2014).

Plants are able to sense external nitrate availability, and nitrate uptake is itself subject to regulation by direct N-signalling (Wang, Xing *et al*. 2007).

NO_3^- induces the expression of several genes related to nitrate transport and assimilation which are conversely activated in the short-term following an initial NO_3^- supply (including *NRT2.1*), but these genes are repressed in the long term after exposure to a prolonged NO_3^-supply (Crawford and Glass 1998, Munos, Cazettes *et al*. 2004, Wang, Tischner *et al*. 2004, Krouk, Tillard *et al*. 2006, Gojon, Nacry *et al*. 2009). The upregulation of genes such as *NRT2.1* in response to NO_3^- starvation is likely to be a direct effect of release from the inhibitory nature of N metabolites on the expression of these genes (Lejay, Tillard *et al*. 1999).

Downstream of nitrate perception by receptors such as the NRT family transceptor NRT1.1, a MADS-box transcription factor, ANR1, is involved in the nitrate-dependent regulation of root development. ANR1 transduces the nitrate signal internally within the plant, with ANR1 overexpression increasing both lateral root numbers and length (Gan *et al.* 2012, Remans *et al.* 2006). ANR1 overexpressing lines are also more sensitive to nitrate, whilst underexpressing lines were not able to elongate lateral roots into locally high nitrate patches (Zhang & Forde 1998). Thus, this transcription factor links external nitrate perception with phenotypical changes known to aid in nutrient foraging in nitrate-rich patches.

1.7.2 Phosphorous

Phosphorous is one of the most common limiting resources in the soil, with concentrations typically <10 μM (Niu, Chai *et al*. 2013). The precise involvement and interaction of various hormones, as well as other factors such as iron (Fe), Calcium (Ca) sugars, and Reactive Oxygen Species/Nitric Oxide in regulating the phosphate starvation response remains to be fully elucidated. For a more comprehensive overview of the contributions of these factors to phosphate starvation response, the reader is directed to the recent reviews Niu, Chai *et al.* 2013 and Peret, Desnos *et al.* 2014.

Phosphorous starvation is known to induce a shallower, more highly branched root architecture phenotype with denser root hairs (Williamson, Ribrioux *et al*. 2001, Lopez-Bucio, Hernandez-Abreu *et al*. 2002, Sanchez-Calderon, Lopez-Bucio *et al*. 2005, Peret, Clement *et al*. 2011, Gruber, Giehl *et al*. 2013). This phenotype arises from the arrest of primary root growth due to decreased cell elongation and reduced cell division rates in the RAM (Sanchez-Calderon, Lopez-Bucio *et al*. 2005) and concurrent promotion of lateral root development. However, it should be noted that these phenotypes have largely been observed as a response to phosphate deprivation in *Arabidopsis thaliana* ecotype Col-0; not all *Arabidopsis thaliana* ecotypes display root growth inhibition, suggesting this response may be largely genetically controlled (Chevalier, Pata *et al*. 2003). Furthermore, many other plant species actually display normal-length or even elongated primary roots in response to phosphate deprivation, including *Oryza sativa* and *Zea mays* (Mollier and Pellerin 1999, Wissuwa 2003, Shimizu, Yanagihara *et al*. 2004, Yi, Wu *et al*. 2005).

This utility of the phosphate starvation phenotype corresponds to the distribution of organic phosphate in the soil, which is primarily concentrated in the upper soil layer due to decomposition of surface organic material combined with the poor soil mobility of phosphate due to its propensity to form insoluble complexes with cations present in soil, such as aluminium, iron and calcium (Tinker and Nye 2000, Lynch and Brown 2001, Rubio, Liao *et al*. 2003, Zhu, Kaeppler *et al*. 2005).

Though the mechanism by which roots sense and respond to phosphate in terms of root architecture is not fully understood, it seems a requirement that the root tip specifically must make contact with phosphate-depleted medium in order for changes to root architecture to occur (Svistoonoff, Creff *et al*. 2007). The promoters of most genes positively or negatively regulated by phosphate starvation contain sequences known as PHR1 BINDING SITES (P1BS). Thus, it is likely that transcription factors which bind to these sites have a role in regulating phosphate response. However, mutants for a MYB-family transcription factor PHOSPHATE STARVATION RESPONSE1 which binds to *P1BS* only display minor alterations in shoot-root-ratios and root hair

phenotype, thus redundancy or a more complex mechanism of regulation is likely (Rubio, Linhares *et al*. 2001). Phosphate uptake from the soil itself is primarily achieved by the PHOSPHATE TRANSPORTER 1:1–9 (PHT1:1–9) family of high-affinity phosphate transporters, of which there are nine members in *Arabidopsis* (Shin, Shin *et al*. 2004).

Transcriptomics approaches have identified several genes up- or down-regulated by phosphate starvation, some of which have been the targets of successful reverse-genetics approaches - such as *PHOSPHATE ROOT DEVELOPMENT* (*PRD*), which encodes a DNA-binding protein with an unclear mode of action which appears to be involved in the primary root growth inhibition response; *prd* null mutants display hypersensitivity to phosphate starvation (Camacho-Cristobal, Rexach *et al*. 2008). In addition, LOW PHOSPHATE ROOT1 and 2 (LPR1/LPR2) encodes a multicopper oxidase which appears to also required for the primary root growth inhibition response under phosphate starvation: *lpr1* and *lpr2* mutants maintain primary root growth on low phosphate media (Svistoonoff, Creff *et al*. 2007).

In terms of driving the increased lateral root branching observed in response to phosphate starvation, auxin seems an obvious candidate. Indeed, evidence suggests phosphorous starvation increases expression of *TIR1*, thus making pericycle cells more sensitive to auxin and increasing the frequency of branching (Perez-Torres, Lopez-Bucio *et al*. 2008). However, auxin is unlikely to be the sole hormone involved in the regulation of the phosphate starvation response; there is evidence from transcriptomic approaches and phenotypical observations that ethylene may also be involved (Borch, Bouma *et al*. 1999, Ma, Baskin *et al*. 2003, Zhang, Lynch *et al*. 2003, Kim, Lynch *et al*. 2008, Thibaud, Arrighi *et al*. 2010, Chacon-Lopez, Ibarra-Laclette *et al*. 2011) and/or abscisic acid (ABA) (Radin 1984, Trull, Guiltinan *et al*. 1997). Nevertheless, there are various data which indeed support a role for auxin in regulation of root branching, though the exact mechanism by which it does so is not clear. For example, the exhaustion of the primary root meristem seen in the *Arabidopsis* response to phosphate starvation could also disrupt auxin circulation in the root-tip and potentially redistribute auxin thus affecting branching (Kramer and Bennett 2006). Three *Arabidopsis* lines that show

resistance to growth inhibition under low phosphorous - *lpr1-1*, *lpr1-2* and *lpr1-3* - have been found to be allelic to BIG, a very large calossin-like protein required for normal polar auxin transport (Gil, Dewey *et al*. 2001, Lopez-Bucio, Hernandez-Abreu *et al*. 2005). In addition, a SUMO E3 LIGASE, SIZ1 (SCAFFOLD ATTACHMENT FACTOR ACINUS PROTEIN INHIBITOR OF ACTIVATED SIGNAL TRANSDUCER AND ACTIVATOR OF TRANSCRIPTION/MSX2-INTERACTING ZINC FINGER 1) has been suggested to potentially regulate AUXIN RESPONSE FACTOR 7 (ARF7) through its sumoylation motif (Overvoorde, Okushima *et al*. 2005), and indeed *siz1* mutants are hypersensitive to phosphate starvation in primary and lateral root phenotypes (Miura, Rus *et al*. 2005); however, this link has not been functionally demonstrated. *Arabidopsis* mutants for POLYNUCLEOTIDE PHOSPHORYLASE (PNPase) also exhibit decreased auxin responsiveness and reduced cell division with highly branched lateral roots when grown on low phosphate media (Marchive, Yehudai-Resheff *et al*. 2009) and mutants constitutively express phosphate responsive genes, as do mutants for PHOSPHATE DEFICIENCY RESPONSE 2 (PDR2), a P5-type ATPase which maintains a transcription factor SCARECROW (SCR) which is known to act in concert with auxin and SHORTROOT in radial root patterning (Ticconi, Delatorre *et al*. 2004). Conversely, cytokinin, (which is known to play an antagonistic role to auxin in various processes) represses many genes involved in the phosphate starvation response (Franco-Zorrilla, Martin *et al*. 2005).

Though the exact molecular components and processes involved in phosphate response are unknown, it seems likely that as with nitrate sensing and response the mechanisms governing phosphate sensing and response are complex and multi-factorial, probably requiring the interaction of several phytohormones, signalling cascades and the initiation and regulation of complex developmental programs controlled by an array of genes.

METHODS

The methods used in this thesis are detailed below in alphabetical order.

All chemicals/reagents are sourced from Sigma-Aldrich (Sigma-Aldrich, St Louis, Missouri USA) unless otherwise indicated.

C

Clearing Protocol (for root tissue)

Place seedlings sequentially in approximately 2 ml each of:

1. Acidified Methanol (20% MeOH, 4% HCL, 76% H_20) for 15 minutes
2. Remove seedlings, then place in 60% ethanol/7% NaOH solution for 15 minutes
3. 40% ethanol – 5 minutes
4. 20% ethanol – 5 minutes
5. 10% ethanol- 5 minutes
6. Sterile distilled water (for storage prior to mounting on slides).

Methanol and Ethanol sourced from ThermoFisher (ThermoFisher, Waltham, Massachusetts, USA).

Confocal microscopy (standard settings used for experiments, unless otherwise stated)

Leica SP5 confocal microscope (Leica Microsystems, Milton Keynes, UK): settings for observing YFP/GFP reporters (e.g. DR5:VENUS, DII:VENUS): 30% power, 514 nm detector using gain value 100%, offset value 28.98. Averaged over 4 frames.

Nikon eclipse Ti-U confocal microscope (Nikon Instruments, Amsterdam, Netherlands): settings for observing YFP/GFP reporters (e.g. DR5:VENUS, DII:VENUS): Full power, 515/30 detector using gain value 110, offset value 127. Averaged over 4 frames. Brightfield gain value 32.

F

Fluorescence quantification (using FIJI)

1. Open Fiji and drag and drop the .ids file from the microscope onto the Fiji toolbar.

2. The ‘memory management’ window should open. Ensure that the drop-down menu is on ‘view stack with hyperstack’ and the ‘use virtual stack’ and ‘split channels’ options are selected.
3. Select OK.
4. 4 windows should come up corresponding to the 4 channels (blue, red, green and transmitted light). Check the transmitted channel for movement/abnormalities etc. by moving the slider at the bottom of the window to scroll through each frame. If satisfied, can close all other windows except the fluorescence window you need.
5. The fluorescent window will be in black and white. If you want, you can add colour: go to menu: image> lookup tables> yellow (or whatever colour you want.)
6. To remove background fluorescence: go to: Image> adjust > threshold
7. A histogram will appear with two sliders below it. Move the bottom slider all the way to the right (the screen will turn red). Then gradually adjust the top slider until what you want to see is clearly defined with minimal background.
8. Press ‘SET’ then on the other window which appears, ‘OK’.
9. To quantify pixels: go to: Analyze> set measurements> make sure ‘integrated density’ and ‘limit to threshold’ options are selected > click OK
10. Click back on the image window to select it, then press ctrl+M
11. This will only give you the measurement for the first frame. To apply it to all frames, you need to go to: Image> stacks> plot z-axis

profile (select ‘no’ when it asks if you want to save 1 measurement(s))

12. The data will each file will be compiled for you (takes a couple of minutes). When it has finished running, press ctrl+a to select all, ctrl+c to copy it, and then you can paste it the data into excel or notepad. (Ignore the graph which appears as the way these are compiled can be quite misleading in terms of axis etc).
13. The raw integrated density is the main thing we are interested in: this is the fluorescence value of the sum of the pixels selected, divided by area.

To generate a more accurate scatter graph, paste the data into an excel spreadsheet, starting with cell A2 (so your first cell of data should be A2). You can then:

1. Get the time in minutes by going to cell H2 and multiplying the number in A2 (which should be 1) by the number of minutes you took your measurements at, then subtracting that same number. For example, if your time course measured with a 2 minute fixed delay (took measurements every 2 mins) the calculation would be A2*2-2
2. For the first cell this should give 0 as this is your start timepoint (select and mouse over the corner of the cell until a plus sign appears, double click the cell using this plus sign and it will automatically repeat the concentration for the whole column)

3. You can do the same to calculate hours by typing this into I2: =H2/60. Apply to the entire column as above.
4. Type this into cell J2: =G2/G2. This will give measurements of fluorescence over time.
5. Select column I. Then press ctrl and select column J. Select 'insert' from the menu, then 'insert scatter graph' (recommended to select the third option it gives you)
6. Your graph will be generated.

G

General growth conditions:

Growth room conditions (seedlings up to 15 DAG):

Unless otherwise stated, all seedlings grown vertically from seed on sugar-free ½ MS media (MS salts 2.15 g/L, Myo-inositol 0.1 g/L, MES 0.5 g/L, plant tissue culture agar 10 g/L, pH adjusted to 5.7) in 24 h light at 21°C. Exogenous treatments added to warm liquid media before pouring plates unless otherwise stated (see 'Media Treatments (exogenous application).

For rosette area quantification and leaf series analysis (Chapter 2), plants were grown horizontally on square Petri plates (12cm × 12cm, Greiner Labortechnik) containing 50ml solid half-strength MS growth medium (per

litre: 2.15g MS salts, 0.1g *myo*-inositol, 0.5g MES, 10g plant tissue culture agar; pH adjusted to 5.7 with KOH) in a growth room at 22 °C under continuous light. Measurements were taken at 18 d after germination.

Glasshouse growth conditions:

For general mature plant growth, seed bulking and analyses of aboveground parts (Chapter 2), plants were grown on Levington M3 compost (Everris, Ipswich UK) in a glasshouse at 20–22 °C under long-day conditions (16/8 light/dark). Measurements were taken after 3 weeks of growth, immediately post-bolt.

Genevestigator analysis

For environmental and hormonal effects on CEP genes, the transcriptome meta-analysis tool Genevestigator (Hruz *et al.*, 2008) was used, with a significance level of <0.05. For cell, tissue, and organ *CEP* expression data, the eFP browser (http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi) was used with standard settings.

GUS Staining

Making X-gluc staining solution from scratch:

1. Make a monobasic buffer stock: 0.2M NaH_2PO_4→ H_2O (7.8 g / 250 ml water)
2. Make a dibasic buffer stock: 0.2M Na_2HPO_4→ $2H_2O$ (8.9g /250ml water)

Use these stocks to make a 0.1M sodium phosphate buffer stock (600 ml):

1. 117 ml monobasic stock
2. 183 ml dibasic stock
3. 300 ml H_2O

You will also need to make these stocks:

1. 10 mg/ml X-gluc (5-bromo-4-chloro—indoyl-β-D-glucoromide cyclohexylammonium salt) dissolved in DMF (N,N-Dimethyl-formamide).
2. 33 mg/ml $K_3Fe(CN)_6$ dissolved in 0.05 M sodium phosphate buffer
3. 44 mg/ml $K_4Fe(CN)_6$ dissolved in 0.05 M sodium phosphate buffer

To make 5ml X-gluc staining solution:

Add the below IN THIS ORDER:

1. 4.245 ml 0.05M sodium phosphate buffer
2. 250 µl of your $K_3Fe(CN)_6$ stock

3. 250 µl of your $K_4Fe(CN)_6$ stock
4. 250 µl of your X-gluc stock
5. 5 µl triton-100

Place your seedlings in enough X-gluc to cover them over, and place them in a 37°C incubator for staining.

Check the progression of the staining over time (recommend every 30 mins) using a microscope.

Once the seedlings have stained a clearly visible blue, stop the reaction by removing the seedlings from the X-gluc and placing them in 70% ethanol.

Mount and photograph the seedlings if required.

M

Media treatments (exogenous application)

Standard concentrations for media treatments were: 5 µM for CEP5 and CEP1 (all variants including $CEP5^{Hyp}$, $CEP5^{Pro}$, $mCEP5^{Hyp}$ etc. and all alanine scanning species), 1 µM for auxins (IAA/NAA), 15 µM for Bikinin, and 50 µM for GA, unless otherwise stated.

'Mock' treatments or 'MS' refer to ½ MS media (see 'growth conditions')

R

RNA extraction and cDNA Preparation

(RNA extraction (RNeasy) and cDNA (SuperScript II) prep protocol)

Keep samples on ice throughout.

RNA extractions:

1. Grind tissue into a fine powder using liquid nitrogen. Maximum of 100 mg tissue per extraction.

2. Add DTT to the RLT buffer to a working concentration of 40 µM (e.g. 40 µl 1M DTT per ml). Allow ~0.5ml of RLT buffer per sample.

3. Add 450 µl buffer RLT to a maximum of 100mg ground tissue in an eppendorf and vortex thoroughly.

4. Transfer lysate to shredder column (lilac). Centrifuge 2 min at full speed.

5. Transfer the flow-through to a fresh tube: do not disturb the pellet

6. Add 100% ethanol at 0.5 volume of the lysate (usually flow through is approx. 400 µl, so add 200 µl ethanol for example). Mix well by pipetting.

7. Transfer the sample to the spin column (pink) and centrifuge at 10,000 rpm (or 8000 x g) for 15 seconds. Discard the flow through from the collection tube.

8. Add 700ml of buffer RW1 to the column and centrifuge at 10,000 rpm (or 8000 x g) for 15 seconds. Discard the flow-through.

9. Add 500 ml of buffer RPE (ensure ethanol has been added to the RPE stock as per pack instructions) to the column and centrifuge at 10,000 rpm (or 8000 x g) for 15 seconds. Discard the flow-through.

10. Add 500 ml of buffer RPE to the column and centrifuge at 10,000 rpm (or 8000 x g) for 2 min. Discard the flow-through.

11. Place the spin column in a new collection tube. Add 30-50 µl RNase-free water to the spin column membrane. Centrifuge at 10,000 rpm (or 8000 x g) for 1 min.

12. Test the concentration of the eluted RNA on the nanodrop (make sure RNA option is selected). Ideally extractions should produce a strong defined peak and a concentration of >400 ng/ µl RNA, though it is possible to work with less (but bear in mind the Nanodrop cannot tell you if your samples are degraded. This website describes good methods of quality checking your RNA: http://www.lifetechnologies.com/uk/en/home/references/ambion-tech-support/rna-isolation/tech-notes/is-your-rna-intact.html).

13. Store any extracted RNA at -80°C.

cDNA preparation:

1. A 20 µl reaction volume is used. Ideally, 500 ng of RNA per reaction should be used, but can use less (e.g. 250 ng). To standardise your samples to 500 ng, divide 500 by the concentration of your sample. For example, if your extraction concentration is 475 ng/µl:

500/475 = 1.05, so add 1 µl of RNA sample to the reaction.

If your extraction was only 250 ng/µl, you'd need to add 2 µl of your sample, and so on.

2. Add the following to a centrifuge tube:

- 1 µl Oligo(dT)12-18
- X µl RNA sample (x = whatever volume you worked out using the sum above)
- 1 µl dNTP Mix (10 mM each NTP)
- Add sterile distilled water to a volume of 12 µl.

3. Heat mixture to 65°C for 5 min, then quick chill on ice. Collect contents of tube by brief centrifugation.

4. Add 4 µl 5X First-strand buffer and 2 µl of the 0.1 M DTT (provided in kit).

5. Mix contents of tube gently and incubate at 42°C for 2 min.

6. Add 1 µl SuperScript II RT and mix by pipetting.

7. Incubate at 42°C for 50 min.

8. Inactivate the reaction by heating at 70°C for 15 min.

9. Can test concentration using the nanodrop, as for RNA (but ensure DNA option is selected).

10. Store cDNA samples at -80°C.

W

Western Blot (Tris-Tricine Schagger gel protocol)

-This is an expanded protocol including the electrophoresis, transfer and ECL protocol used for all western blots contained in this thesis, together with tips and recommendations for others who wish to use this protocol. Electrophoresis protocol adapted from (Schagger 2006). Tris-Tricine Schagger gels are especially recommended for small molecular weight proteins (e.g. CEP5), high proline content proteins (e.g. CEP5) and also recalcitrant membrane proteins.

Buffers and solutions required:

1. Protein extraction buffer: 12% SDS (wt/vol), 30% glycerol (wt/vol), 0.05% Coomassie blue, 150 mM Tris/HCl (pH 7.0)
2. 3 X gel buffer stock: 3 M Tris, 1M Hcl, 0.3% SDS (pH 8.45)
3. 10 X Anode buffer stock: 1 M Tris, 0.225 M HCl (pH 8.9), dilute to 1 X before use.
4. 10 X Cathode buffer stock: 1 M Tris, 1 M tricine, 1 % SDS (pH ~8.25, no need to correct), dilute to 1 X before use.
5. Electrode buffer for semi-dry transfer (prepare fresh each time): 300 mM Tris, 100 mM acetic acid (pH 8.6)
6. PBS (plus PBST: 500 ml 1 X PBS + 0.5 ml Tween-20)
7. TBS (plus TBST: 100 ml 1M Tris pH 7.5, 500 ml NaCl, 400 ml SDW, 1 ml Tween 20)
8. Blocking solution: 5% (for primary incubation) or 1 % (for secondary incubation) skimmed milk powder in TBST or PBST buffer.

1. *Resolving gel preparation:*

For a 16% gel:

For approx 10 ml (enough for two small protein gels, one for transfer and one for Coomassie staining):

- 3.3 ml 40% acrylamide solution, 3.3 ml gel buffer (add as 3 X stock, do not dilute first): add distilled water to 10 ml final volume. Polymerise by adding 33 µl APS 10% and 3.5 µl TEMED.

Pour the gel until the meniscus is approx. 1–1.5cm below the top edge of the plate and overlay it immediately with either sterile distilled water (SDW) or butanol to ensure flat interface between stacking and resolving gel.

- After ~30 mins the gel should be set (can check by seeing if the excess left in the falcon tube has set yet). Pour off the SDW wick up excess with tissue at corner of plate. The stacking gel can then be poured.

2. *Stacking gel preparation:*

Use a 4% gel. For approx 4 ml (enough for 2 gels):

- 333 µl 40% acrylamide solution, 1 ml gel buffer (add as 3 X stock, do not dilute first): add distilled water to 4 ml final volume. Polymerise by adding 30 µl APS 10% and 3 µl TEMED. Pour the gel and immediately add the comb.

- After ~30 mins the gel should be set (check by seeing if the excess left in the falcon tube has set yet).

-The gel can be wrapped in clingfilm (leave the plates and comb in place) and stored in the fridge for a 1-2 days if required.

3. *Protein extraction:*

- Grind fresh plant material in ~100 µL extraction buffer (adjust depending on volume of your sample), either in liquid nitrogen using a pestle and mortar (preferred) or over ice in an eppendorf if sample is <0.5 g.

  -(Can freeze sample solutions at this point if required).

- Treat the samples you will be running with DTT to a final concentration of 0.2M

  -Only need to treat ~20 µl of sample, will be loading approximately 10-15 µl on the gel - e.g. 4 µl 1 M DTT + 16 µl of your sample)

- Vortex and heat samples for 10 min at 50°C.

4. *Electrophoresis set-up:*

- Set up the electrophoresis tank as normal.
- Dilute your ANODE and CATHODE buffers to 1 X

- The 1 X CATHODE buffer needs to be added to the space in-between the glass plates, e.g. sandwiched between the interior sides of the plates to form a centrally enclosed pool.
- The rest of the tank (e.g. on the outside of the glass plates) should be filled with ANODE buffer.
- Warm your chosen ladder (putting it in a 37°C incubator for about about 5-10 mins before you use it is usually sufficient, although some ultralow molecular weight markers require higher temperatures: check manufacturers datasheet).
- Load ~10 µl of your warmed samples in order.

5. *Running the electrophoresis:*

- Start electrophoresis at 30 V. Maintain this voltage until the sample has completely entered the stacking gel.
- Once the sample has entered the gel, increase the voltage (100 V initially, can use up to a maximum of 300 V. Overnight running not recommended).
- After the run is complete, the transfer gel must be immediately prepared for transfer.

- Place the Coomassie gel in a container and stained with Coomassie blue (0.025% Coomassie blue dye in 10% acetic acid) on a shaker to check protein loading uniformity. After a minimum of 2-5 h (but can be left for days if necessary) destain the Coomassie gel twice in 10% acetic acid for 15-60 minutes a time on a shaker.

6. *Semi-dry transfer:*

Use semi-dry system (BioRad Trans-Blot SD).

-At no point touch the membrane with bare hands; use tongs if possible, or gloves if you have to, and hold the corners/edges of the membrane outside the transfer area to avoid ruining the blot.

- Soak filter paper (x 6 for each gel) in electrode buffer for at least 5 minutes. Soak the membrane (Amersham Hybond-P, GE Healthcare, Amersham UK) in methanol for ~30 seconds, rinse with distilled water, and soak in electrode buffer for at least 5 minutes.
- Equilibrate your gel in transfer buffer for 5-15 minutes to remove salts and detergents.

Transfer:

- Remove safety cover and stainless steel cathode assembly
- Place three sheets of pre-soaked filter paper on the platinum anode, and roll a pipette over the surface to remove any air bubbles

- Place the pre-soaked nitrocellulose membrane on the filter paper
- Place the equilibrated gel on the membrane, aligning the gel so it is in the centre of the membrane. Transfer will be incomplete if any of the gel is outside the membrane area.
- Complete the transfer sandwich by placing three sheets of pre-soaked filter paper on the gel, and roll a pipette over the surface to remove any air bubbles
- Remove any excess transfer buffer from the platinum anode using blue towel to ensure current flows correctly. The sandwich should be damp, but not soaking.
- Carefully place the cathode onto the stack and then put the safety cover on
- Transfer at 15 V for 1-2 hours (can check for transfer by carefully checking the membrane to see if the ladder has been transferred onto the membrane - do not disturb the position of the membrane relative to the gel.
- If the ladder has not fully transferred, very carefully replace the membrane onto the gel, and run the electroblot for longer. Also re-wet the sandwich with electroblot buffer if it seems too dried out.
- The membrane can be left in electroblotting buffer at 4°C for storage if necessary.

7. Immunodetection

- Rinse membrane in 1X PBS-T and block with 5% milk power (Sigma) in 1X PBS-T for 1-2 h.
- Remove blocking solution, add primary antibodies (1:2000 is the standard concentration used in this thesis, but can increase or decrease according to specificity and sensitivity) in 1% milk powder 1X PBS-T and incubate with gentle shaking overnight at 4°C.
- Wash membrane 3X for 5 min each with 1X PBS-T.
- Add secondary antibodies (1:20,000 is the standard concentration used in this thesis, but can be increased or decrease depending on sensitivity and background) in 1% milk powder 1X PBS-T and incubate with gentle shaking for 1 h (do not leave for longer as this increases risk of excessive background).
- Wash membrane 3X for 5 min each with 1X PBS-T.
- Briefly wash membrane 3X with 1X PBS (to remove tween which can cause interference with ECL detection).

8. Enhanced Chemiluminescence (ECL)

GE Healthcare ECL plus detection kit (GE Healthcare, Amersham, UK).

Do this part in the dark room under red light.

- Mix an equal volume of detection solution 1 with detection solution 2: 1 ml for each is sufficient for one membrane.
- Drain the excess wash buffer from the washed membrane and place them, protein side up, on clingfilm. Pipette the mixed detection reagent over the membrane (make sure entire transfer area is covered, can agitate to make liquid spread out over surface).
- Incubate for 1 minute at room temperature
- Drain off excess detection reagent by holding the membrane gently and touching the edge against a tissue. Place the blots protein side down onto a fresh piece of (as flat and crease free as possible) clingfilm, wrap up the blots and gently smooth any air bubbles.
- Place the protected membrane in a film cassette with protein side facing up. Turn off all lights except those appropriate for film exposure (red).
- AVOID ALL OTHER LIGHT FROM THIS POINT ON.
- Once all lights are out open the film box and cut a piece of film to fit your membrane (usually one piece of film in cut in half is sufficient). Immediately replace the remaining stack of film in the foil bag to avoid accidental exposure.
- Carefully place a piece of film on top of the membrane, and immediately close the cassette to allow the film to develop. A

recommended first exposure is 60 seconds – optimise to produce good bands (reduce time if background is high or bands are too heavy).

- Place film in a tray of developing solution until bands are visible then wash with water to remove all developing solution
- Place film in fixing solution until all film dark in colour (fixed) then wash with water to remove all fixing solution and then hang to dry.
- Your membrane can be stripped and reprobed if necessary. If not, then wrap it in clingfilm and place in a sealable bag or other appropriate container and leave in the fridge for future reference (e.g. to check size of anything detected against ladder etc).

Q

qPCR protocol

qPCR Master mix (per sample):

- 2 x SYBR Green 6 µl
- Primer Forward 0.1 µl
- Primer Reverse 0.1 µl
- Sterile Distilled Water 0.8 µl (to 7 µl)

1 in 100 diluted cDNA: 4 µl. Total reaction volume 11 µl.

For CEP5 and DII:VENUS expression level analysis:

Arabidopsis RNA was isolated from 20 pooled seedlings (CEP5) or 5-10 F1 seedlings (DII:VENUS) at 7 d after germination using a Plant RNeasy Kit (Qiagen, Germany) according to the manufacturer's instructions. cDNA was subsequently prepared from a minimum of 250ng RNA (determined by UV spectrophotometry) using a SuperScript II reverse transcriptase kit and Oligo(dT)12–18 primers (Invitrogen, USA), according to the manufacturer's instructions. Quantitative real-time PCR (qRT-PCR) was performed in a 384-well white dish format using a LightCycler 480 (Roche Applied Science, USA) with 40 PCR amplification cycles using SYBR Green I fluorescent dye (Quanta Biosciences, USA) and primers (see below). Expression was determined from a minimum of three biological replicates, each with a minimum of four technical repeats, and normalized against ACTIN.

qPCR Primers used:

CEP5:

Forward: 5′-CCATGGACGAACCCTAAAAG-3′

Reverse: 5′-TGCCATCATCGTCTTGCTAT-3′

DII:VENUS (N7 nuclear localisation sequence):

Forward: 5′-GGACTCTGAGGATGGAAACG-3′

Reverse: 5′-TCAGCTTCTGTGTCGTCAATG-3′

ACTIN (reference gene):

Forward: 5′-CTGGA GGTTTTGAGGCTGGTAT-3′

Reverse: 5′-CCAAGGGTGAAAGCAA GAAGA-3′

Chapter 2: Below- and aboveground phenotypical characterisation of the CEP5 peptide.

Parts of this chapter are taken from work performed by the author in Roberts *et al.* 2012.

2.1 Introduction

In *Arabidopsis*, more than 1000 putative small signalling peptides have been predicted, but very few have been phenotypically and/or functionally characterised. In the last 20 years, the importance of small signalling peptides in plant cell-to-cell communication has become increasingly clear, with several families of small signalling peptides known at present to play vital roles in plant growth and development (Butenko, Vie et al. 2009, Matsubayashi 2011, Murphy, Smith et al. 2012).

The majority of small signalling peptides fall into one of two broad groups: the cysteine-rich peptides, which are characterized by a typical mature peptide length of <160 amino acids with a cysteine-rich C-terminal domain; and the small post-translationally modified peptides, which are expressed as longer precursor proteins before undergoing post-translational modifications and subsequent cleavage to form an active, mature peptide <20 amino acids in length (Matsubayashi 2011, Murphy, Smith et al. 2012). Three types of post-

translational modification—tyrosine sulfation, proline hydroxylation, and hydroxyproline arabinosylation—are known to occur in small post-translationally modified peptides, and these modifications appear to be crucial for optimal peptide bioactivity (Matsubayashi, 2011; Shinohara and Matsubayashi, 2013). In CEP peptides, previous mass spectrometry on CEP1 suggests that the prolines contained within the active sequence of the C-terminal motif represent important sites of post-translational modification (namely proline hydroxylation) that are crucial for optimum bioactivity of the peptide (Ohyama, Ogawa et al. 2008).

From the C-TERMINALLY ENCODED PEPTIDE (CEP) family, only one member (CEP1) had previously been shown to be involved in regulating root architecture and development. Specifically, when CEP1 was overexpressed under a constitutive CaMV 35S promoter, *Arabidopsis* seedlings exhibited reduced primary root growth and lateral root elongation (Ohyama, Ogawa et al. 2008). However, it remained unclear if similar/contrasting phenotypical effects would be observed when the levels of other members of the CEP family were perturbed, since different CEP peptides have slightly different sequences and expression patterns. This chapter further explores the role of the CEP1-related peptide CEP5 in primary and lateral root phenotypes, with additional characterisation of potentially related aboveground effects.

2.2 RESULTS

2.2.1 CEP5 overexpression reduces primary root length

It was previously shown that CEP1 overexpression results in a stunted primary root length; microscopic analyses have suggested this is due to a reduced number of cells in the meristematic zone, and reduced cell size in the mature region, without any defect in the quiescent centre (QC) region (Ohyama, Ogawa et al. 2008). Thus, CEP1 appears to affect cell division potential in the primary root. An *Arabidopsis* line with CaMV 35S promoter-driven constitutive overexpression of the related peptide, CEP5, was generated to investigate if this would give rise to a similar phenotype of shorter primary root (Fig. 9) (Roberts, Smith et al. 2013).

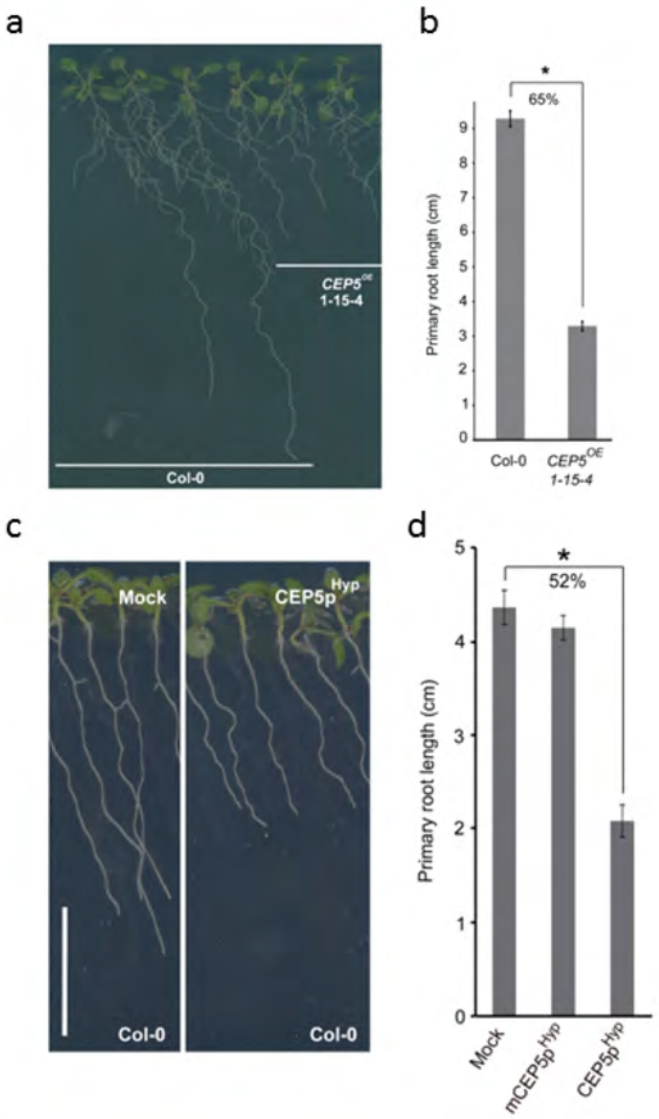

Fig 9. Increasing CEP5 abundance through overexpression or exogenous application of synthetic peptide leads to a reduction in primary root length. (a) Representative picture of a CEP5OE line at 12 days after germination compared with wild type background (Col-0) (b) quantification of primary root length at 12 days after germination. (c) Representative picture of Col-0 *Arabidopsis* seedlings grown on 1 µM CEP5p^{Hyp} for 9 days after germination (g) and quantification of primary root length of Col-0 seedlings treated with 5 µM mCEP5p^{Hyp} compared with mock treatment (1/2 MS) and 5 µM CEP5p^{Hyp} at 12 days after germination ($n = \geq$ 15 seedlings per condition). The % reduction in primary root length is indicated. *, $p = < 0.05$ according to Student's t-test compared to mock. Error bars are ± 1 S.E.

Thus, in agreement with previous observations in CEP1 overexpression lines, CEP5 overexpression led to a clear, dramatic shortening of the primary root at 12 DAG (Fig. 9) (CEP5 expression levels in the CEP5^{OE} 1-15-4 line shown were approximately 30 times more than that of the wild-type background (Col-0) according to qPCR analysis; Ianto Roberts, personal

communication). Other independent CEP5^{OE} lines gave similar phenotypes. However, lines with reduced abundance of CEP5 due to RNAi (35S::CEP5^{RNAi}) gave similar primary root lengths to wild-type background controls (Fig. 10). This may be due to the relatively modest reduction in CEP5 level afforded by RNAi: qPCR experiments suggest expression of CEP5 in 35S::CEP5^{RNAi} is reduced by approximately 40-50% (Fig. 10). Unfortunately, T-DNA insertion lines conferring complete CEP5 knockout were not available for CEP5 due to the small size of the encoding gene.

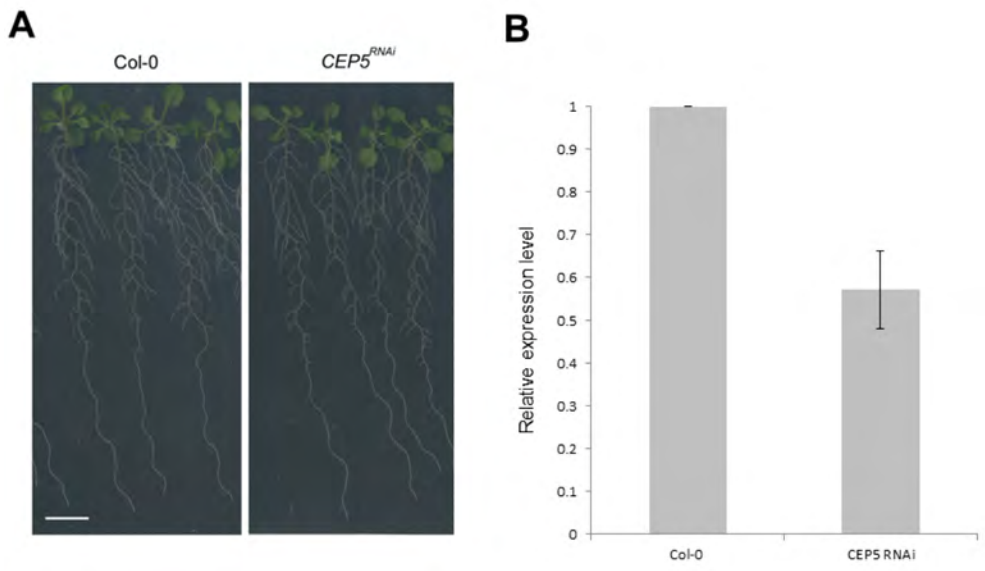

Figure 10. A knockdown line of CEP5, 35S::CEP5^{RNAi}, demonstrates similar primary root lengths to wild type. Comparative analysis of >100 Col-0 and 35S::CEP5^{RNAi} seedlings showed no significant difference in primary root lengths at various stages of development including ~7DAG (A). qPCR analysis of the 35S::CEP5^{RNAi} line showed CEP5 expression is reduced by approximately 40-50%. $N = \geq 4$ biological and 4 technical repeats per condition for the qPCR analysis. Error bars are ± 1 S.E.

2.2.2 Exogenous application of synthetic CEP5 peptide reduces primary root length and can be used as an assay for bioactivity of peptides

Exogenous application of synthetic peptides has been shown for other small signalling peptides to elicit similar phenotypes to peptide overexpression, and thus has previously been used as a straightforward assay to test the activity of small post- translationally modified peptides (Fiers, Golemiec et al. 2005, Ohyama, Ogawa et al. 2008, Kinoshita, Betsuyaku et al. 2010, Delay, Imin et al. 2013, Tabata, Sumida et al. 2014, Czyzewicz, Wildhagen et al. 2015). Indeed, addition of synthetic peptide to media has certain advantages compared with overexpression in that concentration curves can be utilised to observe a phenotypic dose response for the peptide, and thus the bioactivity of different peptide varieties/concentrations can be more easily compared. In addition, seedlings can be transferred from non-treated to peptide-treated media, with similar advantages to that of induced expression lines. Exogenous application of the native but unmodified sequence of CEP5 as a synthetic peptide elicits similar primary root shortening as CEP5 overexpression: thus, primary root length has been used throughout this work as a bioassay to measure bioactivity of differently modified, mutated, and different concentrations of CEP5 on *Arabidopsis thaliana* seedlings.

One criticism of the primary root length bioassay is that it may be not be specific for the action of an individual peptide. To ensure that the primary root length changes observed were not due to a nonspecific stress effect from the simple presence of peptides in the media, seedlings were grown in the presence of CEP5p^{Hyp} , untreated control, or in the presence of a synthetic peptide CEP5 variant with 4 randomly positioned, but not very unlikely amino acid substitutions based on a BLOSUM62 substitution matrix within the 15 amino acid CEP5 peptide sequence while retaining the Hyp residues at the

same positions (mCEP5p^{Hyp}) (Fig. 11) Seedlings grown on mCEP5p^{Hyp} do not replicate the root stunting seen for CEP5p^{Hyp}, thus the primary root shortening effect is not simply due to stress from the presence of peptides in the media (Fig. 11).

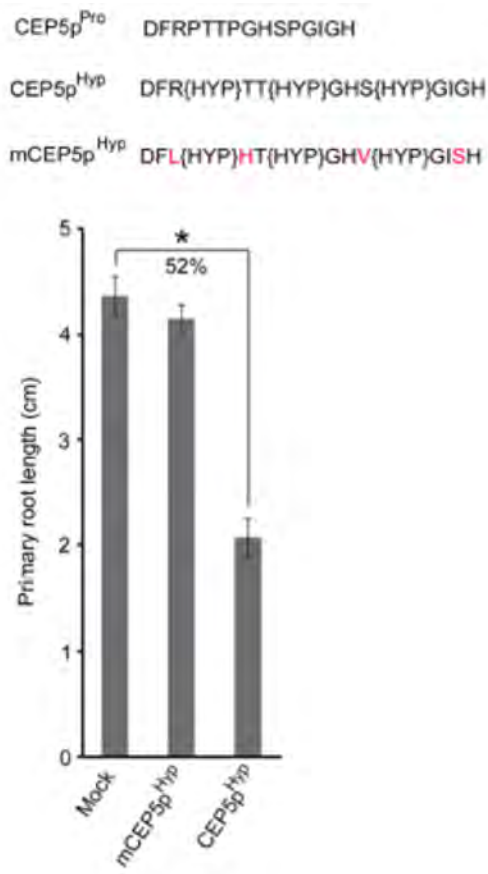

Figure 11. The primary root shortening activity of CEP5 requires the active sequence of CEP5. The active sequence of CEP5 is a 15 amino acid motif (CEP5p^{Pro}) which is hydroxylated (HYP) on each proline residue to form the mature, most bioactive form of the peptide (CEP5p^{Hyp}). Exogenous application of this peptide (via supplemented media) was sufficient to shorten primary root length by 52% in Col-0 seedlings at approximately 7 DAG (p = <0.001). A mutated version of the CEP5 sequence, with four amino acid substitutions rated -1 (evolutionarily unlikely but not highly so) according to the BLOSUM62 amino acid substitution matrix (but with conserved hydroxyproline positions (mCEP5p^{Hyp})) was not able to induce the same primary root length shortening effect. Thus, the primary root length shortening shown in the exogenous CEP5 peptide bioassay is not merely due to a non-specific stress effect from the presence of peptides in the media. $N = \geq 10$ seedlings per condition.

2.2.3 Modification of CEP5 affects bioactivity of the peptide in primary root length assays

Previously gathered mass spectrometry data showed the mature form of CEP5 is a conserved C-terminal CEP domain containing three proline (Pro) residues that gives rise to a small peptide following proteolytic cleavage from a precursor (CEP5p) (Roberts, Smith et al. 2013, Tabata, Sumida et al. 2014). However, small signalling peptides are often post-translationally modified, thereby modulating the binding ability and specificity of peptides to their targets (Matsubayashi 2014). In this context, it was previously shown that members of the CEP family give rise to a peptide containing hydroxyproline (Hyp) residues (Ohyama, Ogawa et al. 2008, Tabata, Sumida et al. 2014, Mohd-Radzman, Binos et al. 2015). To assess the activity of chemically synthesised $CEP5p^{Hyp}$, the primary root growth bioassay was used. To test the bioactivity of differentially modified CEP5 peptides in respect to proline hydroxylation, six synthetic peptides were made: one corresponding to the full length unmodified CEP5 sequence; three peptides which carried a single hydroxylation on the different proline residues respectively; one peptide with the first and third proline residues hydroxylated, and one triply proline hydroxylated variant. Arabidopsis seedlings (ecotype Col-0) were grown on various concentrations (1 nM–10 μM) of the synthetic CEP5 peptide variants for 9 days after germination.

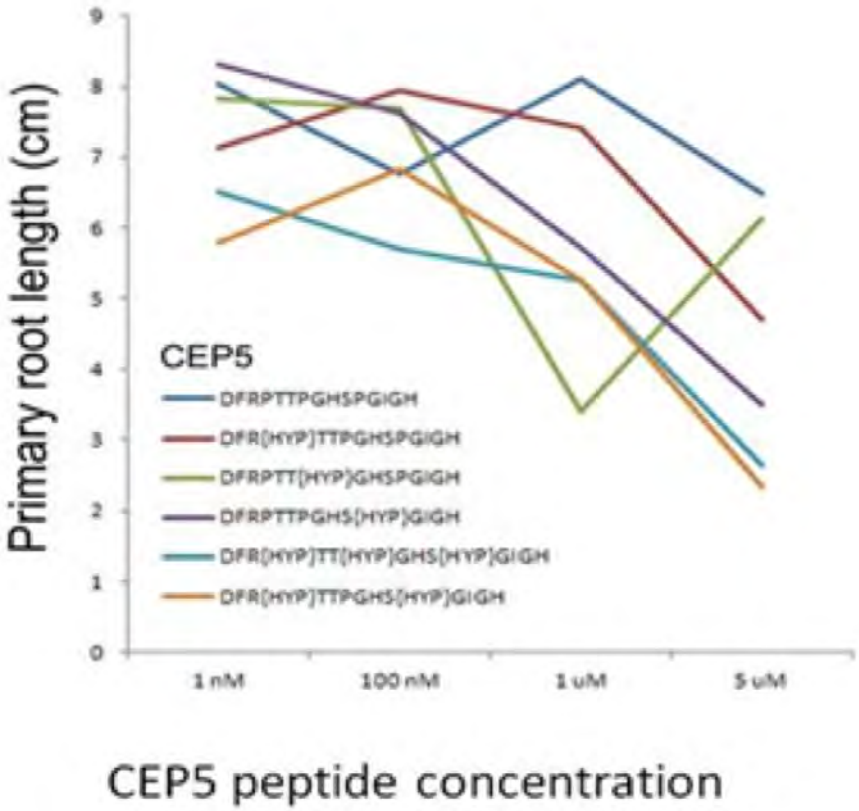

Figure 12. The effect of differentially modified variants of CEP5 on primary root length. Exogenous application of synthetic, differentially modified CEP5 peptide variants over a range of concentrations have differing bioactivities in a primary root length bioassay. Unmodified CEP5 sequence; teal, first proline hydroxylated; burgundy, second proline hydroxylated; green, third proline hydroxylated; purple, all three prolines hydroxylated; cyan, first and third prolines hydroxylated; orange). Triply hydroxyprolinated CEP5 was previously predicted by mass spectrometry to be the bioactive form and in this assay leads to the most pronounced reduction in primary root length, with effects visible within the low-nanomolar range typical for peptide signals. $N = \geq 10$ seedlings per condition. Seedlings ~11 DAG.

The greatest bioactivity in terms of primary root length shortening was observed for CEP5 that had a hydroxyproline modification on at least proline 4 and proline 11, with the strongest bioactivity (reduction of primary root length at lower peptide concentration) observed for CEP5 hydroxylated on all three proline residues (P4, P7 and P11) (orange line and teal line, Fig. 12). Overall, this is in agreement with observations on several other small signalling peptides where bioactivity is increased upon hydroxyprolination (Kondo, Yokomine et al. 2011, Shinohara and Matsubayashi 2013, Butenko, Wildhagen et al. 2014, Czyzewicz, Wildhagen et al. 2015, Mohd-Radzman, Binos et al. 2015).

Next, to gain further insight in the structure-activity relationship within the CEP5p sequence, synthetic peptides where each residue was replaced by alanine (Ala) were tested using the primary root length bioassay (Fig. 13). This revealed that interfering with individual CEP5 residues reduces or abolishes activity in most cases (Fig. 13). In two cases (CEP5p^{Hyp}(Thr6Ala) and CEP5p^{Hyp}(His9Ala)) the synthetic CEP5p variants retained activity and in one case (CEP5p^{Hyp}(Hyp11Ala)) we observed increased activity in our primary root growth assay (Fig. 13). This analysis pinpointed that both the Hyp residues but also several other residues within the CEP5 peptide sequence are important for CEP5p activity.

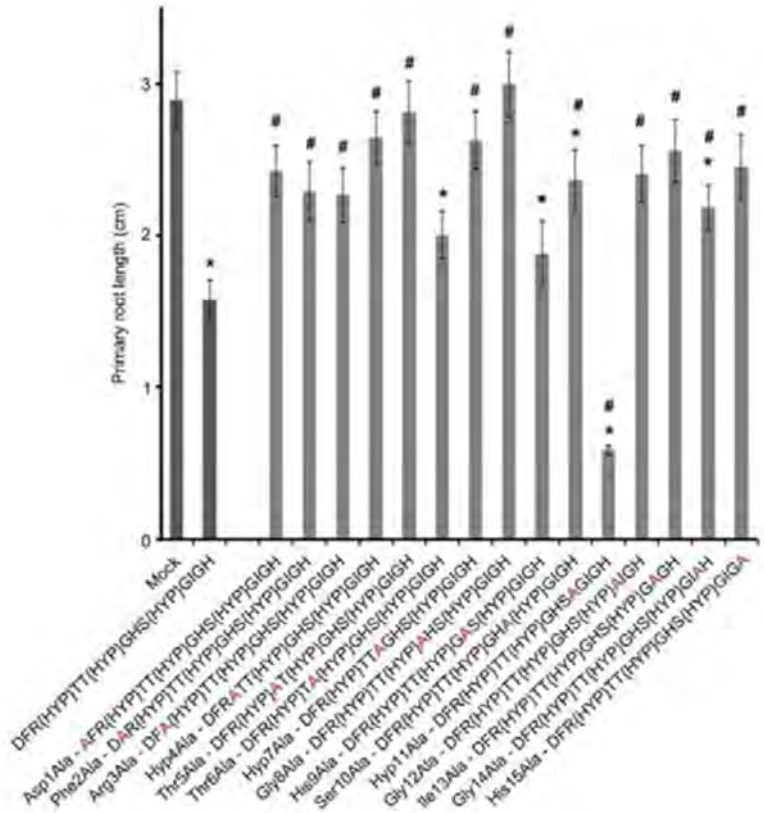

Figure 13. Alanine scanning interrogation of the CEP5 motif sequence. Sequential mutation of each residue in the CEP5 active motif sequence was used to test the contribution of individual CEP5 amino acid residues to overall CEP5 synthetic peptide bioactivity via the primary root length bioassay method. Residues more important to bioactivity result in longer primary root lengths as the bioactivity of CEP5 is reduced or abolished. Unexpectedly, Hyp11>Ala substitution increased the bioactivity of the synthetic peptide species of CEP5. For all, 5 µM synthetic CEP5 peptide variant(alanine scanning) were used and roots analysed at 15 days after germination ($n \geq 6$). *, $p < 0.05$ according to Student's t-test. In all cases, mock refers to medium with water as used to dissolve CEP5p. Error bars are ± 1 S.E.

2.2.4 CEP5 influences lateral root development and density

Since CEP5 expression correlates with lateral root initiation and development, a CEP5 loss-of-function line was analysed in terms of effect on this process. Detailed analyses of lateral root initiation revealed increased stage I lateral root primordia in a CaMV 35S promoter-driven CEP5 RNAi knockdown line ($CEP5^{RNAi}$) (Roberts, Smith et al. 2013) compared to the control (Fig. 14a).

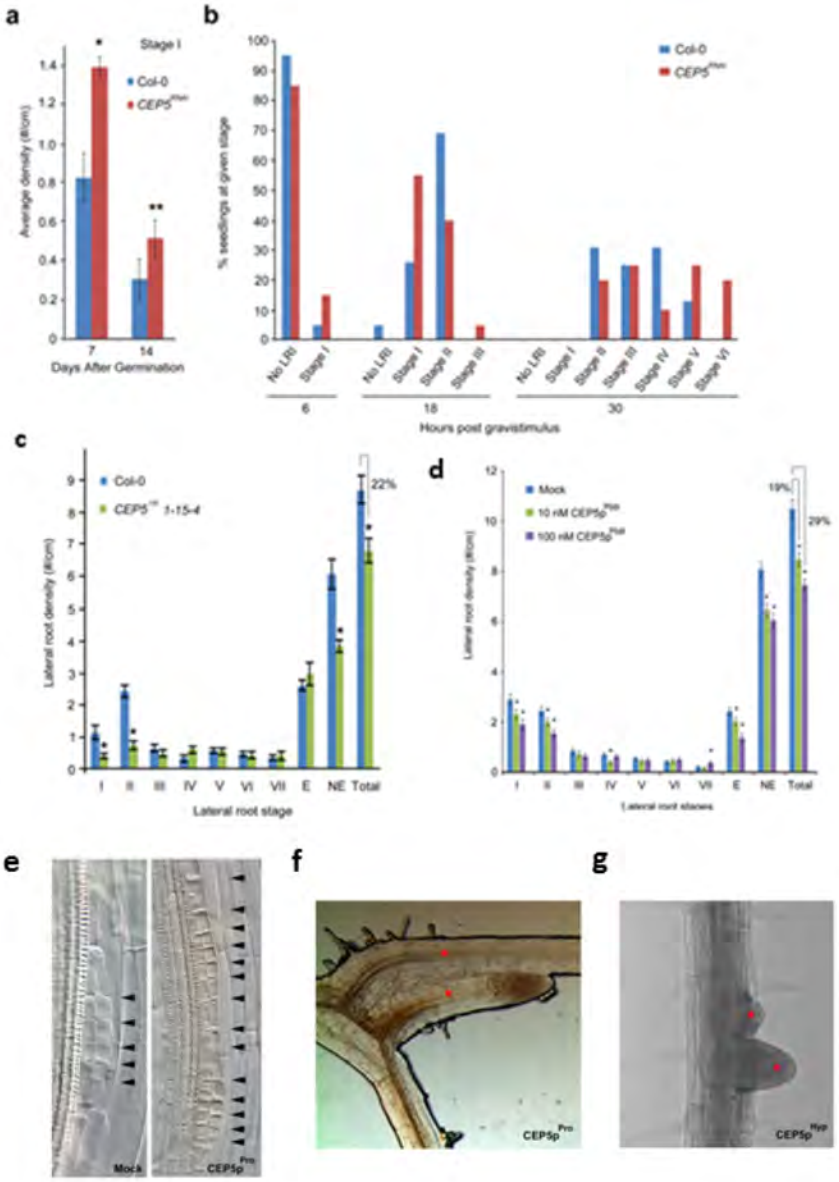

Figure 14. Peturbation of CEP5 abundance corresponds to lateral root number. (a) Knockdown lines for CEP5 (35S::CEP5^{RNAi}) display an increased density of Stage I lateral root primordia at 7 and 14 DAG (n = 10 seedlings per conditions). (b) Progression through lateral root stages at indicated hours post gravistimulus ($n \geq 14$ seedlings per condition). (c) Lateral root stages I to VII (according to Malamy and Benfey, 1997) in CEP5^{OE} line (n ≥ 15) at 7 days after germination. The % reduction in total lateral root density is indicated. E, emerged lateral roots; NE, non-emerged lateral roots; Total, sum of E and NE. (d) Lateral root stages I to VII (according to Malamy and Benfey, 1997) upon mock or CEP5p^{Hyp} treatment at different concentrations at 9 days after germination (data from newly grown root part of 5 day old seedlings transferred to CEP5p^{Hyp} for 4 days, n ≥ 32). E, emerged lateral roots; NE, non-emerged lateral roots; Total, total lateral roots. The % reduction in total lateral root density is indicated (e) Fused early stage primordia in CEP5p^{Pro} exogenous peptide treated Col-0 lines. (f) Abnormal emerged root spacing in CEP5p^{Pro} exogenous peptide treated Col-0 lines. (g) Abnormal late stage primordia spacing in CEP5p^{Hyp} treated Col-0 lines. Error bars are ± S.E.. * represents a p value < 0.05 and **, p < 0.001 according to Student's t-test compared to Col-0. Panels C–E contain work contributed by Ianto Roberts.

Additionally, in a root bending assay (Peret, Li et al. 2012), the

CEP5^{RNAi} line progressed faster through lateral root developmental stages than wild type (Fig. 14b). The CEP5^{RNAi} line did not, however, display a striking difference in primary root length compared to the control. The CEP5^{OE} line displayed a decrease in total lateral root density (22% decrease), with fewer non-emerged lateral roots, compared to wild type (Fig. 14c). Detailed analyses of lateral root developmental stages showed that this was mainly due to fewer initiation events. At later stages of lateral root development, closely spaced lateral root primordia in CEP5^{OE} lines were also observed.

To validate the observed effects on lateral root development in CEP5 overexpression lines, the effect of exogenously applied synthetic CEP5p^{Hyp} on lateral root formation was also analysed. Seedlings grown in the presence of different low concentrations of CEP5p^{Hyp} displayed changes in total lateral root density (19% and 29% decrease for 10 nM and 100 nM, respectively), which is mainly due to a significant reduction in lateral root initiation events (Fig. 14d). Conversely, this did not occur in mCEP5p^{Hyp}-treated seedlings. When lateral root initiation occurred, regions of ectopic and/or aberrant pericycle cell divisions resulting in malformed lateral root primordia or closely spaced primordia in CEP5p^{Pro}/$^{Hyp-}$treated seedlings were observed, which differed from regularly spaced lateral roots in wild type (Fig. 14 e–g).

2.2.5 CEP5 influence on aboveground phenotypes

Since a role for CEP1 in root growth and development has been briefly described previously (Ohyama, Ogawa et al. 2008), the effects of perturbing CEP5 levels on aboveground parts to determine if CEPs also play a role in shoot development were investigated. In the previous work on CEP1 by Ohyama *et al.* (2009), CEP1 overexpression lines has stunted aboveground growth but otherwise appeared normal in terms of leaf number, flowering time and fruit set. To see whether CEP5 expression also correlates with aboveground growth phenotypes, multiple lines with a range of CEP5 expression levels and displaying similar phenotypes were generated and representative knockdown and overexpression lines were photographed. The obtained phenotypes for knockdown or overexpression lines were correlated with reduced or increased CEP5 expression levels, respectively. RNAi knockdown plants for CEP5 after 3 weeks of growth post-bolting demonstrated a slight increase in plant height compared to Col-0 (Fig. 15).

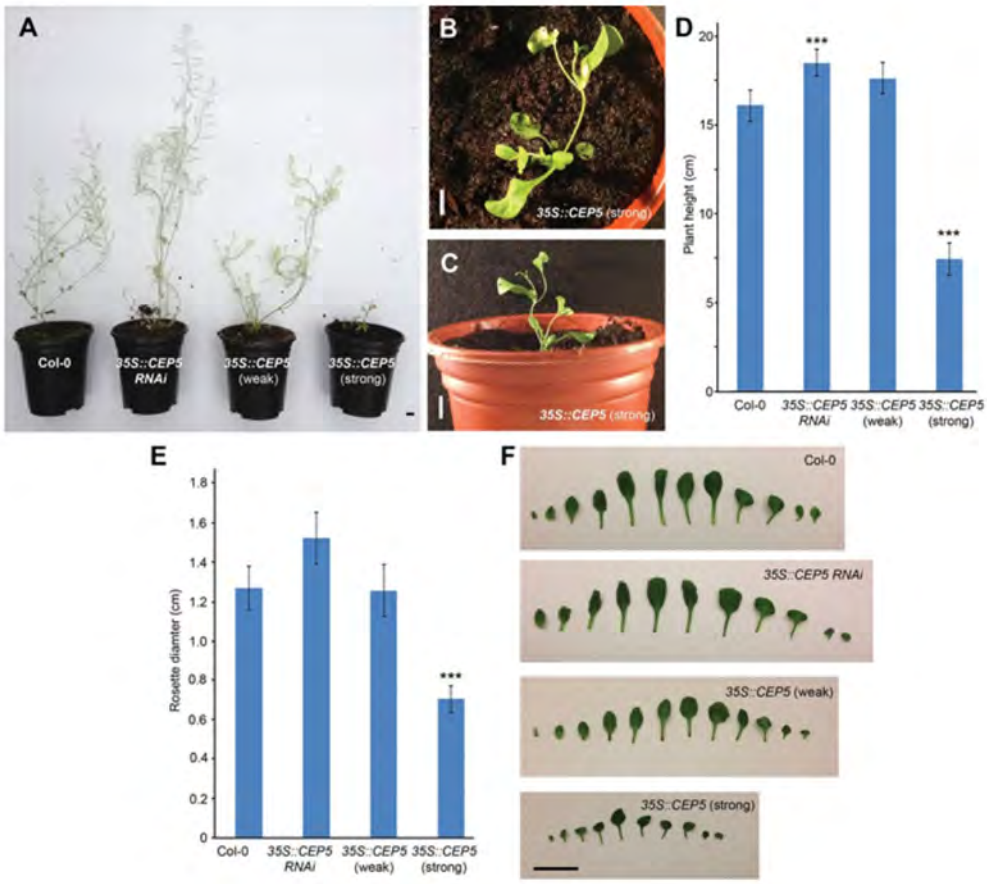

Figure 15. Characterization of aboveground growth in lines with perturbed *CEP5* expression levels. (A) Representative shoot of Col-0 (background line), 35S::CEP5 RNAi knockdown line, and two overexpression lines [35S::CEP5 (weak) and 35S::CEP5 (strong)] after 3 weeks of growth post-bolting. (B and C) Severely stunted growth of 35S::CEP5 (strong) plants after 3 weeks of growth post-bolting. (D) Quantification of the plant height. (E) Rosette area 18 d after germination. (F) Representative leaf series of Col-0 (background line), 35S::CEP5 RNAi, 35S::CEP5 (weak), and 35S::CEP5 (strong). Data in D and E are means± standard errors of at least 20 plants. ***, Student's *t*-test with a *p*-value <0.001. Bars, 1cm.

In contrast, a strong overexpression line for CEP5 was extremely stunted (Fig. 15). A weaker CEP5 overexpression line did not display the same dramatic phenotype but appeared mildly defected in stem gravitropism (Fig. 15). The rosette diameter was significantly reduced in the strong CEP5 overexpression line, compared to the control (Fig. 15). In addition, strong CEP5 overexpression resulted in smaller, often curled, leaves (Fig. 15). However, given the CEP5 expression pattern, the leaf size phenotype might be

due to non CEP5-specific effects and might reflect a role for another, highly similar, CEP peptide.

2.3 Discussion

Previously, one member of the CEP family, CEP1, had already been shown to be involved in regulating root architecture (Ohyama, Ogawa et al. 2008). Using similar primary root length assays and lateral root phenotyping observations for the related peptide CEP5 showed that overexpression of CEP5 resulted in similar phenotypes to CEP1 overexpression, such as stunted primary roots (which like CEP1, appeared to be due to increased meristem consumption) and a reduced number of emerged lateral roots with disorganised lateral root primordia.

The results presented here further establish that a similar phenotype to CEP5 overexpression can be induced by exogenous application of synthetic CEP5 peptide, and furthermore, that severity of phenotype obtained using this method is highly dependent on CEP5 sequence and the presence of hydroxyproline modification on at least proline 4 and proline 11, and probably proline 7 also. This demonstrates that CEP5 has a certain level of specificity of action related to its sequence, since even single amino acid substitutions (including non-proline substitutions) led to reductions in bioactivity in exogenous application bioassay (a detailed analysis of the potential contributions of each residue to CEP5 function and comparisons with other

CEPs can be found in Appendix IV). Furthermore, CEP5 peptides with four amino acid substitutions (outside of the proline residues) were completely abolished in bioactivity and led to phenotypes indistinguishable from wild-type controls in exogenous application assays.

Work performed by others showed differing expression patterns for different CEPs pointing to a role in aboveground tissues (Roberts, Smith et al. 2013). Barely any work had previously been performed assessing the effect of CEP1 on aboveground tissues. The work presented here shows that CEP5 also has aboveground effects on phenotype, including severe stunting in a strong CEP5 overexpressor, and slightly elongated shoots in a RNAi knockdown line, compared to wild-type controls.

These results form the basis for further exploration of the mechanisms underlying this family of peptides and suggest that this family of small signalling peptides has a distinct role associated with developmental events associated with higher plants. Many of the phenotypes reported in this chapter are consistent with CEP5 having an antagonistic relationship with auxin, since auxin generally promotes root growth, lateral root density and various aspects of aboveground growth - whilst mutants in auxin signalling and transport are often impaired in these processes. Thus, the potential for CEP5 antagonising the action of auxin is explored in Chapter 4.

Chapter 3: Elucidating the CEP5 signalling pathway

Parts of this chapter are taken from work performed by the author in Roberts *et al.* 2012.

3.1 Introduction

The CEP peptides have been shown to have a demonstrable effect on plant growth and development through phenotypical observations (See Chapter 2 and (Ohyama, Ogawa *et al*. 2008). However, it remains unclear how CEP peptides physically regulate these processes. Whilst the CEP family was discovered via *in silico* analysis in 2008, it was not established at the time of discovery what the CEP receptor(s) were, or what signalling pathway(s) CEP peptides exert their effects on developmental processes through.

Several plant small post translationally modified peptides have been demonstrated to be perceived via leucine rich repeat receptor like kinases LRR RLKs (e.g. CLV3 and CLV1/2 (Clark, Williams *et al*. 1997, Brand, Fletcher *et al*. 2000, Ogawa, Shinohara *et al*. 2008); TDIF/CLE41/CLE44 and PXY/TDR (Ito, Nakanomyo *et al*. 2006, Fisher and Turner 2007, Hirakawa, Shinohara *et al*. 2008, Etchells and Turner 2010); IDA and HAE/HSL2 (Cho, Larue *et al*. 2008, Stenvik, Tandstad *et al*. 2008), and many others).

Some of these peptide signals and/or LRR RLKs have also previously been shown to cross-talk with classic phytohormonal signalling pathways: The TDIF-TDR/PXY signalling module potentiates auxin signalling independently

from TIR1^{SCF} (Cho, Ryu *et al*. 2014), whilst RGF/GLV/CLEL peptides influence auxin transport during root and hypocotyl gravitropism by modulating PIN2 trafficking dynamics, ensuring that the asymmetrical auxin gradient required for the gravitropic response is maintained. Additionally, GLV1 and GLV2 expression is rapidly induced by auxin, providing an example of an auxin signalling input modulating an auxin transport output via a signalling peptide, thus controlling the initial trigger (Whitford *et al*., 2012). In addition, CLE peptides are regulated through cytokinin signalling during root and vasculature development (Meng and Feldman, 2010). Additionally, other categories of small peptide signals are known to interact with hormonal pathways: for example, POLARIS affects PIN protein localisation (and thus directionality of auxin flux) via ethylene signalling (Chilley, Casson *et al*. 2006).

At the beginning of this PhD project, the receptor(s), signalling pathways, and methods of potential phytohormonal crosstalk for the majority of small peptide signals, including CEP family peptides, remained unknown. Thus, the signalling pathway for CEP5 was investigated further. (This chapter deliberately does not include investigations into potential crosstalk with auxin signalling, as this is covered in depth in Chapter 4).

3.2 Results

3.2.1 ACR4 mutants remain sensitive to CEP5

Receptor-like kinases are known to be important signalling modules in lateral root development: ACR4 has been shown to be required for specifying the fate of neighbouring cells during pericycle cell division in lateral root initiation and restricting the division of neighbouring pericycle cells, thereby controlling the pattern of cell division (De Smet, Vassileva *et al*. 2008). ACR4 likely acts as signal receiver for one, or many, small signalling peptides generated in or surrounding the lateral root initiation site. The phenotype of *acr4* mutants (Fig. 16a) bears a resemblance to CEP5 overexpression (Fig. 16b) and CEP5 exogenous application (Fig. 16c & d) in terms of aberrantly spaced/fused lateral root primordia.

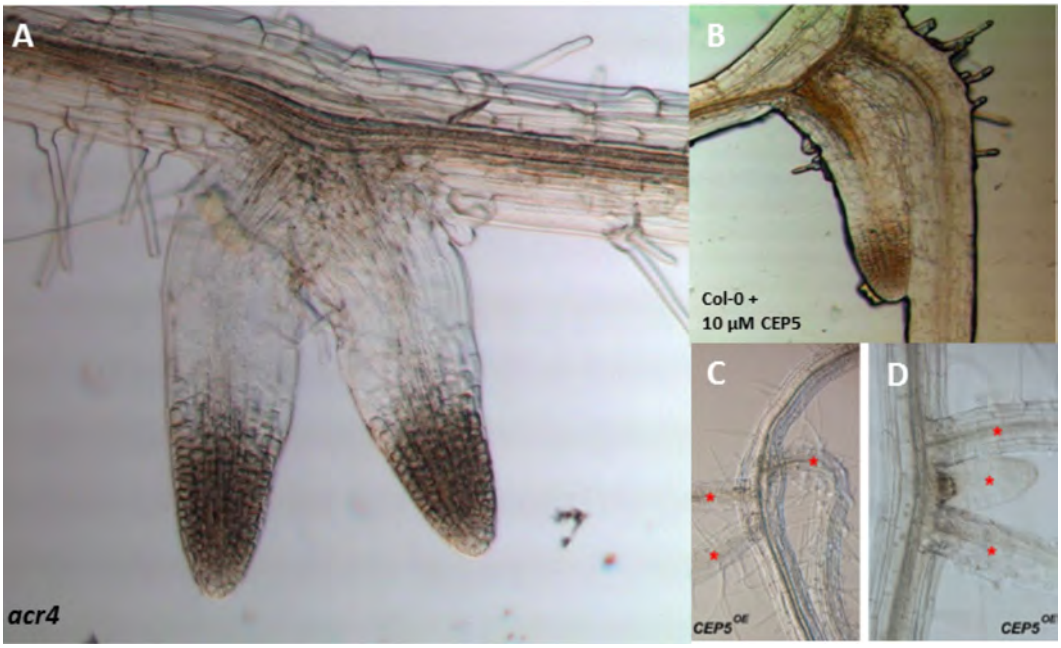

Figure 16. Fused and aberrantly spaced lateral root primordia in *acr4* mutants and under increased CEP5 abundance. Wild-type lateral root intiation typically follows a left-right alternating pattern with evenly-spaced roots on each side. Loss-of-function ACR4 mutants display aberrant lateral root primordia spacing and/or fused primordia (A). This phenotype is also seen when CEP5 synthetic peptide is exogenously applied to wild-type (col-0) seedlings (10 µM $CEP5^{pro}$) (B) or overexpressed in 35S::CEP5 lines (C&D).

Because of these phenotypical similarities, the fact ACR4 is an receptor-like kinase known to regulate formative cell divisions in lateral root organogenesis (De Smet, Vassileva *et al*. 2008) and also because ACR4 is known to bind the small posttranslationally modified peptide CLE41 (which

shares many similarities with CEP family peptides – see Introduction), the possibility of ACR4 representing the receptor to the CEP5 peptide was investigated.

Potentially, these phenotypical similarities between CEP5 overexpression and ACR4 loss-of-function mutants could be indicative of CEP5 acting in an antagonistic fashion to ACR4, with ACR4 restricting formative cell division to regulate formation and spacing of lateral root primordia, and conversely, CEP5 promoting the formation of lateral root primordia.

Exogenous application of CEP5 peptide is known to cause stunted primary root growth via an unknown mechanism causing consumption of meristem, and is not simply caused by the presence of the peptide in the media causing physiological stress to the seedling (see Chapter 2). If ACR4 is indeed required for the perception/action of CEP5, loss-of-function mutants for *acr4* and should display insensitivity to the primary root shortening effects seen in wild-type *Arabidopsis* upon CEP5 exogenous peptide application. Thus, this possibility was investigated.

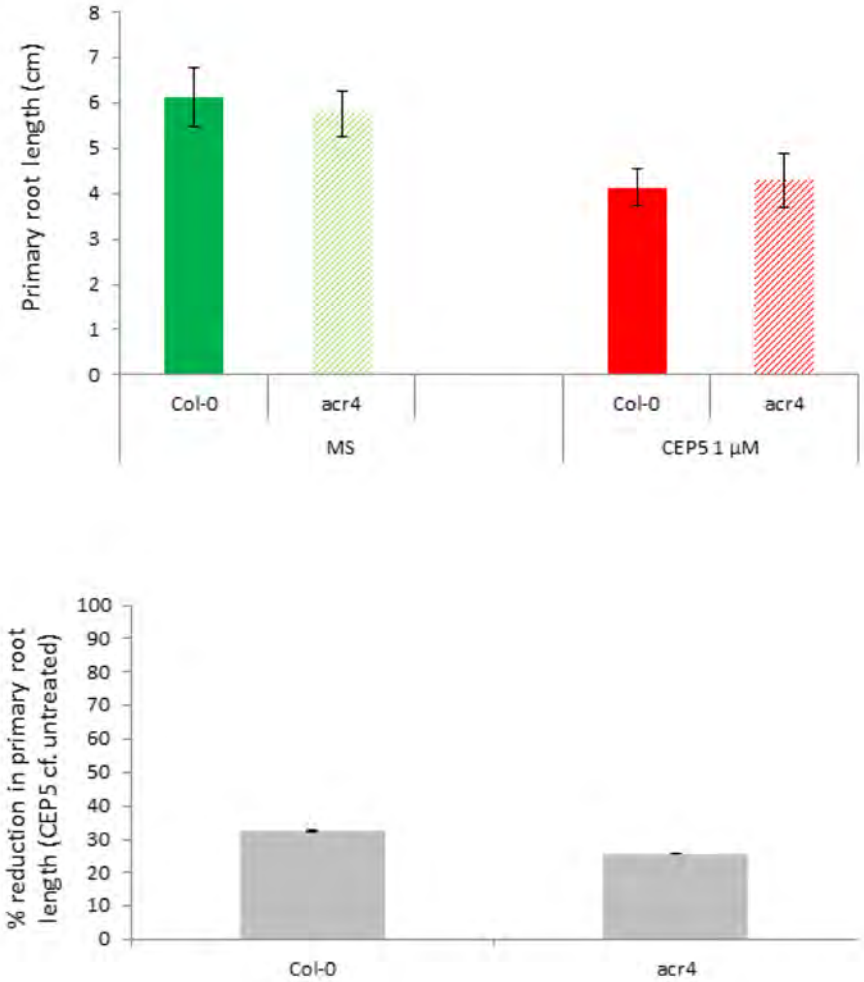

Figure 17. Loss-of-function ACR4 mutants and wild-type controls are equally sensitive to CEP5. Synthetic CEP5 peptide was exogenously applied to *acr4* and wild-type (col-0) *Arabidopsis thaliana* seedlings via planting of seeds on CEP5-supplemented ½ MS media. Primary root length at 7DAG was comparable between both lines under both untreated (MS) and CEP5-treated (CEP5 1 µM) conditions (top panel) with primary root lengths approximately 30% shorter for both Col-0 and *acr4* under CEP5 treatment. $N = \geq 17$ seedlings per condition. p values all = > 0.05 according to Student's T-Test between *acr4* and col-0 primary root lengths. Error bars are ± 1 S.E.

Arabidopsis thaliana loss-of-function mutants for ACR4 remained equally sensitive to exogenous application of CEP5 in primary root length bioassays as wild-type background controls (Col-0), with an average reduction of 25-30% at a CEP5 (concentration of 1 µM) and no significant difference in sensitivity between *acr4* and Col-0.

Therefore, it appears CEP5 does not require the receptor kinase ACR4 for perception/action; however it is still possible that they could both influence the same/related signalling pathways in lateral root primordia formation.

3.2.2 Hormonal and nutritional transcriptional regulation of CEP genes

Hormonal regulation of CEP peptide expression could give clues as to which hormonal pathways CEP peptides could potentially cross-talk with, due to the existence of feedback/autoregulatory networks between the action of a phytohormone and the action of a peptide signal. Thus, a data mining approach was used to investigate potential hormonal and nutrient effects on the regulation of CEP genes in *Arabidopsis thaliana*. Whilst small signalling peptides are often not well represented on available micro-arrays (Murphy, Smith *et al*. 2012) in silico expression data for seven out of 15 CEPs are available. To examine CEP family gene expression changes under several hormonal and nutritional stimuli, this study used the transcriptome meta-analysis tool Genevestigator (Hruz, Laule *et al*. 2008) (Table 1).

Gene	Hormones									Nutrients		
	ABA	Ethylene[a]	BR[b]	GA	Auxin[c]	JA	SA	Strigo	Cyto[d]	N	P	K
CEP1	=	ND	ND	ND	+	ND	=	ND	ND	+	=	=
CEP3	=	=	=	=	+	=	=	ND	=	-	ND	-
CEP5	=	=	-	+	-	ND	-	ND	=	-	+	=
CEP9	=	=	=	=	-	=	=	=	=	+	=	+
CEP12	ND	ND	ND	ND	ND	+	ND	ND	ND	ND	ND	ND
CEP13	=	=	=	+	=	ND	=	ND	=	-	=	=
CEP15	-	=	+	=	=	=	-	=	=	=	=	=

[a–d]Measurements were taken using: [a]ACC (1-aminocyclopropane-1-carboxylic acid); [b]brassinolide; [c]indole-3-acetic acid, 1-naphthaleneacetic acid, and 2,4-dichlorophenoxyacetic acid; [d]zeatin.

Table 1. The effect of hormones and nutrients on CEP expression. All data used were generated on AffymetrixGeneChip ATH1 22K platform. +, Increased expression following stimulus; –, reduced expression; =, no significant effect on expression (defined as P≥0.05 and/or a <1.5 fold change in expression levels compared with control); ND, CEP genes and/or stimuli for which no expression data are currently available in Genevestigator. ABA, abscisic acid; BR, brassinosteroids; GA, gibberellic acid; JA, jasmonic acid; SA, salicylic acid; Strigo, strigolactone; Cyto, cytokinin. Hormones and nutrients in CEP2, CEP4, CEP6, CEP7, CEP8, CEP10, CEP11, and CEP14 are all ND.

Both CEP5 and CEP15 were downregulated by salicylic acid treatment, gibberellic acid induced expression of CEP5 and CEP13, abscisic acid decreased expression of CEP15, whilst jasmonic acid increased expression of CEP12. Contrastingly, auxin (IAA) had opposite effects on the expression of

some CEP family genes, namely an increase in expression of CEP1 and CEP3 but a decrease in CEP5 and CEP9 expression.

Similarly, brassinolide reduced expression of CEP5 and induced CEP15 expression. Other hormones, such as ethylene, strigolactone, and cytokinin, either did not have a significant effect on CEP expression or there was no data available. With respect to nutrients, a high nitrogen level downregulated CEP3, CEP5, and CEP13, and upregulated CEP1 and CEP9. Phosphorus upregulated expression of CEP5. In addition, potassium downregulated CEP3 expression and upregulated CEP9 expression.

3.2.3 Cytokinin receptor mutants remain sensitive to CEP5

Cytokinin is known to influence root patterning and to regulate lateral root organogenesis in an inhibitory manner, with perturbations in cytokinin levels and/or metabolism affecting LR density and development (Sponsel, Schmidt *et al*. 1997, Werner, Motyka *et al*. 2003, Bao, Shen *et al*. 2004, De Smet, Zhang *et al*. 2006, Li, Mo *et al*. 2006, Mouchel, Osmont *et al*. 2006, Laplaze, Benkova *et al*. 2007). The cytokinin signalling pathway is comparable to the prokaryotic two-component signal (TCS) transduction pathways, and is comprised of sensory 'hybrid' kinases, histidine phosphotransfer proteins and downstream response regulators (See Introduction, Figure 6).

Experimental data shows that CEP5 exhibits an antagonistic relationship with auxin (Chapter 4). The phytohormone cytokinin is also known to have many antagonistic actions to auxin, including in lateral root development, and is known to disturb cell division activity and pattern

formation ((Laplaze, Benkova *et al*. 2007, Chang, Ramireddy *et al*. 2015) In particular, the earliest stages of LR formation are uniquely sensitive to cytokinin compared with more developed primordia; possibly owing to the relatively low and high concentrations of antagonistic auxin in these tissues respectively (Bielach, Duclercq *et al*. 2012); phenotypical data suggests that the early stages of LR formation are also where CEP5 is likely to act. Because of the above reasons, the possibility of CEP5 acting within the cytokinin signalling pathway was investigated.

Cytokinin is perceived by the receptor histidine-kinases ARABIDOPSIS HISTIDINE KINASE2 and 3 (AHK2 and AHK3) and CYTOKININ RESPONSE 1/WOODENLEG/AHK4 (CRE1/WOL/AHK4. Thus, the possibility of CEP5 acting upstream of the cytokinin receptors CRE1/WOL/AHK4, AHK2 and AHK3 was investigated using the primary root length/CEP5 exogenous peptide application bioassay (Fig. 18). If these receptors are indeed required for the perception of CEP5, loss-of-function mutants should display insensitivity to the primary root length shortening effects of CEP5 when exogenously applied to seedlings.

Homozygous knockouts for all three cytokinin receptors are not viable, therefore a combination of single (*ahk2* and *ahk 3-3*, homozygous AHK2 and AHK3 knockouts) and higher order (*cre1/ahk2* and *cre1/ahk3* - homozygous knockouts for CRE1/WOL/AHK4 and AHK2 and AHK3 respectively – and *cre1-12/ahk2/ahk3 +/-,* a homozygous knockout for CRE1/WOL/AHK4 and AHK2, heterozygous knockout for AHK3) mutants were used to investigate the importance of cytokinin receptors in CEP5 perception.

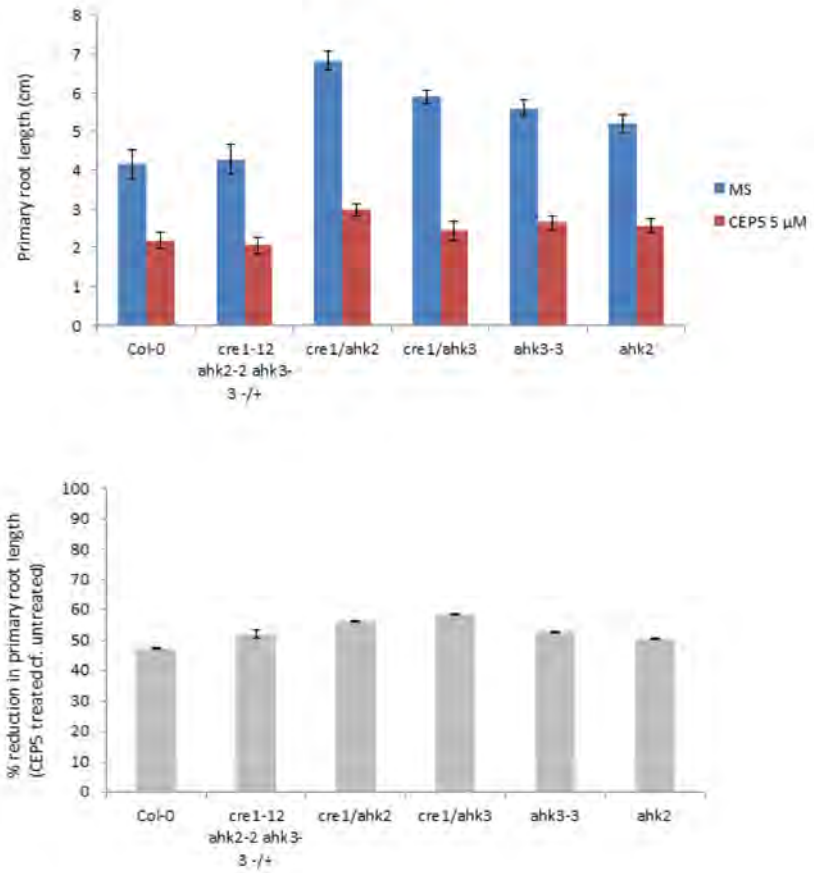

Figure 18. Cytokinin receptor mutants remain sensitive to CEP5. Synthetic CEP5 peptide was exogenously applied to *cre1/ahk2/ahk3* (heterozygous for AHK3 knockout), *cre1/ahk2*, *cre1/ahk3*, *ahk3*, *ahk2* and wild-type (col-0) *Arabidopsis thaliana* seedlings via planting of seeds on CEP5-supplemented ½ MS media. All lines exhibited an approximately 50% reduction in primary root length under treatment with 5 µM CEP5. There was no significant difference in CEP5 sensitivity between all lines according to Student's T-Test (p = > 0.05). N = ≥ 6 seedlings per condition. Error bars are ± 1 S.E.

All cytokinin receptor mutants tested using the primary root length/CEP5 exogenous peptide application bioassay exhibited similar sensitivity to CEP5 as wild-type background controls, with an average reduction in primary root length of approximately 50% at a concentration of 5 µM CEP5 and no significant difference in sensitivity between receptor mutants and wild type (col-0) backgrounds according to T-Test (p = >0.05). Thus, CEP5 does not appear to require CRE1/WOL/AHK4, AHK2 or AHK3 for perception.

3.2.4 CEP5 increases fluorescence in an *Arabidopsis* cytokinin reporter acting downstream of cytokinin receptors

Since CEP5 does not require CRE1/AHK4, AHK2 or AHK3 for perception, the possibility that CEP5 might act downstream of these receptors was investigated.

Downstream signals from the cytokinin receptors are transduced by members of the ARABIDOPSIS RESPONSE REGULATOR (ARR) family. Type A-ARRs are rapidly induced by cytokinin and act as negative regulators of cytokinin signalling, whilst type-B ARRs are positive regulators in cytokinin signalling (Brandstatter and Kieber 1998, D'Agostino, Deruere *et al*. 2000, Jain, Tyagi *et al*. 2006, Argyros, Mathews *et al*. 2008, Tsai, Weir *et al*. 2012).

A cytokinin reporter named Two Component signalling Sensor::Green Fluorescent Protein (TCS::GFP) was used to study the effects of CEP5 on cytokinin responses downstream of the cytokinin receptors. TCS::GFP is a fusion of a typical type-B ARR binding site with a fluorescent reporter (Muller and Sheen 2008, Zurcher, Tavor-Deslex *et al*. 2013). TCS::GFP seedlings were grown on plain ½ MS before being transferred to CEP5 or fresh ½ MS (control) and analysed using confocal fluorescence microscopy; seedlings treated with CEP5 (Fig. 19, red line) showed an approximately 60% increase in TCS::GFP fluorescence compared with the untreated control (Fig. 19, blue line), suggesting a possible increase in cytokinin activity following CEP5 treatment.

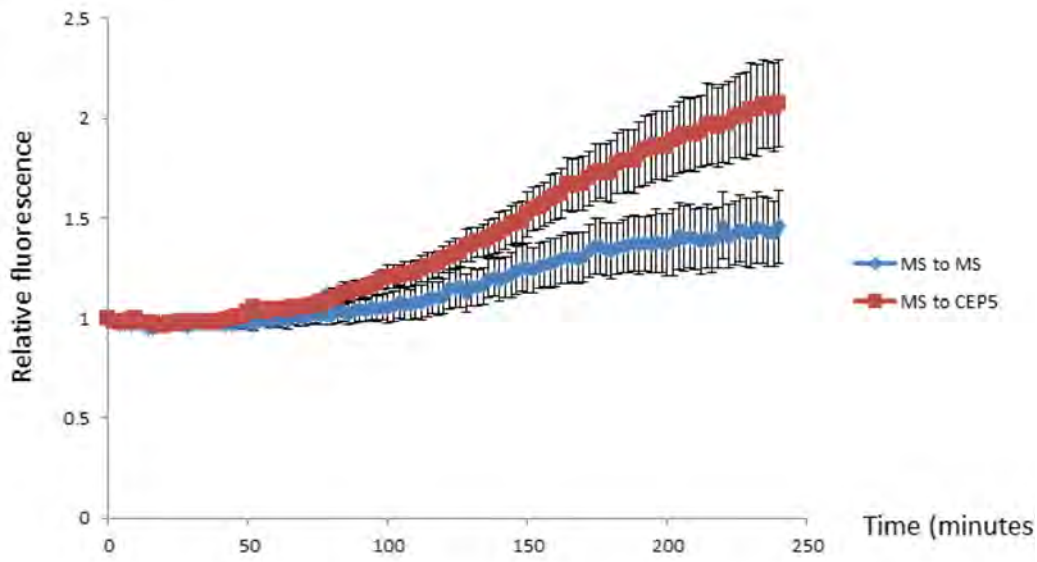

Figure 19. CEP5 increases intensity of fluorescence in a fluorescent cytokinin reporter, TCS::GFP. TCS::GFP seedlings treated with 5 µM CEP5 (red line) had a one-fold increase in relative GFP fluorescence by 240 minutes post-treatment (from a starting value of 1 at time point 0). Controls which were treated with plain ½ MS did not undergo a comparable increase in fluorescence (blue line). The increase in fluorescence following CEP5 treatment was significant according to Student's T-Test (p = <0.001). N = 5 seedlings per condition. Seedlings ~7DAG. Error bars are ± 1 S.E.

Although TCS::GFP fluorescence was higher following CEP5 treatment in this experiment, it is currently unclear why CEP5 treatment leads to this increase in TCS::GFP fluorescence and whether this is a direct effect on cytokinin signalling components downstream of cytokinin receptors, or an indirect effect e.g. through mediating expression of cytokinin signalling components.

3.2.5 RGA::GFP fluorescence is not prolonged by CEP5 following GA challenge

Gibberellic acid (GA) is a phytohormone belonging to a large family of structurally related compounds, with GA_1 and GA_4 being the primary active forms in plants. The DELLA domain protein RGA (repressor of ga1-3) is a

negative regulator of gibberellic acid (GA) signal transduction and is degraded in the presence of GA (Silverstone, Jung *et al*. 2001). A fluorescent reporter, RGA::GFP, is a nuclear-localised negative reporter of GA abundance and is in that respect conceptually similar to the DII::VENUS reporter; RGA::GFP seedlings treated with exogenous GA lose fluorescence in a similar manner to DII::VENUS seedlings treated with exogenous auxin.

Increasing abundance of CEP5 by either exogenous application or crossing CEP5 overexpression lines into DII::VENUS prolongs DII::VENUS fluorescence when challenged with auxin (Chapter 4). Since the gibberellic acid signalling pathway shares similarities with the auxin signalling pathway, and the TCS::GFP and DII::VENUS work via a similar mechanism, investigations were carried out into whether exogenous application of CEP5 to TCS::GFP seedlings might prolong fluorescence when seedlings were challenged with GA and to what degree the antagonistic signalling action of CEP5 is specific to auxin signalling.

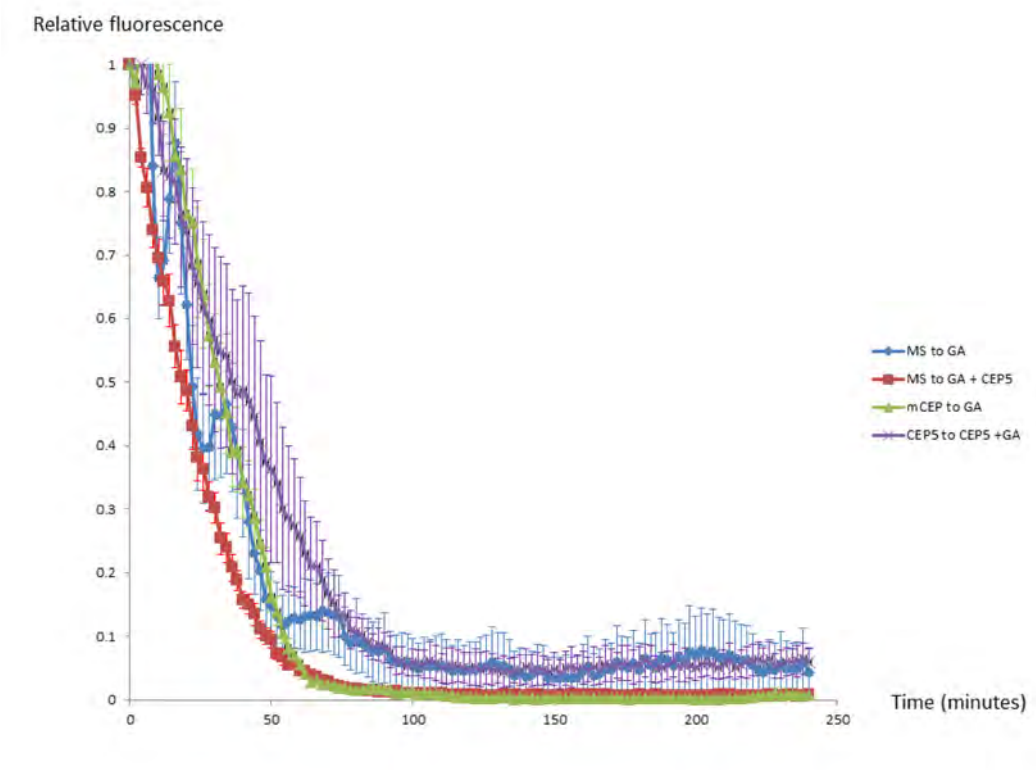

Figure 20. CEP5 does not prolong RGA::GFP fluorescence under GA challenge. RGA::GFP seedlings were grown until ~5-6 DAG on plain ½ MS media before being incubated overnight on either 5 µM CEP5 or mCEP5 (purple and green lines) or plain MS (blue and red lines), then analysed using confocal fluorescence microscopy whilst being challenged with 50 µM GA alone (blue and green lines) or concurrent treatment with both 50 µM GA and 5 µM CEP5 (red and purple lines) for 240 minutes. There was no significant difference in the persistence of fluorescence between treatments ($p = > 0.05$). $N = \geq 3$ seedlings per condition. Error bars are ± 1 S.E.

RGA::GFP seedlings were grown on plain ½ MS media and then transferred and incubated overnight on either CEP5 or mCEP5 (a mutated inactive version of CEP5 – see Chapter 2) or fresh ½ MS (controls) then analysed using confocal fluorescence microscopy. There was no significant difference in the persistence of RGA::GFP fluorescence under GA challenge when seedlings were treated with CEP5, with or without pre-incubation on CEP5 (Fig. 20, red line and purple line) compared with controls (Fig. 20 GA alone, or mCEP preincubated to GA alone; blue line and green line).

3.3 Discussion

In this chapter, potential interactions of CEP5 with the receptor-like kinase ACR4, and potential signalling crosstalk with the classical phytohormonal signalling pathways for cytokinin and gibberellic acid were investigated.

Mutant sensitivity experiments using primary root length bioassays showed that loss-of-function mutants for ACR4 retained sensitivity for CEP5 synthetic peptide, suggesting ACR4 was not required for perception of CEP5. This is in agreement with experiments performed by others that also do not support the hypothesis that CEP peptides bind to ACR4. Photoaffinity cross linking does not show an interaction between ACR4 and CEP1 (Yoshikatsu Matsubayashi, personal communication, see Appendix I) and Fluorescence Recovery After Photobleaching (FRAP) experiments also do not show an increase in recruitment of ACR4 receptor proteins to the cell membrane upon addition of CEP5 - which would be expected if CEP5 was indeed the ligand for the ACR4 receptor (Rudiger Simon, personal communication, see Appendix I). Therefore, this particular angle was not investigated further. However, it is still possible that CEP5 and ACR4signalling overlaps in some other way, e.g. possibly converging on a downstream signalling module. As the downstream signalling pathway of ACR4 currently remains unknown, it was not possible to test this at present, but remains an open possibility of research for the future.

To investigate the role of phytohormones in general CEP peptide regulation, and potentially give clues as to which hormonal pathway(s) CEP5 interacts with, the transcriptomics meta-analysis tool Genevestigator was used. In terms of CEP5, gibberellic acid (GA) increased CEP5 expression, whilst brasssinolide (BR), auxin (IAA, NAA, 2,4-D) and salicylic acid (SA) decreased it. Subsequent investigations focused on CEP5 and potential cross-

talk with the auxin, cytokinin and GA hormonal signalling pathways; however, brassinolide and salicylic acid were not investigated further at this point, and the relationship between these phytohormones and CEP5 may prove a worthwhile avenue of investigation for the future. No data was available for either jasmonic acid or strigolactone on the the expression of CEP5; however a speculative model for a potential CEP5-strigolactone interaction is outlined in the discussion at the end of this chapter.

As well as hormonal regulation, certain nutrients affected the expression of CEP5: nitrogen negatively, and phosphorous positively. Given the role of CEP5 in regulating lateral root branching and the known phenotypical effects on lateral root branching induced by these nutrients (see Introduction 1.7) this is intriguing. The potential nutritional sensing aspect of CEP5 is explored further in the general discussion.

A potential interaction of CEP5 with the cytokinin signalling pathway was investigated. Loss-of-function mutants for the cytokinin receptors CRE1/WOL/AHK4, AHK2 and AHK3 remained sensitive to CEP5, thus these receptors do not appear to act downstream of CEP5 signalling. However, downstream of CEP5, a TCS-based reporter exhibited increased fluorescence when treated with CEP5 - suggesting positive responses to cytokinin signalling may be increased following CEP5 application. This could be indicative of CEP5 acting on cytokinin signalling at modules downstream of the cytokinin receptor(s), such as interactions with AHP proteins or the ARRs themselves. These interactions could be direct, e.g. in terms of regulation of phosphorylation or protein stability - or indirect, e.g. in terms of effects on protein expression, protein localisation, or nuclear import of ARRs. If the

observations from the TCS::GFP were found to be reliably replicated, these possibilities could be investigated. Conversely, whilst cytokinin is known to be involved in vascular patterning, transverse cross sections do not show any difference in vascular patterning in mutants with increased or decreased CEP5 expression and wild-type controls, arguing against an interference of CEP5 with cytokinin signalling (Anthony Bishopp and Ben Goodall, personal communication). Cytokinin also does not seem to have any effect on the expression of CEP5 or other CEP-family peptides according to the transcriptomics meta-analysis tool Genevestigator (Table 1) (Roberts, Smith *et al*. 2013). Thus, the possibility of CEP5 signalling cross-talking with cytokinin signalling looks - on balance - currently unlikely, but is a question that is still not fully resolved (see the discussion at the end of this chapter for further comment).

Data mining analysis using the transcriptome meta-analysis tool Genevestigator previously suggested CEP5 expression is increased by GA, making interference with GA signalling an attractive potential feedback mechanism. In addition, the conceptual similarities of downstream signalling in the GA and auxin pathways gave rise to the possibility CEP5 might act in a general fashion to stabilise both the negative signalling regulators RGA and AUX/IAA proteins, possibly against E3 ubiquitin-ligase mediated proteasomal degradation. However, the effect of CEP5 on prolongation of DII::VENUS fluorescence appears to be specific effect not replicated for RGA::GFP, making it less likely CEP5 is involved in directly attenuating GA signalling or changing GA abundance. Whether CEP5 has any interaction with the GA

signalling pathway in a different/more indirect way and what the connection there is between GA and CEP5 (if any) remains to be investigated.

One potential explanation that has not yet been investigated is a potential link between CEP5 and strigolactone signalling. The potential involvement of strigolactones in CEP5 signalling was not fully investigated here due to time constraints; however, this could represent a worthwhile avenue of investigation for the future. Strigolactones (SL) have been shown to be involved in root growth, lateral root formation, adventitious rooting and stem elongation (amongst other developmental processes) and SL synthesis and secretion into the soil is upregulated by phosphate deficiency (Yoneyama, Xie *et al*. 2007, Yoneyama, Yoneyama *et al*. 2007, Lopez-Raez, Charnikhova *et al*. 2008, Umehara, Hanada *et al*. 2008, Kohlen, Charnikhova *et al*. 2011). SL synthesis is highest in the root tip and SLs can move from the root to the shoot but not vice versa, thus have clear potential to be involved in root-to-shoot signalling (Beveridge, Ross *et al*. 1996, Napoli 1996, Beveridge, Symons *et al*. 1997, Morris, Turnbull *et al*. 2001, Turnbull, Booker *et al*. 2002, Booker, Sieberer *et al*. 2005, Simons, Napoli *et al*. 2007, Sharda and Koide 2008). Whilst the SL signalling pathway has not been fully elucidated, there is some evidence that as for auxin and GA, it could involve SCF-mediated protein degradation of inhibitory proteins, specifically SCF^{MAX2} (reviewed in (Waldie, McCulloch *et al*. 2014). SL mutants display shoot axillary buds resistant to inhibition by auxin, and one potential mechanism for SL action is that SL triggers removal of PIN1 auxin efflux proteins from the plasma membrane (Bennett, Sieberer *et al*. 2006, Crawford, Shinohara *et al*. 2010, Shinohara, Taylor *et al*. 2013). Experiments using PIN1:GFP exogenously

treated with CEP5 potentially show this could be happening: however, the results are inconclusive and would need to be replicated before drawing any conclusions (See Appendix 5).

Chapter 4: CEP5 demonstrates an antagonistic relationship with auxin

Parts of this chapter are taken from work performed by the author in Roberts *et al.* 2012.

4.1 Introduction

Several plant hormones have been shown to affect root architecture, among which the phytohormone auxin has been granted a central role. Lately, small signalling peptides have also emerged as potential molecular components regulating root growth and development. Two members of the CEP family of small posttranslationally modified peptides, CEP1 and CEP5, have been shown to affect various developmental processes known to be driven by auxin – root growth and development, lateral root initiation/ and spacing, and some minor aboveground phenotypes (Ohyama, Ogawa *et al*. 2008, Roberts, Smith *et al*. 2013).

Previously, a search for putative novel early lateral root formation components was performed by screening a dataset obtained through a highly focused transcript profiling analysis on seedling roots treated with the synthetic molecule naxillin, which does not produce the typical pleiotropic effects of exogenous auxin application (De Rybel, Audenaert

et al. 2012). Naxillin works by promoting peroxisomal IBA to IAA conversion in the root cap and leads to an increase in lateral root branching without a global increase in plant auxin levels. This identified CEP5 as differentially regulated between non-treated and naxillin-treated seedling roots (De Rybel, Audenaert *et al*. 2012), suggesting CEP5 has a specific role in lateral root development. Furthermore, data mining analysis using the transcriptomic meta-analysis tool Genevestigator found that CEP5 appears to be negatively regulated by auxin, which gave a potential clue that CEP5 and auxin could negatively regulate each other in a feedback mechanism (see Chapter 3).

Following on from these discoveries and the previously described supporting phenotypical evidence for a role of CEP5 on primary root growth and lateral root development (both representing auxin-mediated processes (Overvoorde, Fukaki *et al*. 2010, Lavenus, Goh *et al*. 2013) and Chapter 2, the relationship between CEP5 and auxin was investigated further.

4.2 Results

4.2.1 CEP5 retards the auxin-driven process of gravitropic response

One well-known auxin driven process is gravitropism, the tropic growth of a plant in relation to a gravity vector; in roots this involves turning towards the gravity vector, allowing downward growth into soil. Following perception of

gravity by specialised starch-containing amyloplasts (statoliths) in the root cap cells, PIN auxin efflux carriers are redistributed, modulating auxin flux. Auxin is directed towards the side of the root most proximal to the gravity vector (e.g. the 'lower' side of a root) resulting in a steeper auxin gradient on this side. Through an unclear mechanism, this inhibits elongation of cells on this side of the root, whilst cells on the gravity-distal 'upper' side continue to elongate. This mismatch in elongation rates on either side of the root causes the root to bend. A gravistimulus can easily be applied to roots by switching them from a vertical to a horizontal growth orientation; following gravistimulation, a bend is induced in seedling primary roots, progressing from a theoretical maximum root bend angle of 180° (vertical growth) to a theoretical minimum root bend angle of 90°. Thus, a gravitropism assay was used to investigate if CEP5 could affect this process, as this could be indicative of a role in auxin transport or auxin signalling.

CEP5pHyp-treated wild type seedlings displayed a slower gravitropism-induced bending response of primary root tips relative to the untreated control, only bending approximately 20° after a period of 3.5 h; mock-treated seedlings displayed approximately double this rate of bending in (Fig. 21). Thus, CEP5 appears to antagonise either auxin transport or signalling in some way.

4.2.2 Analysis of auxin signalling and transport mutants

To investigate a potential role in auxin signalling, several lines mutant for various modules of auxin signalling were investigated; these are detailed in table 2. If any of these mutants displayed differing sensitivity to CEP5^{Hyp} exogenous peptide application compared with the wild-type background (in this case, either Columbia (Col-0) or Landsberg erecta (Ler)) in a primary root length bioassay, it could indicate that that particular auxin signalling and/or transport module was necessary for the antagonistic action of CEP5.

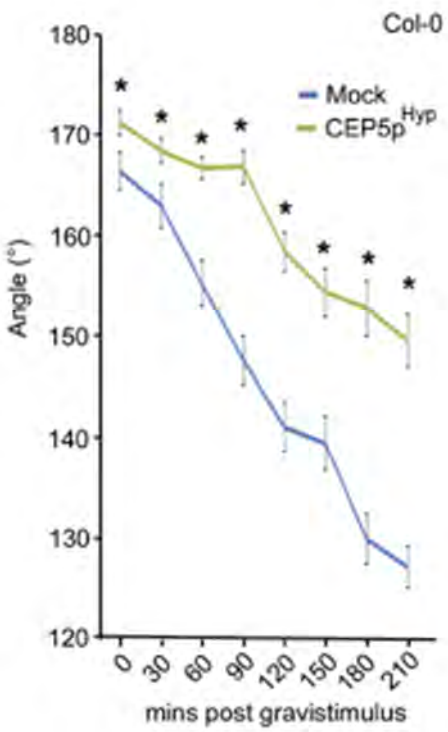

Figure 21. CEP5 slows root gravitropic response. Angle of gravistimulated mock or 5 µM CEP5p^{Hyp}-treated Col-0 root at indicated minutes post gravistimulus. *, $p < 0.05$ according to Student's t-test compared to mock. In all cases, mock refers to medium with water as used to dissolve CEP5p. Error bars are ± 1 S.E.

Name of mutant	Auxin transport (T) or signalling (S) module affected	Type of mutation	Background accession	Additional notes	References
aux1-2	AUX1 (T)	LOF	Ler	Partial LOF	(Bennett, Marchant *et al*. 1996)
aux1-22	AUX1 (T)	LOF	Col-0	Very strong/null LOF	(Bennett, Marchant *et al*. 1996)
shy2-2	IAA3 (S)	GOF	Ler	Semidominant missense GOF mutation	(Tian and Reed 1999)
shy2-3	IAA3 (S)	GOF	Ler	As for *shy2-2*, but less severe	(Tian and Reed 1999)
slr (AKA *slr-1*)	IAA14 (S)	GOF	Col-0	Dominant GOF mutation	(Fukaki, Tameda *et al*. 2002)
pils2.2	PIN-LIKE2 (T)	LOF	Col-0	LOF	(Barbez, Kubes *et al*. 2012)
pils5.2	PIN-LIKE5 (T)	LOF	Col-0	LOF	(Barbez, Kubes *et al*. 2012)
wav5-33	AUX1 (T)	LOF	Ler	Nonsense allele	(Swarup, Kargul *et al*. 2004)

Table 2. List of mutants tested for sensitivity to CEP5. Mutants are impaired in either auxin transport (T) or auxin signalling. (S). GOF: Gain-of-function; LOF: Loss of function. Col-0: Columbia; Ler: Landsberg erecta.

As expected, $CEP5^{Hyp}$ treated wild-type *Arabidopsis* lines had primary root length reduced by ~70% for Col-0 (Fig. 22 A) and ~60% for Ler (Fig. 22 B). The mutants *aux1-22*, *shy2-3* and *wav5-33* all demonstrated similar reductions in primary root length to background controls when treated with $CEP5^{Hyp}$. The partial loss-of-function AUX1 mutant *aux1-2* demonstrated a slight insensitivity to CEP5, with a primary root length reduction of 40%; however, since the stronger loss-of-function AUX1 mutant *aux1-22* did not replicate this, this is not likely to be significant. Interestingly, *slr* and *shy2-2* (both of which are strong gain-of-function AUX/IAA lines) had much-reduced sensitivity to CEP5, with 35% and 16% primary root length reductions respectively. In *slr* and *shy2-2*, AUX/IAA proteins (IAA 14 and IAA3 respectively) are abnormally stabilised as the mutation in the AUX/IAA sequence renders the protein unable to be degraded by the auxin receptor $TIR1^{SCF}$. Thus, the increased levels of AUX/IAA proteins in these lines leads to higher repression of auxin signalling, with various phenotypes corresponding to insensitivity to auxin - e.g. a lack of lateral roots in *slr*. It thus appears CEP5 requires auxin signalling for functionality, and that CEP5 likely acts on or upstream of AUX/IAA proteins.

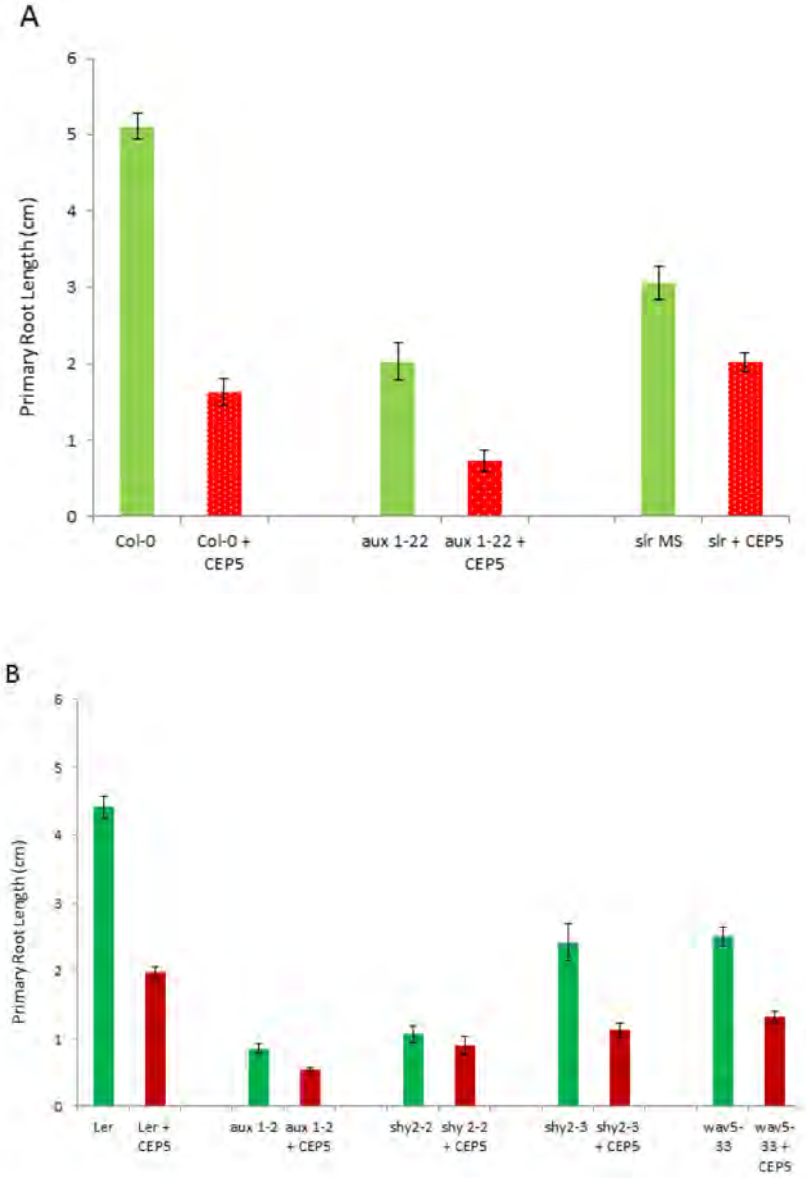

Figure 22. Sensitivity of auxin signalling and transport mutants to exogenous CEP5 application. Primary root length bioassays tested auxin signalling and transport mutants for sensitivity to 5 µM $CEP5^{hyp}$ compared with sensitivity of background lines (Col-0 background lines: panel A; Ler background lines: panel B.) $N = \geq 23$ seedlings per condition. Error bars are ± 1 S.E.

Whilst the auxin transport protein AUX1 does not seem to be required for the action of CEP5 based on the above results, it is possible other auxin transport proteins could act up- or downstream of CEP5. A class of intracellular PIN-like family of auxin transport proteins has been discovered relatively recently called PIN-LIKES (PILS). PILS regulate auxin accumulation in the ER and thus may regulate intracellular auxin homeostasis and metabolism and modulate cellular sensitivity to auxin (Barbez, Kubes *et al*. 2012). PILS overexpressors have reduced levels of free auxin and dampened auxin signalling, whilst the *pils2pils5* double loss-of-function mutant has higher auxin levels and increased lateral rooting. Regulation of these proteins might provide a mechanism by which CEP5 influences auxin responses. Thus, loss-of-function mutants for PILS2 (*pils2.2*) and PILS5 (*pils5.5*) were tested for sensitivity to CEP5 using the primary root length bioassay (Fig. 23).

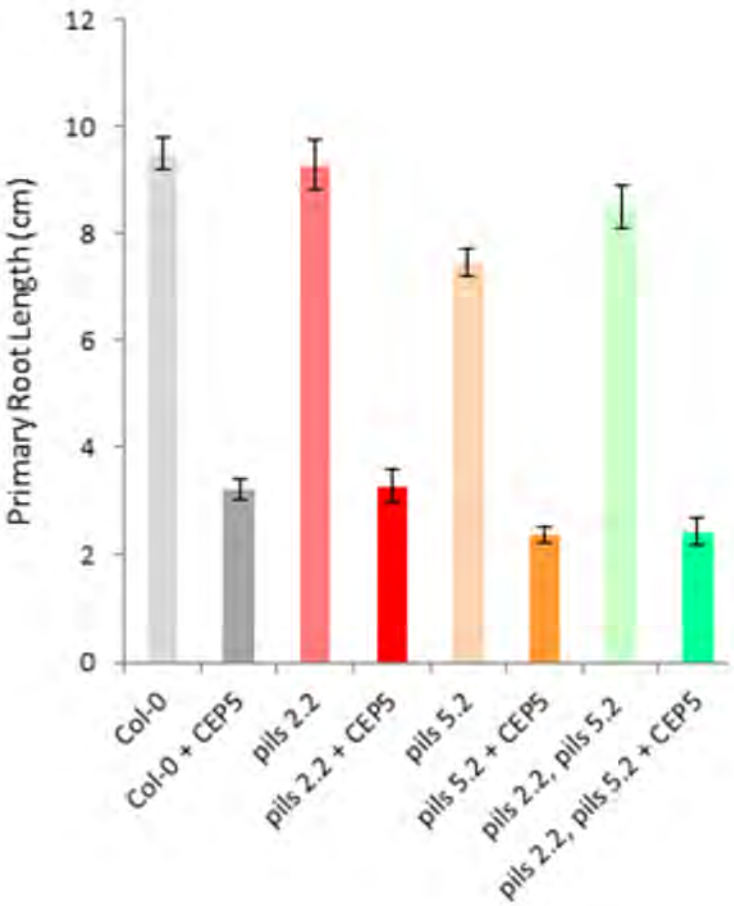

Figure 23. PILS mutant sensitivity to CEP5. Primary root length bioassay for PILS2 (*pils2.2*), PILS5 (*pils5.2*) and single and double (*pils2.2, pils5.2*) mutants for sensitivity to 5 µM $CEP5^{Hyp}$. Error bars are ± 1 S.E. Seedlings ~15 DAG, $N = \geq 10$ seedlings per condition.

As seen previously, wild-type (Col-0) primary root length was reduced ~70% in the presence of $CEP5^{hyp}$. All the PILS mutants analysed also retained comparable levels of sensitivity to exogenous CEP5 peptide application, suggesting that CEP5 does require PILS for activity and does not antagonise auxin action through modulation of PILS-mediated auxin transport.

There are some notable exceptions which were not tested for CEP5 sensitivity here. Unfortunately, attempts to grow loss-of-function mutants for the auxin efflux transporters PIN1 and PIN2 were not successful. This was likely due to poor seed health and/or technical difficulties with growth

facilities, which could not be resolved within the timespan of this project. Investigation of these lines for sensitivity to CEP5 would thus be a high priority for future investigations.

4.2.3 An IAA-28 based sensor, DII::VENUS, is delayed in response to gravity in the presence of CEP5

The above experiments suggest that CEP5 slows auxin responses in terms of gravitropism, and that AUX/IAA gain-of-function mutants might be insensitive to CEP5. Since AUX/IAA proteins are negative regulators of auxin signalling, changes in the stability of AUX/IAA proteins should correlate with changes in the rate of bending in response to a gravistimulus; for example, gain-of-function mutants for AUX/IAA protein SHY2 are agravitropic (Tian and Reed 1999).

Transcriptional responses to auxin depend principally on the auxin-activated SKP1−CUL1/CDC53−F-BOX (SCF)TIR1/AFB-dependent degradation of AUX/IAAs. Auxin concentration and/or activity level of the SCFTIR1/AFB complex can be inferred from the decrease in the AUX/IAA based auxin sensor DII:VENUS fluorescence levels (Brunoud, Wells *et al*. 2012). DII::VENUS (which is based on domain II sequence of IAA28) is able to respond to spatiotemporal changes in auxin distribution elicited by a gravistimulus (Brunoud, Wells *et al*. 2012). In a DII:VENUS root that is growing vertically, fluorescence should be equally distributed on both longitudinal halves of the root. When the root is rotated 90 degrees so it

becomes horizontal, DII:VENUS fluorescence in the bottom longitudinal half of the root (most proximal to the gravistimulus) becomes reduced in response to the relocation of auxin to the lower side. There was a quick change in activity of the DII:VENUS in gravistimulated control primary root tips, which was delayed in the presence of CEP5p^{Hyp} (Fig. 23).

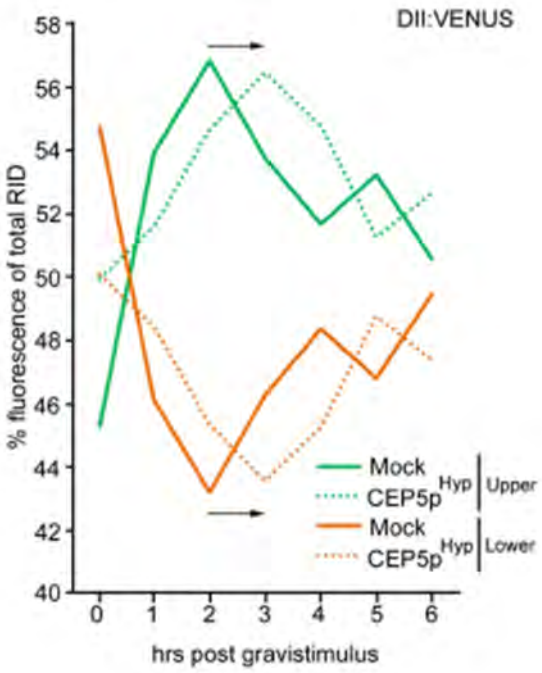

Figure 23. CEP5 slows gravitropism-induced redistribution of DII::VENUS fluorescence. The ratio of VENUS fluorescence in the lower (orange lines) versus upper (green lines) horizontal sections of roots from DII:VENUS reporter line subjected to a gravitropic stimulus and mock or CEP5pHyp-treated. The percentage share of the total root fluorescence at the indicated hours post gravistimulus is shown. RID, raw integrated density. Arrows indicate ~1 hr delay in response. $N = \geq 5$ seedlings per condition/timepoint.

In the mock treated DII:VENUS roots, the ratio of total root fluorescence starts with a roughly even distribution - e.g. 50% of total root fluorescence (Raw Integrated Density, RID) on respective longitudinal halves of the root at timepoint 0 (application of the gravistimulus). Within 2 h post gravistimulus,

the total fluorescence ratio shifts to a minimum of 43% represented by the lower (gravity proximal) half of the root, with the upper (gravity distal) half correspondingly representing 57% of the total root fluorescence, reflecting the shift in auxin distribution known to occur in gravitropic response. 6 h post gravistimulus, total root fluorescence returns to the pre-gravistimulus even distribution.

In DII:VENUS roots treated with $CEP5^{Hyp}$, fluorescence fluctuates in roughly the same way and to the same extent as the control, but in all cases appears delayed by approximately one hour. This could be indicative of CEP5 delaying either the redistribution of auxin (e.g. affecting transport) and thus indirectly affecting the DII:VENUS reporter, or more directly, CEP5 could affect auxin signalling, affecting the stability of AUX/IAA proteins and thus DII:VENUS reporter abundance.

4.2.4 Increasing CEP5 abundances stabilises the IAA-28 based sensor DII::VENUS

If CEP5 stabilises the negative auxin signalling regulators AUX/IAA proteins, this could provide a mechanism to explain the observed auxin antagonistic effects of CEP5 seen in Chapter 2 and in the above results. To investigate whether CEP peptides indeed lead to stabilisation of AUX/IAA proteins, synthetic CEP5 peptides were exogenously applied to DII:VENUS roots and incubated for 18 h (Fig. 24a).

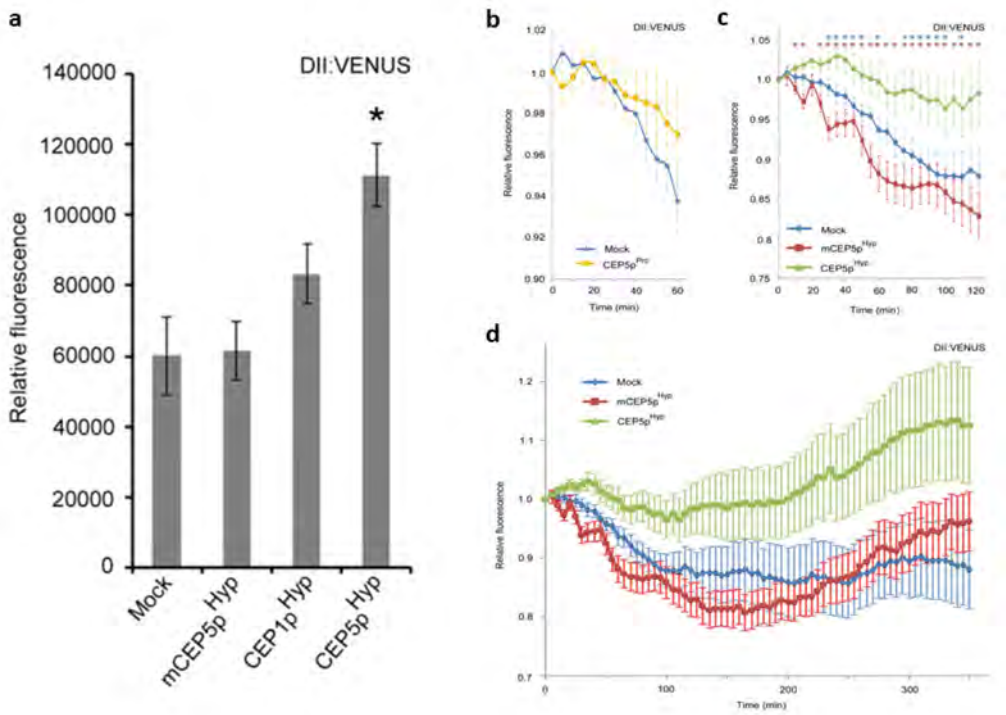

Figure 24. CEP5 increases DII:VENUS fluorescence. (a) Relative DII:VENUS protein fluorescence in 35S::DII:VENUS reporter line following 18 hrs incubation with 5 µM CEP5p^{Hyp}, mCEP5p^{Hyp}, and CEP1p^{Hyp} (DFR[HYP]TNPGNS[HYP]GVGH) compared with mock (medium with water as used to dissolve CEPp) treatment at 5-6 days after germination. $n = 11$. *, $p = < 0.05$ according to Student's t-test compared to mock and mCEP5p^{Hyp}. (b-d) DII:VENUS levels upon treatment with CEP5p^{Pro} or mock (medium with water as used to dissolve CEPp) for 65 minutes (b) and with CEP5p^{Hyp}, mCEP5p^{Hyp} or mock for 120 minutes or 480 minutes *, $p = < 0.05$ according to Student's t-test compared to mock (blue) or mCEP5p^{Hyp} (red). All graphs show average ± standard error. With respect to mock versus mCEP5p^{Hyp} (in c) there was – apart from 15, 30 and 55 min ($p = < 0.05$) – no global significant different. Note: no auxin was used in this experiment. Error bars are ± 1 S.E.

DII:VENUS roots treated with CEP5 displayed increased VENUS fluorescence after 18 h of incubation, suggesting stabilisation of DII::VENUS protein in treated roots. In comparison, the related CEP1p^{Hyp} only had a minor, not significant impact on DII:VENUS fluorescence, and a mutated CEP5 sequence with 4 amino acid substitutions in the sequence (mCEP5^{Hyp}) had no effect on DII:VENUS fluorescence at all (Fig. 24a, c-d). When DII:VENUS seedlings were time-course imaged starting from application of the peptide (e.g. no preincubation with CEP5), CEP5^{Pro} and CEP5^{Hyp} slowed natural loss of DII:VENUS fluorescence over 65-300 minutes compared with mock treated controls (Fig. 24b-d); mCEP5^{Hyp} was again not able to do this.

Because fluorescence in the CEP5 treated DII:VENUS seedlings was particularly bright in the epidermal tissues of the root and the method and/or efficiency by which CEP5 peptide is taken up by the root, it was investigated whether crossing CEP5 perturbed expression lines into DII:VENUS lines would replicate the increase in VENUS fluorescence seen following exogenous application of synthetic peptide, relative to Col-0 x DII:VENUS controls.

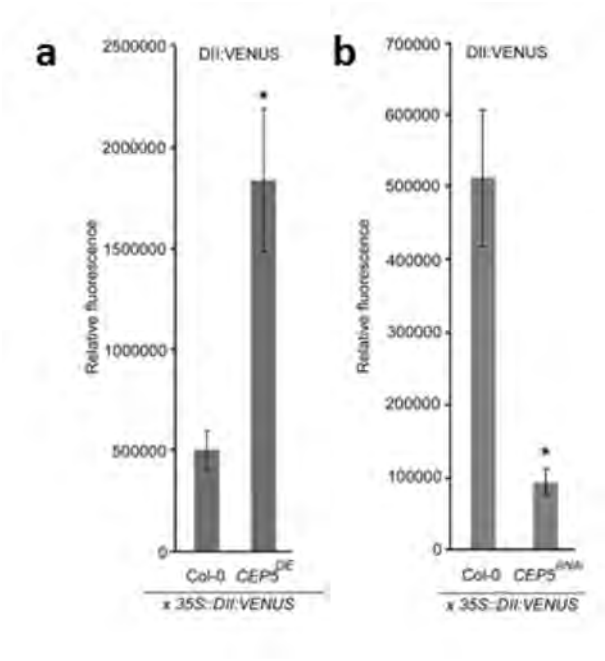

Figure 25. DII:VENUS fluorescence in F1 DII:VENUS crosses. Crosses were generated and analysed for relative DII:VENUS fluorescence: Col-0 x 35S::DII:VENUS, CEP5RNAi x 35S::DII:VENUS, and CEP5OE x 35S::DII:VENUS. *N* = pool of 5-10 seedlings (6 days after germination). Error bars are ± 1 S.E.

In agreement with what was observed by increasing CEP5 abundance via exogenous application, $CEP5^{OE}$ x DII:VENUS lines had greatly increased VENUS fluorescence (Fig. 25a) and this corresponded with an equally significant decrease in VENUS fluorescence in CEP5 knockdown ($CEP5^{RNAi}$) lines (Fig. 25b) compared with Col-0 x DII:VENUS controls. Furthermore, the fluorescence observed in these lines was more typically distributed as for

DII:VENUS homozygous lines. qPCR analysis also showed that the changes in DII:VENUS fluorescence were not merely caused by upregulation of DII:VENUS in CEP5^{Hyp} treated and CEP5^{OE} lines, or by downregulation of DII:VENUS in CEP5^{RNAi} x DII:VENUS lines.

4.2.5 Increased CEP5 abundance delays DII:VENUS signal degradation under auxin challenge

If CEP5 stabilises AUX/IAA proteins, DII:VENUS fluorescence should be more resistant to degradation in CEP5 increased-abundance seedlings when challenged with auxin. Ordinarily, exogenous application of auxin leads to rapid loss of DII::VENUS fluorescence (Fig. 26), with 1 μM of exogenous auxin treatment (IAA or NAA) sufficient to cause fluorescence to fall to levels barely detectable by the naked eye within as soon as 60 minutes of application based on previous trials.

Co-incubation of DII:VENUS seedlings with auxin (IAA or NAA) with CEP5p^{Hyp} resulted in a significant delay of DII:VENUS degradation compared to auxin alone, while the similar, but mutated mCEP5p^{Hyp} does not slow DII:VENUS degradation, confirming this effect is due to the CEP5^{Hyp} sequence rather than a nonspecific peptide effect (Fig. 27 e & f).

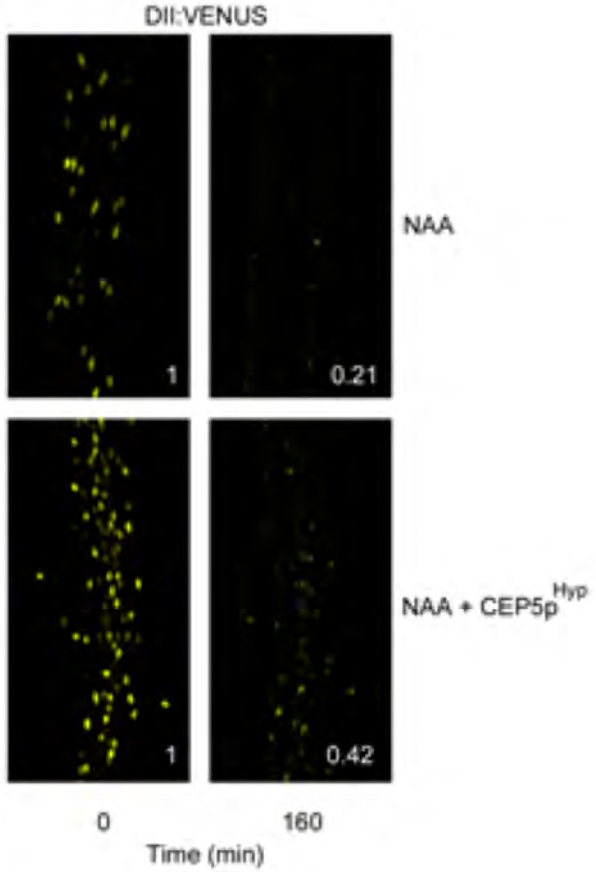

Figure 26. CEP5 stabilises DII:VENUS fluorescence under auxin challenge. 1 µM NAA causes DII:VENUS fluorescence to fall from a relative starting value of 1 to 0.21 within 160 minutes, whilst $CEP5^{Hyp}$ treated DII:VENUS fluorescence degradation is slowed by approximately half.

Further investigation showed that the effect of CEP5 upon DII:VENUS fluorescence was even more pronounced when seedlings were pre-incubated with $CEP5^{Hyp}$ for 18 h prior to auxin challenge (Fig 27c), presumably due to the greater level of peptide uptake afforded by this timeframe.

It was also investigated whether a similar effect of CEP5 prolonging DII:VENUS fluorescence could be observed in the CEP5 perturbed expression line crosses with DII:VENUS, thus providing a more direct, endogenous assessment of whether CEP5 levels correlate with DII:VENUS fluorescence.

CEP5 overexpression x DII:VENUS lines had strikingly prolonged fluorescence compared with Col-0 x DII:VENUS controls when challenged with auxin (Fig. 27d).

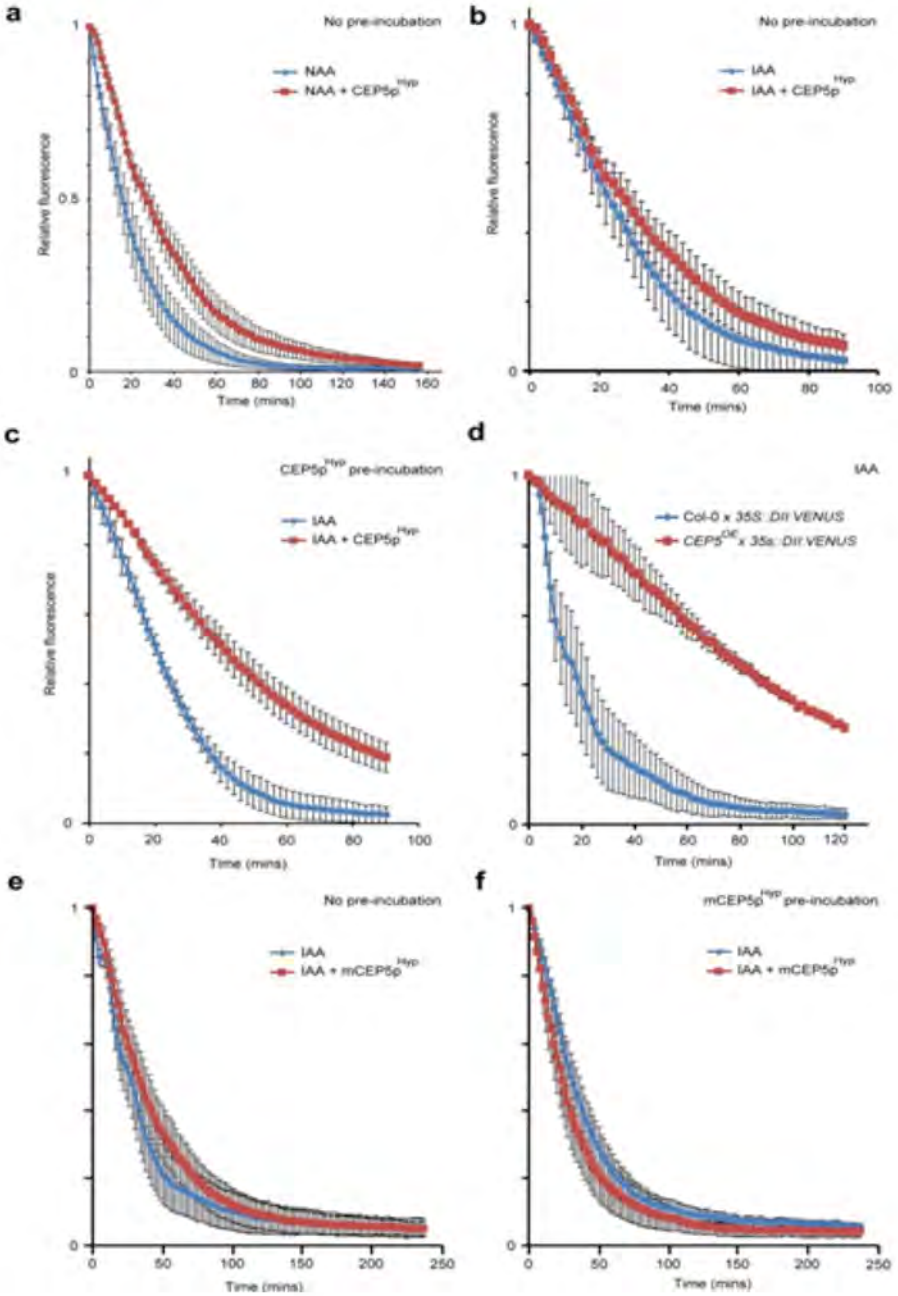

Figure 27. DII:VENUS signal degradation curves under auxin challenge. (a & b) Co-treatment with exogenous synthetic CEP5^{Hyp} slows auxin-mediated DII:VENUS signal degradation over 180 mins (NAA) (a) and 90 mins (IAA) $p = <0.05$ according to Student's T-Test. (b). (c) Preincubation of DII:VENUS seedlings for 18 h with CEP5^{Hyp} dramatically prolongs DII:VENUS fluorescence over 90 minutes (IAA). $p = <0.05$ according to Student's T-Test. (d) Endogenously increased CEP5 levels also prolong DII:VENUS fluorescence under auxin challenge in CEP5OE x DII:VENUS F1 crosses over 120 mins (IAA). $p = <0.05$ according to Student's T-Test. (e & f) Exogenous application with a CEP5 peptide containing 4 amino acid substitutions was not able to extend DII:VENUS fluorescence under auxin challenge over 240 mins in either co-treated (e) or 18 h pre-incubated (f) conditions (IAA). In all cases, peptide concentration is 5 µM and auxin (IAA or NAA) concentration is 1 µM. N = ≥ 4 seedlings per condition. Error bars are ± 1 S.E.

4.2.6 Localisation of CEP5 peptide

In order for CEP5 to directly act on stabilising AUX/IAA proteins, CEP5 would have to be localised to the nucleus. Whilst it has not been possible to conclusively prove this, several pieces of evidence suggest this is possible. The precursor to CEP5 contains a putative N-terminal secretion sequence; whilst it might seem counterintuitive for a protein to contain both a secretion signal and a nuclear localisation signal, there are in fact several biological examples of this phenomenon, particularly in proteins which undergo proteolytic processing (reviewed in (Arnoys and Wang 2007)).

Two separate bioinformatics approaches were used to query possible localisations for CEP5. Balanced Subcellular Localization Predictor (BaCElLO) uses algorithims based on sequence and alignment profiles to predict five classes of subcellular localisation (secretory pathway, cytoplasm, nucleus, mitochondrion and chloroplast) in a kingdom-specific manner (animals, plants or fungi) with an approximate accuracy score of 67% in plants.

Full-length precursor and cleaved motif sequence for CEP5 was queried using BaCelLO. CEP5 full length and/or N-terminal precursor sequence was predicted to be targeted to the nucleus. The full length precursor sequence of CEP5 was predicted to be targeted to the nucleus, whilst the cleaved portion was predicted to enter the secretory pathway (Fig. 28a).

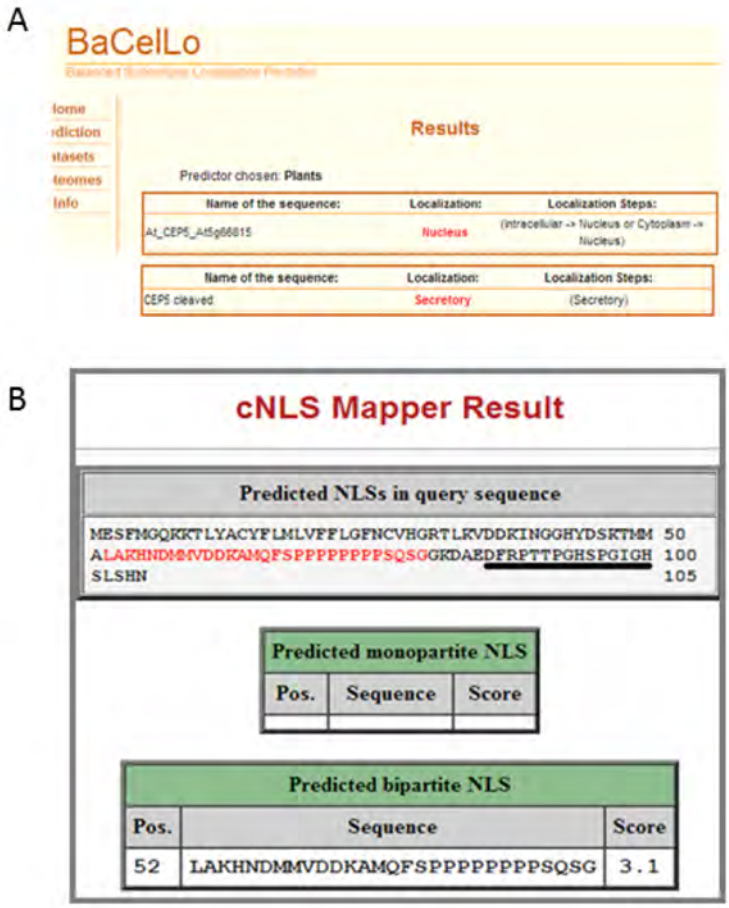

Figure 28. Bioinformatical predictions of CEP5 localisation. (A) BaCelLo predicts a nuclear localisation for the full length precursor sequence of CEP5 (represented by accession number At_CEP5_At5g66815) and a secretory pathway localisation for cleaved CEP5 sequence. (B) cNLS Mapper predicts a nuclear localisation in part of the CEP5 precursor sequence (highlighted in red). The cleaved portion of CEP5 is underlined in black. Overall cNLS predicted CEP5 to have dual nuclear cytoplasmic localisation.

Another bioinformatics program, cNLS Mapper, predicts whether a protein is likely to be targeted to the nucleus by extracting putative NLS sequences with a score equal to or more than the selected cut-off score. Higher scores indicate stronger NLS activities: thus a GUS-GFP reporter protein fused to an NLS with a score of 8, 9, or 10 is likely to be exclusively localised to the nucleus, a score of 7 or 8 represents partial localisation to the nucleus, a score of 3, 4, or 5 is localised to both the nucleus and the cytoplasm, and a score of 1 or 2 is likely to be localised exclusively to the cytoplasm (Kosugi, Hasebe *et al*. 2008, Kosugi, Hasebe *et al*. 2009, Kosugi, Hasebe *et al*. 2009). The full length/precursor sequence of CEP5 is predicted to have a bipartite NLS sequence shortly before the cleaved CEP motif. Overall, CEP5 was given a

score of 3.1, suggesting a dual localisation in agreement with the prediction obtained from BaCelLo (Fig. 28b).

To see if CEP5 could be directly detected in the nucleus, nuclear protein extracts were prepared from Col-0, CEP5^{RNAi} and CEP5^{OE}. Bands corresponding to the predicted cleaved molecular weight of CEP5 (~1.6 kDa) were subsequently detected in the nuclear extracts. The two band sizes observed are expected to represent hydroxyprolinated and non/differentially hydroxyprolinated CEP5 peptide species (Fig. 29a).

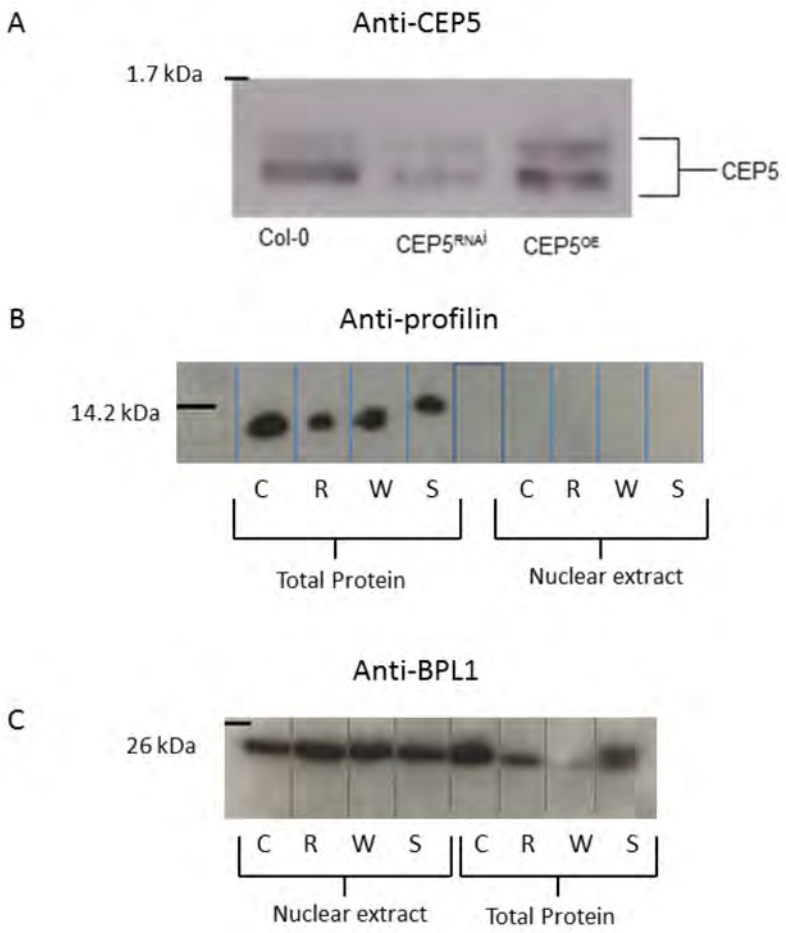

Figure 29. CEP5 nuclear extract western blots. (A) Anti-CEP5 antibody western blot performed on nuclear protein extractions from Col-0, CEP5^{RNAi} and CEP5^{OE} lines. Bands are thought to correspond to the cleaved unmodified and cleaved modified size of CEP5 peptide respectively (~1.6 kDA). (B) Anti-profilin antibody western blot performed on total protein and nuclear protein extractions from Col-0 (C), CEP5^{RNAi} (R) and a weak (W) and strong (S) CEP5 overexpression line. Cytoplasmic profilin contamination was not detected in the nuclear extracts. (Profilin 1: 14.2 kDa. (C) Anti-BPL1 antibody western blot performed on total protein and nuclear protein extractions from Col-0 (C), CEP5RNAi (R) and a weak (W) and strong (S) CEP5 overexpression line. BPL1 was detected in all samples, thus ER contamination of nuclear extracts cannot be ruled out at this point. (BPL1: 24.6 kDa).

Whilst CEP5 was detected in nuclear extracts, the possibility remained that the nuclear extracts could be contaminated with cytoplasmic protein, giving an inaccurate nuclear localisation. To exclude this, the nuclear extracts were tested using antibodies specific to the exclusively cytoplasm-localised cytoskeletal protein profilin. Profilin was detected in total protein but not nuclear protein extracts (Fig. 29b), confirming the purity of the nuclear extracts from cytoplasmic protein contamination.

One other possible source of contamination is the endoplasmic reticulum, which could potentially remain connected to the nuclear membrane during the nuclear protein extraction protocol and contain CEP5 peptide. To test for this, nuclear extracts were tested using an antibody specific for BPL1, a protein specifically localised to the ER (Ferguson 2012). Bands corresponding to the expected size of BPL1 were found in all samples for both total protein and nuclear protein extracts. This may be indicative of endoplasmic reticulum contamination. Whilst methods such as subcellular fractionation could potentially remove endomembrane contamination from nuclear extracts to more conclusively address the potential nuclear status of CEP5, the lack of a CEP5 knockout for verification purposes meant this approach was not a particularly useful option at this current time and thus was not performed.

4.3 Discussion

The results presented in this chapter suggest that the small posttranslationally modified peptide CEP5 antagonises auxin signalling, and this antagonism is

dependent on the stabilisation of AUXIN/INDOLE ACETIC ACID (AUX/IAA) transcriptional repressors, with the potential existence of an additional control mechanism through which plants can attenuate auxin signalling in a developmental context.

In addition to the auxin-antagonistic phenotypes seen in genetic approaches in Chapter 2 (Roberts, Smith *et al*. 2013), CEP5 is demonstrated to slow the auxin-driven process of gravitropism. On the face of it, this could be due to either an effect on auxin transport or an effect on auxin signalling; analysis of auxin signalling and transport mutants, as well as experiments using the AUX/IAA-sequence based reporter DII:VENUS, in this chapter suggests the latter to be more likely (data from collaborators have shown no differences in free auxin levels between CEP5 expression perturbation lines and wild type, thus any effect on synthesis or global-level auxin conjugation is unlikely) (Karin Ljung, personal communication).

The potential involvement of the PIN1 and PIN2 auxin transporters was not adequately interrogated here due to technical difficulties and time constraints, and thus a potential effect of CEP5 on auxin transport cannot be dismissed at this point. There are many other aspects of auxin transport, e.g. ABCB transporters and the dual-affinity nitrate-auxin transporter NRT1.1, which have also not been investigated here by any genetic, biochemical or pharmacological methods. Notwithstanding this, the effect of NAA and IAA (which have different transport properties) on CEP5-mediated stabilisation of DII:VENUS fluorescence is similar, suggesting CEP5 is unlikely to have any direct effect on auxin uptake or transport.

As well as DII:VENUS, CEP5 was tested on the auxin reporter DR5:VENUS, which is a transcriptional reporter whereby VENUS fluorescence corresponds (non-linearly) to auxin-driven transcriptional output. As would be expected if the negative AUX/IAA auxin signalling regulators were stabilised, a small decrease in DR5:VENUS fluorescence was observed in the CEP5-treated line (see Appendix III); however, the difference was not statistically significant, and due to significant levels of fluorescence fluctuation observed even between untreated seedlings in this line, this experiment should be improved and repeated before drawing any firm conclusions.

Whilst CEP5 is shown to lead to stabilisation of the AUX/IAA protein based DII:VENUS reporter here, the mechanical basis of this stabilisation is unknown; it is unclear whether this effect is direct AUX/IAA stabilisation (for example, through slowing ubiquitin mediated proteasomal degradation) or if this effect is downstream of a general reduction in auxin perception by receptors such as $TIR1^{SCF}$. Data from collaborators also shows no change in expression of auxin receptors such as TIR1 (see Appendix X, Figure S14), so a simple reduction of auxin receptor-driven AUX/IAA degradation seems unlikely. In addition, CEP5 does not appear to directly compete with auxin for binding to TIR1 (Stefan Kepinski, personal communication). Additionally, unlike for the similar small posttranslationally modified peptide TDIF, no effect on ARF7 phosphorylation was detected for CEP5 (Cho, Ryu *et al*. 2014) (Appendix X).

Although DII is based on the degron sequence from IAA28, other full-length AUX/IAAs were also stabilised by CEP5 (IAA19, IAA12/BODENLOS) (See Appendix X, Figure 5f and S13). Future work

should examine the extent to which other full-length AUX/IAA proteins are stabilised by *CEP5* overexpression or exogenous application (possibly including non-canonical AUX/IAAs lacking DII domains, such as IAA20 or IAA30 for comparison).

Another experiment which is a priority for future work is quantification of cell size in exogenous peptide treated or CEP5-peturbed expression lines, as a change in cell size could lead to more or less fluorescent nuclei being included in the analysis (whilst the area of root analysed for the DII::VENUS fluorescence quantifications was the same in all conditions, the exact number of nuclei within the area were not counted or standardised). Whilst looking at DII:VENUS images does indeed suggest individual nuclei are brighter under CEP5 treatment and that this -rather than mere differences in number of nuclei- is responsible for the differences in overall fluorescence (Fig. 26), future experiments should investigate whether cell size differs and quantify DII::VENUS fluorescence in individual and/or set numbers of nuclei.

For a direct stabilisation of AUX/IAA proteins to occur, CEP5 would need to be localised to the nucleus. The bioinformatical and biochemical data presented in this chapter suggest this could be the case; however this cannot be proven conclusively. The lack of availability of a CEP5 loss-of-function knockout currently precludes the ability to adequately verify CEP5 localisation using western blots. There are several approaches which could help to address this. Firstly, a CEP5 knockout could be generated using alternative methods to T-DNA insertion, for example CRISPR-Cas9. Secondly, CEP5 could be tagged using a fluorescent probe. Previous attempts to make traditional CEP5-fluorescent protein reporters have failed (Ive De Smet, personal

communication). There are several potential reasons for this. Firstly, the CEP5 precursor protein is cleaved in several places to produce the mature motif, and fluorescent proteins may be cleaved off in this process. One potential solution could be to make a construct using only the mature sequence of CEP5 driven by the native promoter; however, this still suffers from another problem with using fluorescent protein reporters, which is that at 26 kDa, GFP (and similar proteins) are considerably larger than the 1.6 kDa mature CEP5 peptide, and thus could adversely affect CEP5 localisation, especially if CEP5 requires secretion into the apoplast for function as is the case for most small posttranslationally modified peptides studied thus far (Matsubayashi 2014).

Therefore, attempts were made to fluorescently label CEP5 using low molecular weight Succinimidyl esters (Alexa Fluor 488 NHS ester). These attempts were initially unsuccessful. However, attempts to label the similar small posttranslationally modified peptide IDA were also unsuccessful until the addition of an N-terminal valine residue to the sequence (Butenko, Wildhagen *et al*. 2014). Addition of this residue led to successful labelling with only minor effects on bioactivity, and thus a similar strategy might prove successful for CEP5 in the future. Alternative approaches that also could be tried are immunofluorescence techniques using anti-CEP5 antibodies, or immunogold/radiolabelling.

CHAPTER 5: GENERAL DISCUSSION

The data presented in this thesis shows that the small posttranslationally modified peptide CEP5 modifies root architecture in *Arabidopsis* - most noticeably through negatively regulating lateral root development – and that this phenotype may be due to CEP5 antagonising auxin signalling through an as-yet not fully determined mechanism (Chapter 2 and 4). CEP5 also has minor effects on aboveground plant architecture in *Arabidopsis* (Chapter 2). Shortening of the primary root upon exogenous peptide application or overexpression was shown to occur for CEP5 as it does in CEP1 and other small peptides such as the CLE family (Fiers, Golemiec *et al*. 2005, Ohyama, Ogawa *et al*. 2008, Czyzewicz, Shi *et al*. 2015), and this effect was shown to not be due to nonspecific peptide stress on the plant - thus confirming that primary root length can be used as a surrogate indication of bioactivity for peptides (Chapter 2). In addition, information regarding the importance of individual residues in the CEP5 sequence was obtained, as well as the importance of hydroxylation of the proline residues of CEP5 to bioactivity (Chapter 2). The potential interaction of CEP5 with various signalling pathways and phytohormones such as cytokinin and giberellin was explored (Chapter 3), and the original hypothesis that CEP5 might represent the ligand for ACR4 was dismissed based on experimental data.

Many of the phenotypes and bioactivities for CEP1 reported by (Ohyama, Ogawa *et al*. 2008) were also observed for CEP5; however, additional specific observations were made for CEP5 – such as increased

stabilisation of DII:VENUS fluorescence – supporting the idea that the diversity of CEP peptides is not merely due to redundancy and instead are indicative of specialised physiological processes. The distinct expression patterns and transcriptional regulation for the CEP family peptides reported in (Roberts, Smith *et al*. 2013) also supports this, as does the striking differences in bioactivity levels observed for many CEP5 peptide species where even a single residue differs (Chapter 2).

The findings above have certain limitations; the role and exact mechanism of CEP5 in the natural biological context is still unclear at this stage. The developmental process of lateral root organogenesis is a complex, tightly controlled process - the overarching regulator of which is the phytohormone auxin. Lateral root branching is an adaptive mechanism influenced by several abiotic factors, including soil nutrient status. It is admittedly unexpected that a phloem pole pericycle-expressed signal would have such an effect on a process which initiates at the xylem pole pericycle: it is possible that during lateral root formation, CEP5 might assist in dampening auxin responses in neighbouring tissues of xylem pole pericycle cells undergoing lateral root initiation, thereby repressing cell division in the flanking cells. The antagonistic relationship between auxin and CEP5 could be important in regulating auxin response thresholds and fine-tuning (very sensitive) auxin responses during growth and development, potentially through negative regulation of auxin signalling - either directly or indirectly.

The data presented in Chapter 2 show that CEP5 has a negative regulatory effect on lateral root branching, whilst the data presented in Chapter 4 show CEP5 has an antagonistic effect on auxin signalling. Given the

importance of auxin in driving lateral root formation, it is not surprising that these two findings should be connected. The question remains how these findings fit within the wider field of research, as during the course of this project, several surprising new discoveries have been made regarding CEP peptides. Firstly, that CEP peptides may be involved in communicating nitrate starvation (Tabata, Sumida *et al*. 2014). Secondly, that CEP peptides are involved in nodulation in *Medicago* (Imin, Mohd-Radzman *et al*. 2013, Mohd-Radzman, Binos *et al*. 2015). Thirdly, that CEP-motif peptides are produced by root parasitic nematodes and are involved in the creation of feeding sites (Eves-van den Akker, Lilley *et al*. 2016). Whilst these findings may seem to be unconnected at first glance, it is possible that all in fact represent aspects of integration between nutrient sensing, auxin, and root architecture via control of cell division.

In terms of nitrate perception, CEP peptides have been proposed to act as root-derived ascending N-demand signals to the shoot, where they are perceived by CEP receptors (CEPRs/XIP), leading to the production of a putative shoot-derived descending signal that up-regulates nitrate transporter genes in the roots, including NRT1.1 (Bryan, Obaidi *et al*. 2012, Tabata, Sumida *et al*. 2014). However, the downstream mechanism remains largely elusive and other potential mechanisms have not been explored. The work presented in (Tabata, Sumida *et al*. 2014) and the findings of this thesis in fact are not mutually exclusive: nitrate has opposing influences on lateral root development dependant on the concentration and distribution of nitrate within the soil. For example, globally high nitrate is repressive of lateral root elongation, whilst localised nitrate-rich patches in otherwise nitrate poor soil

promotes lateral root elongation within the nitrate-rich area. Thus, the idea that CEP5 both communicates nitrate starvation and negatively regulates lateral root formation makes sense, as this would prevent the plant wasting resources on the formation of lateral roots in locally nitrate-poor areas. Furthermore, this process is likely to involve auxin for several reasons, which are discussed below.

One obvious potential area of nitrate and auxin overlap is the dual nitrate/auxin transporter NRT1.1, which Tabata *et al.* (2014) found to be upregulated downstream of CEP1. NRT1.1 facilitates uptake of the phytohormone auxin at low NO_3^- availability and is highly expressed in the primary root tip and lateral roots. NRT1.1 has a repressive effect on lateral root formation due to NRT1.1 promoting basipetal transport of auxin out of lateral roots following initiation and early primordia formation, causing arrest of LR elongation (Krouk, Lacombe *et al*. 2010) (Fig, 30). Considering the role of NRT1.1 in transporting auxin out of lateral root primordia, the auxin antagonism and repression of lateral roots by CEP peptides such as CEP5 could potentially be due to NRT1.1-mediated increased basipetal auxin transport away from lateral root tips. Furthermore, this upregulation of NRT1.1 could change the flux of auxin within the root as a whole, and lead to differences in lateral root distribution similar to those observed for other peptides known to elicit changes in auxin distribution, such as CLE26 and CLE45 (Czyzewicz, Shi *et al*. 2015, Rodriguez-Villalon, Gujas *et al*. 2015). In agreement with this model, *NRT1.1*, *AtCEP5* and *MtCEP1* have overlapping expression patterns in root tips and young lateral roots, and MtCEP1-induced

root nodule formation is resistant to high N availability, a condition which is normally strongly repressive of nodulation (Imin, Mohd-Radzman *et al*. 2013).

Whilst CEP5 was not shown to be a likely candidate ligand for ACR4 based on the work performed in Chapter 3 and Appendix I, photoaffinity labelling does show binding of CEP1 to XIP1/CEPR1 and CEPR2 (Tabata, Sumida *et al*. 2014). Whilst CEP5 was not demonstrated by photoaffinity experiments to bind to these receptors in the study by Tabata *et al*., CEP5 was sufficient to destabilise CEP1-XIP1/CEPR1 interaction, suggesting an element of competitive binding to receptors by different CEPs. If this assumption is correct, differential binding of CEP peptides to the same receptor could also explain differences in CEP bioactivities, for instance the difference in DII:VENUS fluorescence stabilisation seen between CEP1 and CEP5 in Chapter 4.

Interestingly, loss-of-function XIP1/CEPR1 mutants demonstrate reduced auxin-inducibility of several lateral root initiation-associated genes including *PIN1*, *LBD16*, *LBD29* and *ARF19*, and thus it fits that CEP5 requires perception by XIP1/CEPR1 in order to exert the auxin antagonistic effects shown in Chapter 4. Furthermore, whilst *CEP5* overexpression lines have an expected increase in the abundance of proteins associated with nitrate/nitrogen compared with controls, they are also enriched in proteins associated with various hormones, including auxin (Appendix X).

Downstream of the CEP-XIP/CEPR1 and/or CEPR2 interaction, Tabata *et al.* (2014) suggest that a putative shoot-descending signal is required for the upregulation of nitrate transporter genes in the roots – based on the findings presented in Chapter 4 and above, this could potentially be auxin. Microarray data supports *NRT2.1* expression being upregulated by auxin (Kiba, Kudo *et al*. 2011), though it is less clear whether the expression of *NRT3.1* and *NRT1.1* also are. Whilst *NRT1.1* expression has been reported to be strongly induced by auxin (Guo, Wang *et al*. 2002), this finding was not replicated by other collaborators (Ive De Smet, personal communication).

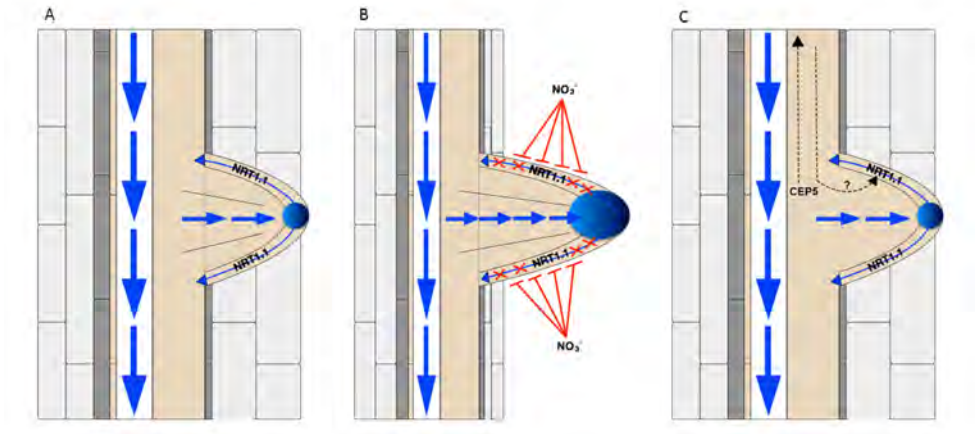

Figure 30. Comparison of NRT1.1 function in low nitrate and high nitrate conditions, and a model of possible CEP5 action. In low soil nitrate abundance (A) NRT1.1 promotes basipetal transport of auxin (blue arrows) away from the lateral root tip, inhibiting root elongation. In high nitrate abundance (B) NRT1.1-mediated transport of auxin is inhibited, leading to accumulation of auxin and increased lateral root elongation. (C) CEP5 is proposed by Tabata *et al.* (2014) to be perceived by the shoot-expressed receptors XIP1/CEPR1 and CEPR2: a putative shoot-descending signal then upregulates NRT1.1 expression, potentially directing auxin flux away from the lateral root tip and inhibiting root elongation. Figure based on Beeckman & Friml 2010.

In terms of linking the phenotypes seen in Chapter 2 with the phenotypes observed by Tabata *et al.*, loss of function XIP1/CEPR1 and CEPR2 mutants have enhanced lateral root elongation - which corresponds with the negative effect of CEP5 on this process, further supporting that XIP1/CEPR1 and

CEPR2 represent the receptors for CEP5. However, *xip1/cepr1* and *cepr2* also have typical nitrate starvation phenotypes - namely pale green leaves, dwarfism and anthocyanin accumulation. Based on this observation, an opposing phenotype might be expected in *CEP5* overexpression lines: this does not seem to be the case from our observations in both weak and strong overexpression lines (Chapter 2) (Roberts, Smith *et al*. 2013). Furthermore, no nitrate starvation phenotype was observed in CEP5 RNAi-knockdown lines or reported for CEP3 knockout lines (Delay, Imin *et al*. 2013, Roberts, Smith *et al*. 2013). However, since very excessive nitrate abundance can also lead to some of the same phenotypes as nitrogen deficiency (such as stunting), it will be interesting to quantify overexpression lines of either *CEP* peptides or *XIP1/CEPR1* and *CEPR2* for other phenotypes associated with excessive nitrate abundance, such as 'burning', darker leaves, and reduced flower and fruit number, comparisons of nitrate sensitivity/tolerance in *CEP* knockdown/knockout/overexpression lines, and/or direct measurements of nitrate uptake through quantification of $^{15}NO_3^-$ uptake.

It is possible that certain CEP peptides are more important in nitrate starvation response than others: the fact that CEP peptides appear to be differentially regulated in response to nitrate supports this (Chapter 2) (Roberts, Smith *et al*. 2013). For example, based on transcriptomic meta-analysis, *CEP1* and *CEP9* expression is upregulated by nitrate, whilst *CEP3*, *CEP5*, and *CEP13* expression is downregulated by it. Tabata *et al*. also found different levels of CEP peptide expression in response to nitrate starvation (Tabata, Sumida *et al*. 2014). Interestingly, CEP5 expression was found by this analysis to be upregulated by phosphate (Chapter 2) (Roberts, Smith *et al*.

2013), leading to the tantalising possibility that CEP peptides are involved in sensing and/or regulation of root architecture responses to nutrients besides nitrate. In support of this idea, the level of *CEP* expression in response to nitrate depletion varies considerably: at 6, 24 and 48 hours post nitrate depletion: by 12 hours, *CEP3* and *CEP7* expression increases ≥10 fold, whilst expression of *CEP4*, *CEP5* and *CEP11* changes slightly, or not at all (Tabata, Sumida *et al*. 2014). Microarray analysis found that auxin also regulates AtCEP peptides differently, with *CEP1* and *CEP3* expression induced by auxin, whilst *CEP5* and *CEP9* expression is downregulated by auxin (Chapter 2, Roberts *et al.* 2013, Table 2.) It is not yet known if auxin also upregulates *CEP7*, but it would be interesting to see if there is overlap between CEP genes known to be strongly induced by nitrate depletion with *CEP* genes induced by auxin, providing further evidence for the existence of crosstalk between CEP-mediated N-demand signalling and phytohormones. The lack of induction by nitrogen depletion and/or auxin for certain CEPs also suggests that some CEP peptides might play bigger roles in communicating nitrate status/crosstalk with auxin than others, with the other CEPs perhaps more involved in developmental or other signalling roles.

Tabata *et al.* (2015) propose that CEP peptides act as root-derived ascending N-demand signals which are perceived by CEP receptors in the shoot: however, *CEP* peptides are not exclusively expressed in the root: in fact many *CEP* peptides are expressed in aboveground tissues including shoots, inflorescences, siliques and cotyledons/leaves (Roberts, Smith *et al*. 2013). The CEP receptors themselves are also not exclusive to the shoot: *XIP1/CEPR1* is expressed in the phloem and procambium regions in roots

(Bryan, Obaidi *et al*. 2012). In light of this, it would be interesting to examine whether ectopic overexpression of *CEP* peptides in the shoot or exogenous application of synthetic CEP peptides to shoot tissues only, could also induce *NRT* overexpression and lateral root repression. Considering the proximity of *NRT1.1* with *XIP1/CEPR1* and *CEP* expression in the root, the benefit of the proposed CEP peptide translocation to the shoot is unclear. In *Medicago* MtCEP1 was able to inhibit lateral roots and promote circumferential cell proliferations (CCPs) in root cell cultures lacking intact shoots, providing further evidence that CEP peptides may act locally (Imin, Mohd-Radzman *et al*. 2013).

The specificity of spatiotemporal expression and downstream responses to individual CEP peptides is reminiscent to the actions of other specific peptides from the same family leading to distinct fates, such as EPFL4 and EPFL6 (Lee, Hnilova *et al*. 2015). EPFL4 and EPFL6 exert. Future studies assessing the binding/binding affinities of different CEP-family peptides to the XIP1/CEPR1 and CEPR2 respectively, including whether both receptors are necessary and sufficient to perceive all CEP-family members or whether they exhibit specificity (or involve further co-receptors) could prove valuable. This should be coupled with downstream analysis of gene induction and developmental/phenotypical responses. Taken together, these future enquiries could explain the observed diversity of CEP family members, as neither simple redundancy or gene duplication currently appear to be entirely satisfactory explanations (Roberts, Smith *et al*. 2013, Vie, Najafi *et al*. 2015).

Whilst it appears increasingly certain that CEP5 is perceived by XIP1/CEPR1 and CEPR2, and that downstream of this interaction there is

antagonism of auxin signalling which is a likely driver in the negative regulatory effect on lateral root development seen in Chapter 2, there are still many gaps in our understanding of the pathway downstream of CEP peptides, and at what stage or signalling module auxin is involved. Several other similar small peptides have also been shown to interact with auxin and other phytohormones; potentially, CEP5 might act through similar pathways to these. The small posttranslationally modified peptide TDIF/CLE41/CLE44 was shown to directly affect auxin signalling via a TDIF-TDR-BIN2 signalling pathway (Cho, Ryu *et al*. 2014); however, no ARF phosphorylation was observed following CEP5 treatment, so this auxin antagonistic mechanism may not apply for CEP5 (Appendix X). Elsewhere, several peptides have been shown to have an effect on auxin transport: RGF/GLV/CLEL peptides regulate the formation of auxin gradients in a PIN2-dependant manner (Whitford, Fernandez *et al*. 2012), whilst auxin distribution is also altered by CLE26 and CLE45 (Czyzewicz, Shi *et al*. 2015, Rodriguez-Villalon, Gujas *et al*. 2015). The possibility of CEP5 affecting PIN based auxin transport should be a priority for future work, especially since recent data has shown reduced auxin-inducibility of *PIN1* in loss-of-function XIP1/CEPR1 mutants. Furthermore, there is strong evidence for cross talk between CEP peptides and auxin in species beyond *Arabidopsis*: in *Medicago* plants co-treated with NAA and MtCEP1 D1 have 10-fold fewer lateral roots than plants treated with NAA alone. Additionally, the auxin-responsive GH3:GUS reporter showed stronger staining of vascular tissues in MtCEP1 D1 and D2 peptide treated plants compared with untreated controls and also strong staining in underlying vascular tissue/surrounding inner cortical cells at circumferential cell

proliferation sites (Mohd-Radzman, Binos *et al*. 2015). Whilst strong GH3:GUS staining was observed in lateral root primordia in *Medicago*, these auxin maxima were not sufficient to promote lateral root elongation in the presence of MtCEP1 D1; suggesting CEP peptides can alter auxin sensitivity or distribution. According to microarray analysis, auxin also regulates AtCEP peptides differently, with *CEP1* and *CEP3* expression induced by auxin, whilst *CEP5* and *CEP9* expression is downregulated by auxin (Chapter 2) (Roberts, Smith *et al*. 2013). The fact *CEP5* is downregulated by auxin whilst also antagonising auxin signalling also suggests the possible existence of a regulatory feedback loop. Furthermore, expression of key auxin signalling and transport proteins are reduced in both *CEP5* overexpression lines (reduced auxin-induced expression of *IAA18*, *LBD16*, *LBD29* and *PIN1*) and in XIP1/CEPR1 loss-of-function mutants (reduced auxin-induced expression of *PIN1*, *LBD16*, *LBD29* and *ARF19*) supporting that altered auxin response and/or distribution is an important aspect of CEP5 function (Appendix X).

In addition to roles in lateral root architecture and/or nitrate sensing, CEP peptides have been shown to be involved in nodulation and in the formation of feeding sites by parasitic nematodes (Bobay, DiGennaro *et al*. 2013, Imin, Mohd-Radzman *et al*. 2013, Mohd-Radzman, Binos *et al*. 2015, Eves-van den Akker, Lilley *et al*. 2016). This intriguing role in rhizosphere plant-organism interactions could hold clues to the molecular mechanism of CEP peptides, in particular pointing to a role upstream of cell division.

CEP peptides have been shown to play a role in promoting nodulation in the leguminous model organism *Medicago truncatula*. Both overexpression of MtCEP1 and exogenous application of MtCEP1 resulted in inhibition of

lateral root formation, enhancement of nodulation, and the induction of periodic circumferential root swellings (Imin, Mohd-Radzman *et al*. 2013, Mohd-Radzman, Binos *et al*. 2015). Similarly to lateral roots and nematode-induced syncytia, some of these circumferential roots swellings arise from pericycle tissues, although cortical and epidermal tissues also contribute. In agreement with a role for CEP peptides in communication of nitrate status, enhancement of nodulation by MtCEP1 occurs even under high nitrate conditions, where nodulation is normally supressed.

Peptides containing a CEP motif are known to be secreted by several species of plant parasitic nematodes, including root knot nematodes and cyst nematodes of the *Meloidogyne* spp. and *Heteroderidae* spp. CEP peptides are not unique in this regard: peptides functionally analogous to CLAVATA3/ESR peptides have been found in cyst nematodes (Olsen and Skriver 2003, Lu, Chen *et al*. 2009) and crucially, these peptides can complement *Arabidopsis* lines with loss-of-function mutations in endogenous CLE3 - whilst overexpression of the nematode peptide induces a phenotype reminiscent of WUSCHEL downregulation (Wang, Mitchum *et al*. 2005). Certain plant parasitic nematodes such as *Heterodera schachtii* are known to induce syncytial feeding sites in host plant roots. Like lateral roots, these syncytia initiate within tissues proximal to the vasculature – normally a single pericycle, procambial or inner cortical cell. Subsequently these cells fuse with surrounding cells and the site undergoes hypertrophy (Wieczorek and Grundler 2006, Eves-van den Akker, Lilley *et al*. 2016). The syncytia-forming plant-parasite *Rotylenchulus reniformis* produces C-terminally Encoded Peptide (CEP) plant hormone mimics (RrCEPs): intriguingly, exogenously applied

synthetic peptide based on domain 1 of RrCEP1 also reduces primary root elongation and upregulates AtNRT2.1 expression - as reported for plant endogenous CEPs by Tabata *et al*. Additionally, when the syncytia-forming parasitic nematode *Heterodera schachtii* - which does not produce CEP peptides - infects *Arabidopsis* in a CEP-rich environment, a smaller feeding site is produced. The reason for this encouragement of nitrate uptake and concurrent restriction of feeding site size is proposed to be due to the requirement of the parasitic nematode to obtain adequate nutrition, whilst minimising the risk of killing the host (Eves-van den Akker, Lilley *et al*. 2016).

In all three processes of lateral root formation, nodulation, and nematode feeding site formation, control of cell division is a crucial aspect. Cell division is dependent on the interactions of cyclins and cyclin-dependant kinases (CDKs); Kip-related proteins (KRPs) are inhibitory to CDKs, with docking of KRPs to CDKs inducing cell cycle arrest or delaying cell cycle progression and thus reducing cell division rate. In *Arabidopsis*, KRP4 has been found to negatively regulate root architecture downstream of the glutamate receptor-like protein GLR3.6. Loss-of-function mutants for GLR3.6 show many of the same phenotypes as CEP5 overexpression lines, such as reduced primary root length, reduced lateral root density and a smaller root apical meristem (Singh, Chien *et al*. 2016). This is interesting in terms of linking nitrate perception with a lateral root phenotype, because in nitrate sensing mechanisms in various organisms, nitrate requirement is not sensed directly but via perception of the abundance of glutamate (which requires nitrate for synthesis) (Reviewed in (Chantranupong, Wolfson *et al*. 2015)).

Thus, high glutamate corresponds with low nitrate demand, and vice versa. This could represent a model whereby glutamate perception by glutamate receptors leads to inhibition of KRP4, which in turn inhibits cell division leading to reduced root growth, providing an elegant mechanism connecting nitrate demand with root architecture. Additionally, GLRs are thought to have channel-forming properties which contribute to Ca^{2+} flux and signalling (Kim, Kwak *et al*. 2001, Singh, Chien *et al*. 2016); Ca^{2+} signalling is known to affect PIN polarity and thus auxin flux and changes in cytosolic Ca^{2+} affect lateral root initiation (Benjamins, Ampudia *et al*. 2003, Zhang, Vanneste *et al*. 2011). Thus, if CEP peptides work by increasing expression of nitrate transporters and this in turn leads to an increase in nitrate uptake, this could explain both the lateral root phenotype and the antagonistic relationship with auxin seen in Chapters 2 and 4. In support of this idea, MtCEP peptides –known to promote nodulation and the acquisition of nitrate whilst simultaneously inhibiting lateral root formation in Medicago - altered the auxin distribution pattern seen in GH3:GUS lines when exogenously applied (Mohd-Radzman, Binos *et al*. 2015).

5.1 Other Future Work

In addition to the work proposed to address the limitations of this thesis in the discussions at the end of Chapters 2-4 and in the general discussion above, there are several other aspects of CEP processing and function on a biochemical/structural level which could be interrogated by further work. CEP family peptides require enzymatic cleavage from a precursor protein and posttranslational modification in order for activation. The enzymes and target sequences involved in these processes are not well characterised for the majority of small posttranslationally modified peptides (See Introduction 1.6.2 'Peptide signal processing and maturation'), and the specific enzymes involved in cleavage of CEP motifs from the precursor protein remain unknown.

Plants express many subtilisin-like enzymes which might represent candidates for peptide processing. A subtilisin-like Ser protease, SBT1.1, is required for processing of the PSK4 precursor (Srivastava, Liu *et al*. 2008), whilst overexpression of *SBT5.4* gives a *clv*-like phenotype - though it was not demonstrated to cleave CLV3 in vitro (Liu, Srivastava *et al*. 2009). Furthermore, SITE 1 PROTEASE (S1P) has been shown to be required in the processing of RALF23 (Srivastava, Liu *et al*. 2009). Recently, METACASPASE-9 and CDR1 have also been suggested as potential processing enzymes for AtPEP1 (Bartels and Boller 2015). Due to the large number of subtilisin-family and other proteases - and the pleiotropic actions of many of these processing enzymes - identification of which are involved in CEP5 processing may prove difficult. One approach would be to use mass

spectrometry to analyse different CEP5 species in candidate protease loss-of-function and/or expression perturbation lines to see if there are obvious shifts in the ratio of cleaved to uncleaved CEP5 product compared with the wild type.

CEP-family peptides are generally hydroxylated on 2–3 prolines. Proline hydroxylation is catalysed by PROLYL-4-HYDROXYLASE (P4H) (Myllyharju 2003); whilst thirteen genes for P4H have been identified in *Arabidopsis*, no substrate consensus sequence for proline hydroxylation has yet been established (Matsubayashi 2012), and it is unclear which one(s) are involved in modification of CEP5. If single and higher order mutants can be obtained for these P4H genes, it would be of interest to analyse their phenotypes (particularly in light of lateral root phenotypes) and observe whether exogenous application of hydroxylated CEP5 can rescue any observed mutant phenotypes: this may help identify which P4H genes are involved in modifying CEP5. Alternatively, mass spectrometry approaches could be used to examine single P4H mutants for CEP species deficient in hydroxylation.

In MtCEP1, a small proportion of the peptide species were found to be arabinosylated as well as hydroxyprolinated. It is suggested that hydroxyproline arabinosyltransferases (HPATs) are a likely limiting factor in the creation of arabinosylated peptides, explaining their relatively low frequency (6.5%) (Mohd-Radzman, Binos *et al*. 2015). However, the purpose of the arabinosylation and what effects this has on the structure of CEP peptides is unclear. Synthetic arabinosylated peptides are not currently commercially available: if this changes in the future, the relative bioactivity of these arabinosylated CEP species compared with hydroxyprolinated versions

could be tested. One way to investigate this angle presently would be to overexpress HPAT candidates and analyse through mass spectrometry whether this results in detectable arabinosylated CEP5 species in *Arabidopsis,* and potentially also informative phenotypical changes.

Finally, several CEP-family peptides and other similar peptides have been shown to encode multiple active motif sequences; intriguingly, there is increasing evidence that different CEP peptides and even different motifs cleaved from the same precursor can have opposing functions. For example, the N-terminal and C-terminal motifs respectively of MtCEP and AtCLE18 both have opposing inhibitory/promoting effects on lateral root elongation (Ito, Nakanomyo *et al*. 2006, Meng, Buchanan *et al*. 2012). In *Medicago*, *MtCEP8* was the most down-regulated transcript in *MtCEP1* overexpressing roots, suggesting the presence of a negative feedback mechanism (Imin, Mohd-Radzman *et al*. 2013). It would therefore be interesting to establish whether other AtCEP family peptides have contrasting functions to CEP5 and/or are expressed differently/show different protein abundance in *CEP5* overexpression lines or following exogenous application of synthetic peptide.

5.2 Concluding remarks

The work presented in this thesis shows that CEP5 modulates *Arabidopsis thaliana* root architecture – specifically lateral root regulation - and that CEP5 demonstrates an antagonistic relationship with auxin, with these two findings

potentially linked through an as-yet unknown pathway. This pathway may also link with recent findings for roles of CEP peptides in nitrate sensing, nodulation and creation of nematode feeding sites. Fine control of auxin responses could represent a novel way of fine-tuning auxin-driven developmental processes, particularly lateral root branching and organisation of general root architecture but also potentially, other auxin-driven processes. Future work may establish the precise molecular mechanism for the auxin antagonism shown by CEP5, the molecular mechanisms involved in nitrate sensing, the function of nematode-secreted CEPs, and other CEP-family peptide regulatory pathways and interactions.

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APPENDIX I

ACR4 is unlikely to perceive CEP5

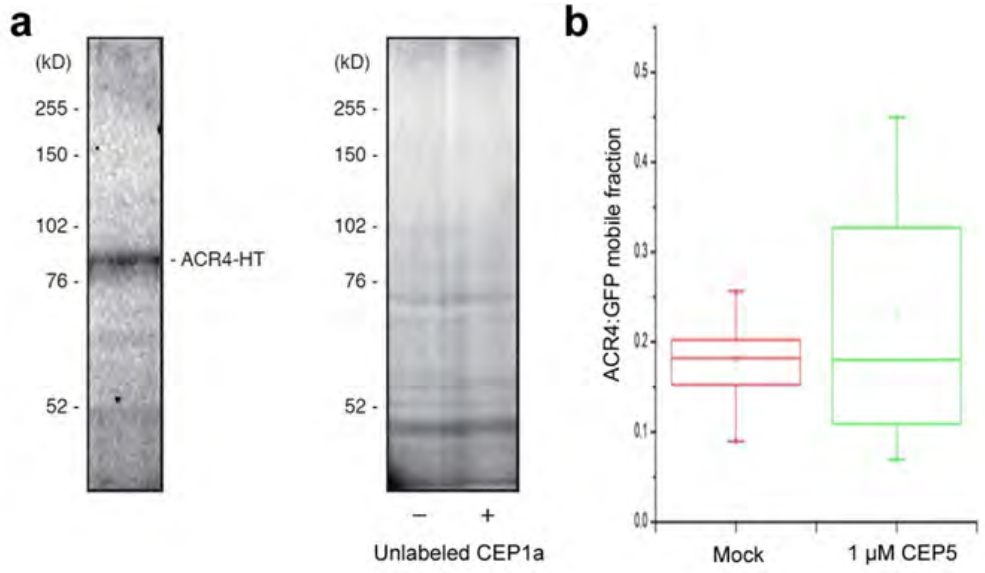

Figure A1.

(a) Unpublished data from Yoshikatsu Matsubayashi. Photoaffinity cross linking using a HaloTag (HT) labelled ACR4 protein does not show interaction between CEP1 peptide and ACR4. ACR4: ~98 kD.

(b) Unpublished data from Rudiger Simon. Fluorescence Recovery After Photobleaching (FRAP) experiments show no difference in the mobile fraction of ACR4:GFP expressed in Tobacco cells when CEP5 peptide is added compared with mock treatment.

APPENDIX II

CEP5 does not change cell specificity in the pericycle

In order to understand how CEP5 fits into the lateral root regulatory network, CEP5 peptide application was tested on J0121 and PPP (phloem pole pericycle) enhancer trap fluorescent lines (these lines emit GFP fluorescence in the xylem pole pericycle (J0121) and phloem pole pericycle tissues (PPP) respectively). CEP5 peptide application at both 5 μM and 10 μM did not change the fluorescence pattern in these lines, suggesting that CEP5 does not exert its influence through changing cell specificity in the pericycle tissues.

Method:

Arabidopsis GFP enhancer trap line seedlings for xylem pole pericycle (J0121) and phloem pole pericycle (PPP) tissue identity (Laplaze, Parizot et al. 2005) were grown vertically until approximately 5DAG on either plain ½ MS media or media supplemented with either 5 or 10 μM CEP5 peptide. Seedlings were analysed for fluorescence pattern using a Leica SP5 confocal microscope.

APPENDIX III

DR5:VENUS fluorescence is not significantly different following CEP5 treatment

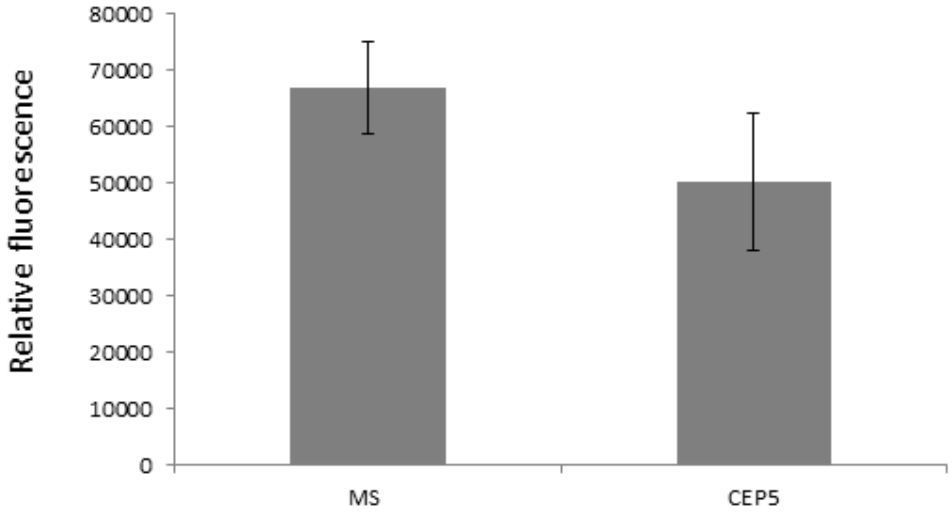

Figure A2. DR5:VENUS fluorescence in mock-treated (MS) or CEP5p^{Hyp} treated (5 µM) seedlings. There is no significant difference in VENUS fluorescence in DR5:VENUS seedlings treated with CEP5 and controls, P = >0.05 according to Student's T-Test. Seedlings analysed at ~6-7DAG, N = ≥ 10 seedlings per condition, mature root sections (above basal meristem) analysed. Error bars are ± 1. S.E.

APPENDIX IV

A speculative analysis of the sequence and structure of CEP5

In Chapter 2, the importance of proline hydroxylation posttranslational modification to the bioactivity of CEP5 was shown using primary root length bioassays and alanine scans. However, the reason why proline hydroxylation increases the bioactivity of CEP5 and related CEP peptides is still an open question. Several small posttranslationally modified peptide signals (such as the CEP, IDA, CLE, PSY1 and RGF families) contain a higher proportion of proline than would be expected from the length of the active motif (typically 3–5 prolines in a 12–18 residue sequence). Due to the geometry of proline caused by the five-membered ring side chain structure, proline is restricted in the conformations it can adopt: for this reason, prolines are known to induce kinks in protein secondary structures, with one well-known example being the in the formation of collagen alpha-helices in animals (Barnes and Gray 2003). CEP5 has three proline residues: P4, P7 and P11; mass spectrometry data, bioactivity assays and comparisons with other CEP peptides support CEP5 being hydroxylated on at least two (probably three) of its proline residues: based on CEP1 and comparisons with other CEPs these are most likely to preferentially include proline 4 and proline 11 (Ohyama, Shinohara *et al*. 2009, Bobay, DiGennaro *et al*. 2013). Hydroxyprolination of CEP5 is likely to be important for structural conformation and receptor binding (as has been demonstrated with hydroxyproline arabinosylation of CLV3 in CLV1 receptor binding (Shinohara and Matsubayashi 2013). In collagen, hydroxylation of proline residues serves to reinforce structural rigidity and promote protein-protein interactions with other collagen strands (Deber, Brodsky *et al*. 2010). This raises the intriguing possibility that hydroxyproline modification of CEP5 might promote protein-protein interactions, either with receptors or potentially with other proteins.

APPENDIX IV

Recently, Mohd-Radzman *et al.* (2015) found that differentially hydroxylated MtCEP1 peptides exist in *Medicago.* As observed for AtCEP5, proline hydroxylation patterns were linked to bioactivity, with P4 and P11 hydroxylation of MtCEP1 D1 peptide conferring the strongest lateral root inhibition activity (unlike AtCEP5, hydroxylation on all three prolines reduced bioactivity: despite this, the triply hydroxylated form of MtCEP1 D1 was the most abundant form isolated in *Medicago*). Interestingly, the second CEP motif derived from MtCEP1 (D2) was only found isolated in one form: in this form, P11 was the only proline residue hydroxylated, and this had the opposite effect of D1 peptide on lateral root formation. This suggests the tantalising possibility that differential post translational modification of CEP peptides is a means of regulating their specific (and even opposing) functions. Furthermore, in a minority of the D1 MtCEP1 peptides (6.5%), P11 carries a triarabinosylation modification in addition to proline hydroxylation. The bioactivity of these triarabinosylated CEP species is unknown due to the unavailability of commercially synthesized arabinosylated peptides: however, it would be of interest to see if similar arabinosylated species exist for AtCEP5 and other CEPs in *Arabidopsis*.

The proline residues at positions 4, 7 and 11 are highly conserved in Group I CEP motifs across a wide range of plant families and interestingly, also in the CEP-like peptides secreted by nematodes (Bobay, DiGennaro *et al*. 2013, Ogilvie, Imin *et al*. 2014). Proline 4 is the most N-terminal proline residue, and in common with the other residues located in the N-terminal portion of the group I CEP motif, is generally less conserved across plant families than the C-terminal proline 11, often being substituted with other residues in the *Poaceae*, *Solanaceae* and *Pinaceae* families (Ogilvie, Imin *et al*. 2014). Group II CEP motifs do not have a conserved proline at position 4 at all (Delay, Imin *et al*. 2013). Nevertheless, P4 is absolutely conserved in *Brassicaceae* and alanine scanning analysis using synthetic CEP5 variants found that mutation of P4 to alanine (P4>A) to be sufficient to almost completely abolish bioactivity in *Arabidopsis* root length assays (see Chapter 2, Figure 13).

APPENDIX IV

Proline 7 is situated centrally within the CEP motif and is moderately conserved across plant species, often being substituted with a serine residue in *Pinaceae* and in group II CEP motifs. A conserved proline bioactivity is found at position 7 of other similar small posttranslationally modified peptides including IDA, CLV3 and the majority of CLE peptides. P7 represents the site of a hydroxyproline-arabinosylation modification in CLV3; structural analyses revealed this modification causes C-terminal residues distal from the arabinosylation site to adopt a new conformation, introducing a sharp upward bend and sideways twist at the C-terminus. The distribution of proline residues in CLV3 and CEP active motifs are similar; thus there is the potential that CEP proline residues also represent the sites for key modifications involved in the final structural conformation of CEP peptides: future NMR structural analyses may confirm this. P7 is more commonly unmodified than P4 and P11 in CEPs analysed so far (Ohyama, Shinohara *et al*. 2009, Bobay, DiGennaro *et al*. 2013, Mohd-Radzman, Binos *et al*. 2015), and is highly but not absolutely conserved across *Brassicaceae*, however, alanine scanning analysis found AtCEP5 P7>A mutation to be sufficient to almost completely abolish bioactivity in *Arabidopsis* root length assays (see Chapter 2, Figure 13).

Proline 11 is found in the most highly conserved C-terminal region of the CEP motif and is almost universally conserved across all plant families where CEP peptides are found, (including both angiosperm and gymnosperm families); in both group I and group II CEPs, and also nematode CEP peptides (Bobay, DiGennaro *et al*. 2013, Delay, Imin *et al*. 2013, Ogilvie, Imin *et al*. 2014). This high level of conservation may be linked to the fact that in contrast to P4 and P7, P11 is invariably hydroxylated in all CEPs analysed to date, and in some cases, also triarabinosylated (Ohyama, Shinohara *et al*. 2009, Bobay, DiGennaro *et al*. 2013, Imin, Mohd-Radzman *et al*. 2013, Mohd-Radzman, Binos *et al*. 2015). In MtCEP1 D1, removal of hydroxyprolination from P11 reduced lateral-root inhibiting bioactivity, and completely abolished circumferential cell proliferation (CCP)-promoting activity (Imin, Mohd-Radzman *et al*. 2013). In MtCEP1 D2, P11 was the only proline modified: the fact that MtCEP1 and MtCEP2 were found

to have opposing effects on lateral root elongation suggests the possibility that P11 modification might be crucial for mediating receptor binding, whilst modification of the other proline residues determines other specific functions and bioactivity. In support of this, under nitrogen-limiting conditions, only MtCEP1 D1 hydroxylated on P4/P11 or P4/P7/P11 was able to inhibit lateral root formation: the D1 P7/P11 species did not (Mohd-Radzman, Binos *et al*. 2015).

Unexpectedly, in CEP5 substitution of P11 with an alanine residue increased bioactivity (see Chapter 2, Figure 13). The reasons for this are currently unclear: it may be that alanine confers the peptide more flexibility than a proline/hydroxyproline residue in the same position, allowing more flexibility in protein-protein interactions, including peptide-receptor interactions (Charlie Hodgman, personal communication) or it could be that alanine in this position behaves like a 'modification mimic' again, increasing the efficiency of the peptide further. A similar effect was observed for P7>A in CLE26 (Czyzewicz, Shi *et al*. 2015).

As well as the prolines, several other CEP motif residues show high degrees of conservation and importance in bioactivity assays. Serine 10 and Glycine 14 are almost absolutely conserved amongst all CEPs (all families group I and group II CEPs and nematode CEPs) whilst Glycine 8, Glycine 12 and Histidine 15 are very highly conserved across all CEPs. Given the structural importance of glycine residues, it is perhaps not surprising that all three glycine residues are highly conserved across CEP peptides: as the smallest amino acid and the only one containing hydrogen rather than carbon as a side chain, glycine is often found in areas of protein structure requiring high conformational flexibility - such as following tight turns. This may indeed be the case for CEP peptides, since two of the conserved glycine residues immediately follow kink-inducing proline residues. NMR structures for MtCEP1 and MhCEP11 show that residues between positions 9–12/8–13 respectively in the CEP motif form a distinct alpha helix: this structural feature is likely important for function and could explain the pattern of conserved residues within this region. Furthermore, if the C-terminal portion of

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CEP peptides bends in a similar way as the CLE motif of CLV3 following posttranslational modification (Shinohara and Matsubayashi 2013), the position of the glycine residues may be crucial in allowing the conformational flexibility to achieve this. Similarly with S10, the small size of serine also means it is commonly found in tight turns (Barnes and Gray 2003).

Alanine scans suggest a particular importance for G8 in the CEP5 sequence: mutation of this residue to an alanine completely abolished bioactivity in primary root length assays, whilst MtCEP1 with G8>A substitution also greatly reduced its LR-inhibiting and circumferential cell proliferation (CCP) -promoting activity. As well as potentially forming part of an alpha-helical structure, this may be also be due to the fact that the predicted position of G8 within the protein structure would enable the formation of ionic bonds across a V-shaped cleft with the polar side chain of the S10 residue (which is also absolutely conserved in *Brassicaceae*) thus constricting the molecule into a more compact form than initially would be expected.

Threonine 5 is also absolutely conserved in *Brassicaceae* and alanine scans found T5>A mutation sufficient to completely abolish bioactivity in root length assays. Threonine is not particularly flexible in terms of conformations and thus the fact that two threonine residues span the sequence from P4 to P7 is perhaps surprising. The importance of T5 may thus be less structural and more to do with reactivity/chemical properties. Conversely, the second threonine, T6, is the least conserved residue in *Brassicaceae* CEP peptides and T6>A mutation only conferred a minor reduction in bioactivity in root length assays (Chapter 2, Figure 13).

The N-terminal and C-terminal residues of CEP peptides are also both apparently important for bioactivity. Primary root length stunting bioactivity was reduced for both D1>A and H15>A substitutions in CEP5 (Chapter 2, Figure 13) whilst in MtCEP1, deletion of A1 and H15 greatly reduced/abolished lateral root inhibition and CCP proliferation respectively (Mohd-Radzman, Binos *et al*. 2015). In both CLV3 and IDA, removal of the C-terminal Asn residue also completely

abolishes bioactivity, and the ability of IDA to bind to its receptor HSL2 (Kondo, Sawa *et al*. 2006, Butenko, Wildhagen *et al*. 2014). Whilst the C-terminal residue of CLV3 and IDA is an asparagine in contrast to CEP-family peptides which have a histidine in this position, Asn and His are conserved substitutions according to the BLOSUM62 substitution matrix (Henikoff and Henikoff 1993) suggesting that the C-terminal H15 might play an important part in peptide processing and/or receptor recognition in CEP peptides also.

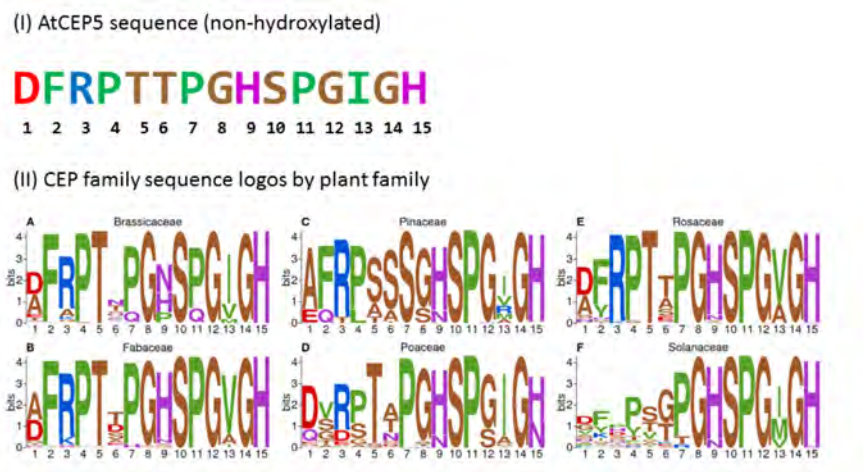

Figure A3. Comparison of AtCEP5 motif with consensus sequence of CEP motifs across plant families. (I) AtCEP5 CEP motif sequence (basic sequence without post-translational modification) Amino acids colour coded by chemistry: small/non-polar (brown), hydrophobic (green), polar (purple), negatively charged (red), positively charged (blue). (II) Sequence logos of CEP domains by plant family. Sequence logos, which visualize the distribution of AAs at each position of a short motif, are based on weighted CEP domain sequences identified in the genomes of the plant families *Brassicaceae* (A), *Fabaceae* (B), *Pinaceae* (C), *Poaceae* (D), *Rosaceae* (E) and *Solanaceae* (F). Taken from Figure 3 from Ogilvie *et al*., 2014.

Structural prediction for CEP5 (residues T6-G12)

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Although no CEP5 NMR structure is available, similarities with existing peptides with similar regions which have available structures enable predictions of limited sections of CEP5. Below is a SwissPDB model representing the CEP motif between residues T6-G12, based on the structures for MtCEP1 and MhCEP11. The position of certain residues may allow projection of side chains into the solvent to increase reactivity (e.g. on P7 and P11) or potentially to form bonds with other residues in the sequence thus stabilising the overall protein structure further (e.g. between the nonpolar and polar side chains of G8 and S10).

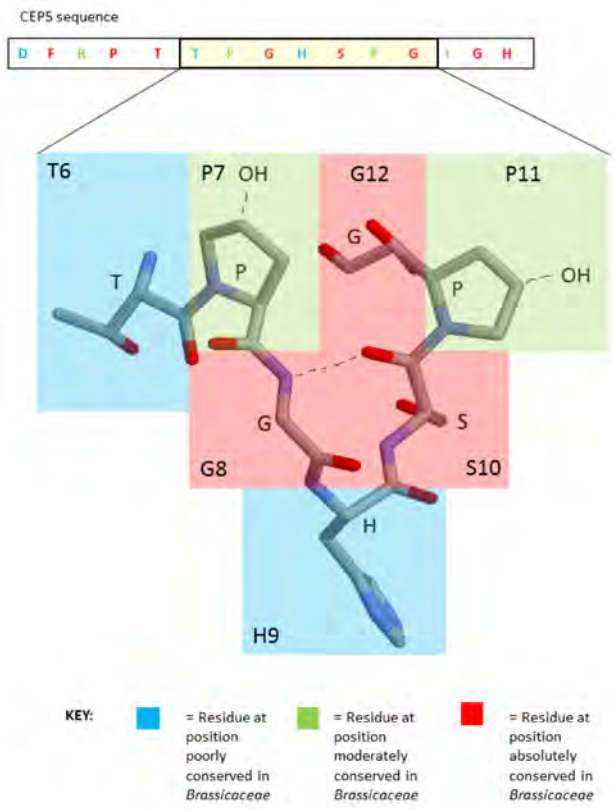
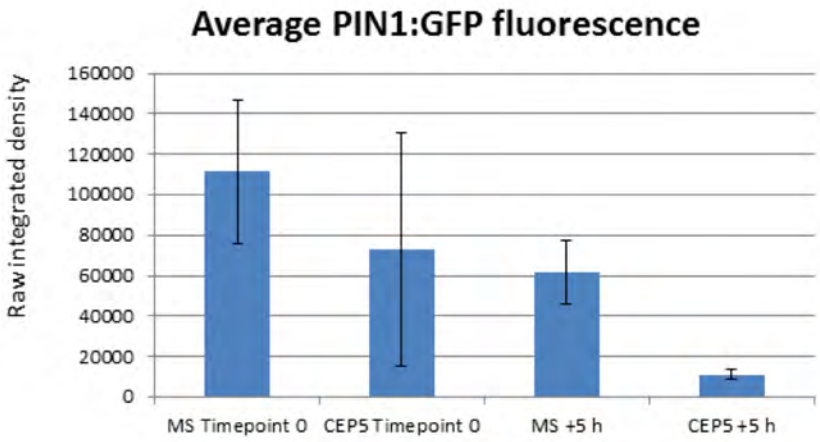


Figure A4. Structural prediction of CEP5 residues T6-G12 based on NMR structures of MtCEP1 and MhCEP11. Residues are colour coded according to the degree of conservation in *Brassicaceae*. Figure generated by SwissPDB.

APPENDIX V

Comparison of PIN1:GFP +/- CEP5p^{Hyp}



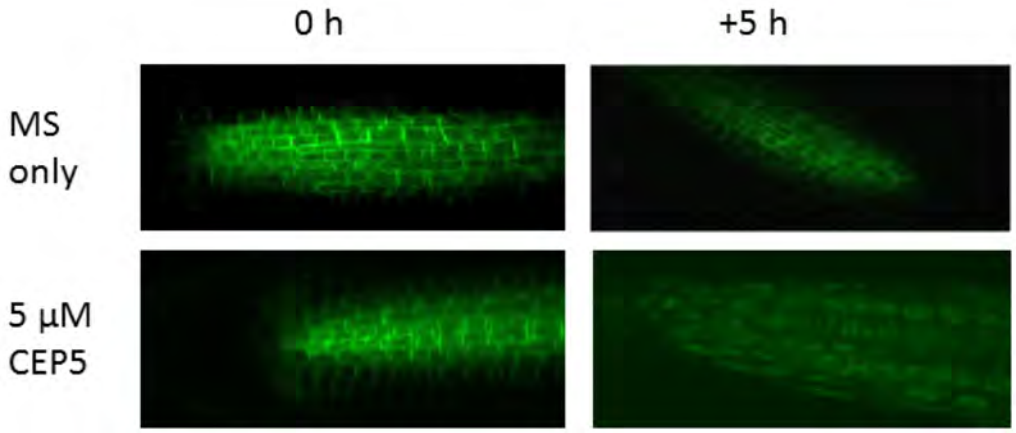


Figure A5. PIN1:GFP +/- exogenously applied CEP5p^{Hyp}. The root tips of PIN1:GFP seedlings (7DAG) were incubated with either plain ½ MS (control) or 5 µM CEP5p^{Hyp} and imaged at timepoint 0 and then again after 5 h. Fluorescence after 5 h was generally lower in all seedlings, but appeared much lower in CEP5 treated seedlings. In addition, CEP5 treated seedlings seemed to lose the normal polarized distribution of PIN1:GFP in the cells. However, none of these differences are statistically significant according to Student's T-Test (p = >0.05). $N = \geq 3$ seedlings per condition.

APPENDIX VI

Root system architecture: insights from *Arabidopsis* and cereal crops

Stephanie Smith and Ive De Smet

2012. Philosophical Transactions of the Royal Society B **367:** 1441–1452.

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Introduction

Root system architecture: insights from *Arabidopsis* and cereal crops

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Roots are important to plants for a wide variety of processes, including nutrient and water uptake, anchoring and mechanical support, storage functions, and as the major interface between the plant and various biotic and abiotic factors in the soil environment. Understanding the development and architecture of roots holds potential for the exploitation and manipulation of root characteristics to both increase food plant yield and optimize agricultural land use. This theme issue highlights the importance of investigating specific aspects of root architecture in both the model plant *Arabidopsis thaliana* and (cereal) crops, presents novel insights into elements that are currently hardly addressed and provides new tools and technologies to study various aspects of root system architecture. This introduction gives a broad overview of the importance of the root system and provides a snapshot of the molecular control mechanisms associated with root branching and responses to the environment in *A. thaliana* and cereal crops.

Keywords: root; *Arabidopsis*; maize; rice; hormones; environment

1. INTRODUCTION

Plants are undeniably important to humans and indeed, to most life on Earth. The benefits to humankind provided by plants include food, fuel, fibres, medicines and materials as well as ornamental and leisure uses. It is easy to think of plants purely in terms of the clearly visible, above-ground parts of plants, such as leaves, flowers, fruits and stems; however, this is only half the story. Plants also demonstrate a considerable degree of variability in the less visible underground elements: root systems. Root system architecture (RSA) varies between species, and also within species, subject to genotype and environment [1]. The importance of root systems combined with the inherent difficulty of studying them has led to roots being described as 'the hidden half' [2].

Roots are important to plants for a wide variety of processes, including nutrient and water uptake, anchoring and mechanical support. In addition, many plants use roots for storage functions, and some root structures serve as human food sources, including root vegetables (such as carrots, parsnips, turnips and many others) and spices (such as ginger, arrowroot, liquorice and others). Roots serve as the major interface between the plant and various biotic and abiotic factors in the soil environment: by both sensing and responding to environmental cues, roots enable plants to overcome the challenges posed by their sessile status. This is seen in the many ways plants can dramatically alter their root architecture to optimize growth in a large variety of environmental and soil nutrient conditions. Root architecture has traditionally been largely ignored by plant breeders in terms of potential yield increases, and was not a major selection criterion as part of the crop development programmes of the 1960s' green revolution [3]. Understanding the development and architecture of roots thus holds potential for the exploitation and manipulation of root characteristics to both increase plant yield and optimize agricultural land use [4].

2. WHY BOTHER?

With the global population projected by the UN to rise to over 9 billion by 2050, improvement of crops is becoming an increasingly pressing issue. Crop plants, particularly cereals, are a vitally important food supply, both directly and through use in animal feed: an estimated 75 per cent of the human energy demand is fulfilled by cereal starch [5]. The 'green revolution' of the 1960s and 1970s helped agriculture to meet the food demands of a rapidly growing global population, through the creation of dwarfed cereal cultivars, better irrigation and the application of fertilizer. This resulted in a doubling of cereal yields in developing countries between 1960 and 1990 [6]. Fifty years on, and the benefits of this revolution appear to have peaked: the global population since the 1960s has more than doubled, fresh water shortages hinder large-scale irrigation, and further fertilizer application would be damaging in both crop

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One contribution of 18 to a Theme Issue 'Root growth and branching'.

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yield and environmental terms (as well as being economically unfeasible in many third-world countries without considerable subsidies) [7]. The demand for biofuels (in such circumstances where the production of such fuels directly competes with arable land) can also reduce the area of arable land that can be dedicated to food production [8]. The challenge for the 'second green revolution' is to solve the current and future obstacles to maintaining food security through higher crop yields.

Drought is the most common environmental condition impeding crop growth and productivity [9]; furthermore, currently 70 per cent of the world's fresh water demand is for agricultural use [10]. Drought is a threat which is likely to become more pressing with the predicted future global increase in temperature [11]. Improved access to deep soil water, reducing the need for irrigation, is one potential benefit that could be achieved by exploitation of RSA. Already, modelling techniques suggest that rising yield trends in the US Corn Belt can be attributed to the selective breeding of maize with increasingly deeper, more vertically orientated root architectures, leading to more efficient soil water use and increased crop density [12]. Various studies have also evaluated breeding of root phenotypes for increased phosphorus acquisition and carbon sequestration [13,14], and the latter is also addressed in this issue by Kell [15].

Application of inorganic fertilizer, containing mineral nutrients such as nitrogen, phosphorus and potassium, has traditionally been used to increase yield in cereals and other crop plants. However, these fertilizers are expensive, often have negative impacts on soil and waterway ecosystems, and fertilizers (particularly nitrogen fertilizers) require large amounts of energy—mainly derived from fossil fuels—to produce [16]. In addition, global reserves of nutrients such as phosphorus are limited, with supplies projected to be depleted in 50–100 years time [17]; and nutrient acquisition by roots is rather inefficient, with acquisition rates of approximately 33 per cent for nitrogen and 20 per cent for phosphorus [18,19]. Climate change adds further pressure to develop crops that can cope with higher temperatures, increased water salinity and denser pest loads [8]. Root system exploitation and modification in crops may enable plants to make more efficient use of existing soil nutrients and increase stress tolerance, improving yields while decreasing the need for heavy fertilizer application.

Many of the advances in understanding RSA to date have come from studies on the model plant, *Arabidopsis thaliana* (referred to as *Arabidopsis*). For example, the description of the cellular structure of the *Arabidopsis* root by Dolan *et al.* [20] provided the basis for future developmental and genetic work. Because RSA can have major influences on both plant yield and the ability of a plant to be grown in 'marginal' environments, understanding RSA in cereals and biofuel plants is a necessity. RSA in cereal plants has been a comparatively little-studied area thus far, but newer studies are starting to address this neglected area—the first step in translating research on the molecular level into the tangible benefits of exploiting RSA to improve crop biomass and yield.

3. ROOT SYSTEM ARCHITECTURE

RSA on a macroscale describes the organization of the primary and lateral roots, and also accessory roots (including other root types found in cereals) where they are present, and is a key determinant of nutrient- and water-use efficiency in plants. On a microscale, this includes root hairs that increase the surface area, aiding with uptake of water and nutrients [21–25]. In both dicots and monocots, adventitious roots are also sometimes formed post-embryonically at the root–shoot junction, for example to explore and exploit the phosphorus-rich upper soil strata (figure 1*a*,*b*). Here, we will mainly describe root branching and we refer to recent reviews on (molecular) control mechanisms of primary root [26,27] and root hair development [25,28].

Primary roots are the first root to emerge in both dicots and monocots, and are derived from embryonically formed meristematic tissue. The root itself broadly consists of xylem and phloem within a central vascular column and pericycle to constitute the stele, surrounded by concentric layers of epidermal, cortical and endodermal tissues (figure 1*c*,*d*). Primary and mature roots contain meristematic tissue at the tip (the root apical meristem), which forms the basic stem cell pool for other cell types in the root. Within the root apical meristem, there is an area of rarely dividing cells, the quiescent centre, which signals to surrounding cells to organize and maintain the population of initial stem cells [20].

In terms of the organization of the root tissue itself, there are some substantial differences between *Arabidopsis* and other species, including cereals. The organization of root tissues for many other dicot species, e.g. *Brassica* species, is generally not well characterized at present. The majority of root architecture research in monocots has focused on the species *Zea mays* and *Oryza sativa* [29], thus these will henceforth be used as models for monocot root systems for the purpose of this review. Overall, cereal root tissues tend to be larger and more complex; the primary root of cereals such as maize or rice can have 10–15 cortical cell layers (figure 1*d*) compared with the single layer typically found in *Arabidopsis* (figure 1*c*), and often the root quiescent centre population is drastically larger in size: 800–1200 cells in maize, compared with 4 in *Arabidopsis* [30]. For a more detailed comparison, the reader is directed to Hochholdinger & Zimmermann [30].

At the very tip of the root is the root cap, and columella cells, which are involved in root gravitropism—an important growth aspect which causes roots to grow deeper into the soil. Some columella cells, the statocytes, contain specialized amyloplasts (organelles containing dense starch). When a gravity stimulus is applied, sedimentation of amyloplasts on a cell edge is thought to result in a change in the distribution of auxin transporters in the cell membrane. This leads to changes in auxin flux and a differential growth response within the root, which in turn bends towards the gravity stimulus [31]. In this issue, Guyomarc'h *et al.* [32] discuss the importance and control of lateral root gravitropism.

Root branching is essential to increase the surface area of the root system, enabling the plant to tap more distant reserves of water and nutrients and improve soil anchorage. In contrast to primary roots,

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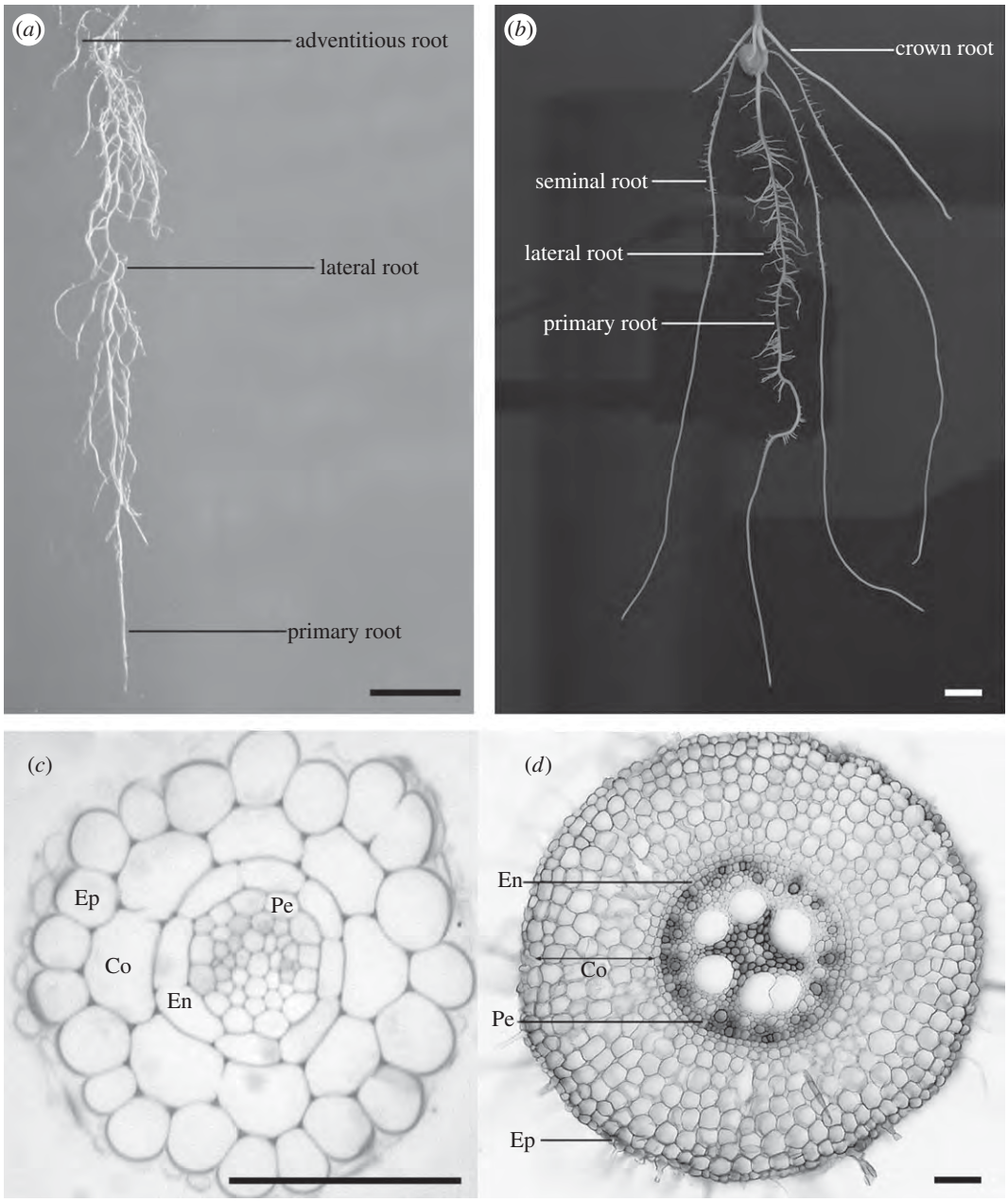

Figure 1. *Arabidopsis* and maize root system. (*a*,*b*) Macroscopic view of 14 day old *Arabidopsis* (*a*) and 10 day old maize root system (*b*), with the major root types indicated. (*c*,*d*) Transverse section through an *Arabidopsis* (*c*) and maize root (*d*). The epidermis (Ep), cortex (Co), endodermis (En) and pericycle (Pe) are indicated. Maize pictures in (*b*) and (*d*) were kindly provided by Leen Jansen. Scale bars: (*a*,*b*) 1 cm and (*c*,*d*) 100 μm.

lateral roots are formed post-embryonically. In *Arabidopsis* and most dicots, the pericycle—a tissue layer located between the central vascular cylinder and the endodermis—represents the initiation site for lateral root branching (figure 1*c*). Specifically, the pericycle cells adjacent to the xylem pole represent the precursors to lateral root founder cells [33]. Lateral root initiation is the result of auxin-dependent cell cycle progression in a specific subset of the pericycle cells, to form 'founder cells'. These initial cells subsequently go through several rounds of cell division to form the new lateral organ, which emerges perpendicularly to the parent primary root [34,35]. In contrast to the formation of shoot lateral organs, the later stages of lateral root emergence require the *de novo* creation of a new apical meristem within the emerging lateral root.

The reliance on the embryonically derived primary or 'taproot' system is a feature typical of dicots, such as *Arabidopsis* (figure 1*a*). Other dicots, for example *Brassica*, usually have a broadly similar root system in terms of development and morphological layout to *Arabidopsis*, though their comparatively longer life cycles usually result in a larger and denser root system, especially for perennial species. It should also be noted that considerable variety exists in root architecture between many dicot species, and *Arabidopsis* is (as in many areas of research) a simplified model.

Although cereals and other monocots form primary and lateral roots in a manner roughly similar to dicots, overall root architecture is more complex in monocots, forming a 'fibrous' root system of many types of branched root. In cereals, shoot-borne 'crown' and

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'brace' roots, sometimes together with 'seminal' roots, constitute the majority of the monocot root system (figure 1*b*). These monocot-unique roots (particularly crown roots) are sometimes described as adventitious roots, but this is somewhat inaccurate as they are formed during the normal developmental programme of cereals [36]. The maize primary root can be seen from 2–3 days post germination, shortly followed by seminal roots by approximately 7 days after germination (figure 1*b*); similar to the primary root, these are formed embryonically, and emerge from the scutellar node [36]. Post-embryonically formed cereal roots include crown roots and brace roots. Crown roots start to emerge from below-ground stem nodes by 5–10 days post-germination. By contrast, brace roots emerge from above-ground stem nodes much later, approximately 6 weeks after germination [36,37]. Lateral root initiation also differs somewhat in cereals: both pericycle and endodermal cells can progress to become lateral root cells, and lateral roots form at the phloem poles rather than the xylem poles [38]. Furthermore, *Arabidopsis* has only two xylem poles (figure 1*c*), with lateral roots initiated in an alternating left–right pattern at each pole, but in cereals there can be as many as 10 or more phloem poles (figure 1*d*), resulting in a much more radial branching pattern around the parent root than observed in *Arabidopsis* [39]. In this issue, Jansen *et al.* [40] and Babé *et al.* [41] further explore lateral root initiation in cereal crops.

4. CONTROL OF ROOT BRANCHING IN *ARABIDOPSIS*

(a) *Hormones*

Lateral root initiation requires that primed pericycle founder cells undergo several rounds of cell division to form the lateral root primodium that gives rise to the new lateral root. The overriding influence in cell cycle regulation during root formation is the phytohormone auxin; many factors which influence lateral root branching can be observed to do so either directly via auxin, or by having a synergistic or antagonistic interaction with auxin (e.g. brassinosteroids and cytokinin). Indeed, simple exogenous application of auxin alone is sufficient to initiate lateral roots [42–44].

Lateral root initiation sites are created following the concentration of auxin in defined pericycle regions; the pericycle cells encompassed by these maxima are then 'primed' to undergo the divisions necessary for lateral root primordia formation [45]. Auxin promotes the interaction of members of the auxin/indole-3-acetic acid (AUX/IAA) family of repressor proteins with TIR1/AFB family auxin-receptor proteins. TIR1 is the F-box component of an ubiquitin ligase complex, which ubiquitinates AUX/IAAs marking them for destruction by the 26S proteasome. This frees the transcriptional regulators AUXIN RESPONSE FACTORS (ARFs) from repression and allows them to bind to genes that contain auxin response elements, which are involved in controlling the organogenesis cell division programme [46–48]. Predictably, because the auxin-signalling pathway is so crucial for lateral root formation, several mutants for the various modules of this pathway display altered root phenotypes. For example, studies have isolated various mutants for the AUX/IAA and ARF modules of the auxin response pathway, such as the *solitary root* (*slr*) mutant, which has a complete absence of lateral roots owing to a gain-of-function mutation that increases the repressive activity of IAA14 [49]. In addition to *slr*, other gain-of-function *aux/iaa* mutants also exhibit lateral root phenotypes: the *short hypocotyl2-2* (*shy2-2*)/*iaa3* and *iaa28 Arabidopsis* mutants both have reduced lateral root numbers [50,51]. Conversely, the gain-of-function *auxin resistant2-1* (*axr2-1*)/*iaa7* mutant demonstrates an increased number of lateral roots, suggesting that different members of the AUX/IAA family may have varying responses to auxin [52].

Simple initiation of cell division is, however, not enough; cell divisions must be tightly spatially and temporally controlled to give rise to correctly patterned primordia. Inducing pericycle cells to re-enter the cell cycle through overexpression of D-type cyclins or the G1–S transition-promoting transcription factor E2Fa/DPa is not sufficient to induce lateral root organogenesis in the absence of further cues determining cell patterning [53]. This control is achieved by fine-tuning the auxin response pathway through AUX/IAA–ARF pairs. Different combinations of these partially account for the diversity in phenotypic responses generated in response to a generic auxin signal [54,55]. SLR/IAA14 paired with ARF7–ARF19 is one such auxin response module, which directs transcription of the early auxin response *LATERAL ORGAN BOUNDARIES/ASYMMETRIC LEAVES-LIKE* (*LBD/ASL*) genes, which encode transcription factors for a set of genes controlling cell proliferation and patterning. In turn, these genes direct the initial asymmetric pericycle cell divisions during lateral root formation [56]. Another factor required for correct cell patterning downstream of the SLR/IAA14–ARF7–ARF19 auxin response module is the BODENLOS (BDL)/IAA12-MONOPTEROS (MP)/ARF5-dependent module, which has both lateral-root-promoting and -inhibitory mechanisms [57]. The ARF protein MP was recently demonstrated to control both its own expression and the expression of its repressor, the AUX/IAA protein BDL, subject to a threshold level of auxin, suggesting that the ARF–AUX/IAA auxin response may be self-regulating [58]. In this issue, Goh *et al.* [59] also highlight the roles of multiple AUX/IAA–ARF modules in lateral root development.

It has traditionally been thought that the pericycle cells require a de-differentiation stage with exit from an arrested G_2 phase before renewal of cell division can occur [60,61], but observations suggest that the period between pericycle cell exit from the root apical meristem and the formation of founder is relatively short [62], thus allowing cells to remain division-competent without the requirement for de-differentiation [63,64]. However, it does seem to be the case that a subset of pericycle cells remain in G_2 phase for an extended period of time after exit from the meristem, and it is these cells which are most susceptible to lateral root initiation [64]. The founder pericycle cells can thus be considered to belong to an 'extended meristem' of not-yet fully differentiated pericycle cells [65,66]. In this issue,

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Parizot *et al.* [67] discuss this and highlight the heterogeneity of the pericycle.

The brassinosteroid regulatory pathway appears to act in synergy with the auxin pathway, with brassinosteroid-insensitive mutants exhibiting reduced lateral roots [68]. It is thought that brassinosteroids promote lateral root development through enhanced acropetal auxin transport, although the exact mechanism by which it does so is unclear.

Cytokinin is primarily synthesized in the root cap [69] and has a largely antagonistic effect to auxin in terms of root architecture: cytokinin has an inhibitory effect on lateral root branching, with mutants for cytokinin biosynthesis and sensitivity demonstrating increased numbers of lateral roots [70–73]. Auxin is synthesized primarily in young aerial tissues and undergoes polar transport towards the root tissues [74]. It is then distributed in the root tissues via strategic positioning of auxin carriers, particularly the auxin efflux carrier PIN-FORMED (PIN), which directs auxin flux enabling concentration in specific regions [75,76]. The inhibitory effect of cytokinin on lateral root branching appears to be due to cytokinin-mediated downregulation of *PIN* gene expression. PIN family auxin efflux facilitators are required to generate the targeted auxin maxima necessary for priming pericycle cells to form correctly patterned lateral root primordia [77]. In this issue, Bielach *et al.* [78] identified various mutants that are impaired in the auxin–cytokinin crosstalk.

Increased synthesis or signalling of the gaseous phytohormone ethylene has an inhibitory effect on lateral root formation. Similar to cytokinin, this is thought to occur by altering auxin dynamics in the root, for example by modulating the action of the auxin influx carrier AUX1 [79,80]. Recently, treatment of *Arabidopsis* root tissues with the ethylene precursor 1-aminocyclopropane-1-carboxylic acid was also found to increase expression of the *PIN3* and *PIN7* auxin efflux transporters, thus promoting auxin transport towards the root apex and preventing the localized auxin accumulations needed to initiate lateral root formation [81].

Abscisic acid (ABA) is a phytohormone that has an important role in stress, particularly in stress responses to drought, as well as dormancy. In addition to closure of stomata to reduce water loss by transpiration, one ABA-mediated response to drought is the elongation of the primary root; more recently, evidence is growing that ABA also regulates lateral root branching, including in normal, non-drought conditions [82]. ABA signalling appears to connect many factors known to inhibit lateral root number and elongation to the resultant phenotype: for instance, lateral root inhibition in response to homogeneously high soil nitrate [83,84] and the inhibitory effect on lateral root development observed as a result of high (sugar) carbon : nitrogen ratios in the soil [85,86].

Strigolactones are a novel class of phytohormones, which are proposed to be synthesized from carotenoids in the root tissues [87,88]. Strigolactones are known to inhibit shoot branching, as well as being involved in interactions with root parasitic plants and symbiotic arbuscular mycorrhizal fungi [89]. However, it has recently become apparent that strigolactones are also involved in root morphogenesis: plants which are insensitive to strigolactones or deficient in strigolactone biosynthesis exhibit reduced primary root lengths with increased root branching, while application of a synthetic strigolactone analogue rescues the root phenotype of synthesis-deficient plants. The action of strigolactones on root phenotypes is thought to be due to modulation of auxin flux in the root, via strigolactone-regulated membrane cycling of PIN auxin efflux carrier proteins [90,91].

(b) *Other signalling mechanisms*

Various other root architecture-affecting mechanisms have been described, and we will highlight a few examples here. Although much emphasis has traditionally been placed on the roles of hormone signalling in root development, there are also many non-hormonal signalling pathways involved, such as peptide signalling [34,92,93].

The most prominent signalling system is through receptor-like kinases (RLKs). One of these is the RLK ARABIDOPSIS CRINKLY 4 (ACR4), thought to be a receptor for an as-yet undetermined small peptide signal. Following the first asymmetric pericycle cell division in lateral root formation, the small daughter cells formed can be observed to express *ACR4*; the expression of *ACR4* continues in daughter cells following the second division, leading to a core-specific expression pattern [34]. In a cell autonomous fashion, ACR4 appears to play a role in promoting the early formative pericycle cell divisions required to initiate lateral roots; conversely, in a non-cell-autonomous fashion, ACR4 restricts supernumerary cell divisions in pericycle and columella cells once organogenesis has begun. This dual function, therefore, forms the basis of a homeostatic mechanism to prevent the unregulated division of pluripotent cells. More details on the role of peptide–receptor kinase signalling in root development will be highlighted further on in this issue by Stahl & Simon [94].

There are many other factors driving and controlling lateral root development, such as ABERRANT LATERAL ROOT FORMATION 4 (ALF4) and ARABIDILLO-1 and -2. *Arabidopsis* ALF4 is a nuclear-localized protein, the expression and subcellular localization of which is independent from auxin influence. ALF4 appears to function by keeping xylem pole pericycle cells in a mitotically competent stage, possibly by preventing terminal differentiation of these cells [95]. ARABIDILLO-1 and 2 are *Arabidopsis* proteins with homology to the animal Armadillo/beta-catenin proteins: *arabidillo-1/2* mutants have fewer lateral roots than wild-type, whereas *ARABIDILLO* overexpressing lines produce more. How ARABIDILLO precisely regulates lateral root branching is unclear, but it is possibly achieved through targeting of a lateral root inhibitor for ubiquitin-mediated destruction via the F-box of ARABIDILLO. This suggests a signalling pathway in lateral root initiation entirely separate from other known pathways [96].

(c) *Impact of the environment on the root system*

The environmental factors affecting root growth, such as nutrient and water availability and distribution

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status, soil density, salinity and temperature, and microorganism interactions, are variable and complex; thus roots are necessarily plastic in their architecture. This plasticity is particularly well demonstrated in soil nutrient foraging. Nutrients such as nitrates (particularly NO_3^- but also NH_4^+) and inorganic phosphates are distributed heterogeneously in the soil: indeed, root activity itself exacerbates soil nutrient heterogeneity. Plants, therefore, exploit concentrated nutrient reserves through modification of root architecture: for example, through the preferential elongation of lateral roots in both locally concentrated nitrate, and even more markedly, in phosphate-rich patches, respectively [65,97].

Nitrogen, sourced from the plant-available form of soil nitrates, is required by crops for the formation of essential macromolecules, including DNA and proteins. Many studies have demonstrated a relationship between soil nitrate levels and root architecture: lateral root growth is inhibited at low concentrations of soil nitrate, whereas locally nitrate-rich soil patches stimulate elongation of lateral roots in order to increase N uptake [98]. However, it is important to disentangle nutritional effects from signalling effects when considering soil NO_3^- in root phenotypes.

Recently, a mechanism to explain the inhibitory effect of low soil NO_3^- concentration on lateral root elongation has been described, via the NRT1.1 nitrate transporter [99]. NRT1.1 has a dual function: it is a sensor for soil NO_3^- concentration, and perhaps surprisingly, it also facilitates auxin uptake, thus coupling nutrient sensing with a hormonal response [100–102]. At low NO_3^- concentrations, NRT1.1 modifies auxin transport, promoting basipetal transport away from the lateral roots and thus inhibiting their formation and growth (although low nitrate concentrations can also inhibit growth simply owing to N starvation, independently from the signal-based effect of NO_3^- on NRT1.1). Furthermore, high NO_3^- levels also inhibit the auxin transport activity of NRT1.1, explaining the inhibition of lateral root formation and growth also observed when a high concentration of NO_3^- is distributed homogenously in the soil. Conversely, primary root growth in *Arabidopsis* appears largely insensitive to soil NO_3^- concentration [97], though increased primary root elongation in maize roots exposed to moderately high nitrate levels has also been reported [103].

Phosphorus is an important micronutrient that is often limiting because of the tendency of phosphorus to form insoluble compounds in the soil. In contrast to nitrate, which is highly mobile in soil and tends to leach easily, phosphorus also has poor soil mobility and tends to be concentrated in the upper soil strata [13]. Plants grown in low phosphorus conditions typically exhibit reduced elongation of the primary root and increased lateral root density and length [104]. This is thought to be an adaptive response to aid nutrient foraging. Indeed, another type of root, 'cluster roots', comprising a morphology of dense clusters of rootlets from a parent root, can be observed in some species, most notably members of the Proteaceae family. These cluster roots are formed as part of the normal root developmental programme rather than as a responsive change [105], and are an example of a root structure which appears to have evolved primarily for the maximization of phosphorus acquisition from the soil [106,107]. In this issue, Nussaume *et al.* [108] discuss a novel imaging technique for the visualization of nutrient uptake.

Drought has a major effect on root architecture, with many plants preferentially increasing primary root elongation and suppressing lateral root branching in response to drought. Many plants adapted to drought, such as sorghum, have a naturally more vertically orientated root structure [109]. Soil salinity also has an effect on root architecture owing to the infliction of osmotic and ionic stress on the roots, but in contrast to drought stress, high soil salinity inhibits primary root elongation while promoting lateral root emergence in glycophytes such as *Arabidopsis* [110,111]. Drought is often associated with high temperatures; in this issue, Hund *et al.* [112] discuss the effects of high soil temperature on root growth in maize.

The effects of soil pH and soil strength are less well understood. High soil acidity can solubilize and thus increase the availability of aluminium in the soil, to a level where aluminium becomes toxic to plants; studies in grasses and cereals have reported generally reduced root elongation in response to aluminium toxicity owing to acid soil [113,114]. Similarly, hard and compacted soils generally also impede root growth, particularly lateral root growth [113,115,116]. Soil strength is a complex factor in root growth: as well as increasing resistance and thus decreasing root growth, other stresses such as water stress, nutrient stress and root hypoxia may be induced as the soil is increasingly compacted [115]. The structure of soil is also important: an absence of pores in compacted soil can present problems, as roots typically use pores in the soil to facilitate growth, anchorage and root hair expansion [117].

5. MOLECULAR CONTROL OF ROOT BRANCHING IN (CEREAL) CROPS

While a lot of progress has been made on the level of the molecular control mechanisms of RSA, and specifically root branching, in *Arabidopsis*, comparatively less is known in cereals. The majority of research conducted in cereals has focused on the species *O. sativa* and *Z. mays*.

The *rootless concerning crown and seminal roots* (*rtcs*) maize mutants, and their rice homologues, *adventitious rootless 1* (*arl1*) and *crown rootless 1* (*crl1*), completely lack all shoot-borne roots and also seminal roots in maize, leading to a reliance on the primary and lateral root system more reminiscent of that found in dicots. In the rice mutants, emergence of lateral roots from the primary root is also greatly reduced. Without support from shoot-borne roots, lodging (flattening) of the mutant plants is a major issue. Both maize *rtcs* and rice *arl1*/*crl1* have been found to be closely related to the *Arabidopsis* genes *LBD16* and *LBD29*, two lateral organ boundary genes activated by ARF7/ARF19 [56,118,119]. In this issue, Majer *et al.* [120] describe the control of RTCS on *ARF34* expression in maize.

Lateral rootless 1 (*lrt1*), *rootless with undetectable meristems 1* (*rum1*), *short lateral roots 1* (*slr1*) *and slr2* are

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four maize mutants demonstrating abnormal lateral root development. The *lrt1* [121] and *rum1* [122] mutants fail to initiate lateral roots (in *rum1* at least this appears to be due to disrupted auxin transport) whereas *slr1* and *slr2* [123] are affected after initiation, leading to reduced lateral root elongation.

Mutants for crown root elongation have also been found in rice: the *crl5* mutant for an AP2/ERF family transcription factor and as such is unable to induce the cytokinin type-A response regulator OsRR1, which usually negatively represses cytokinin signalling. In the absence of this repression, cytokinin inhibits crown root initiation in rice—similar to the inhibition of lateral roots by cytokinin observed in *Arabidopsis* [124]. OsGNOM is a guanine nucleotide exchange factor for a GTPase—ADP-ribosylation factor (ARF)—involved in polar auxin transport, and has a high degree of homology to the GNOM protein found in *Arabidopsis*. Mutants for OsGNOM also have defects in crown root development owing to disrupted distribution of auxin in the shoot tissues at the site of crown root primordia initiation [125].

Rtcs, *arl1*/*crl1* and *Osgnom* are selected examples of the translatability of *Arabidopsis* research into crop plants, but the added complexity of the monocot root system means the translation is not straightforward. The inadequacy of *Arabidopsis* as a model organism in the context of studying shoot-borne root developmental processes is clear: all mutants discovered thus far for lateral root phenotypes in monocots are affected only in the embryonic roots [126,127]. This demonstrates that the mechanisms controlling architecture must be different for the post-embryonic and embryonic roots, respectively. However, despite some knowledge of shoot-borne root developmental processes in cereals such as maize and rice, there are currently no known mutants that demonstrate aberrant lateral root formation from shoot-borne roots in cereals.

One possible candidate model that may help bridge the gaps between *Arabidopsis* and current cereal models is *Brachypodium distachyon*. This species has many of the same benefits of *Arabidopsis* as a model species, such as small size, relatively short life cycle and small genome size; but crucially it has the root architecture typical of monocots, because it belongs to the family Poaceae, which also includes rice, maize, wheat and barley. Pacheco-Villalobos *et al.* [128] and Ingram *et al.* [129] further discuss the suitability of *Brachypodium* as a model for root studies.

6. VISUALIZING COMPLEX ROOT SYSTEM ARCHITECTURE

The study of root architecture has always been compromised by the inherent difficulties in studying a system that necessarily operates in a below-ground environment. Extracting an entire root system from soil, while maintaining completeness and avoiding damage to the finer elements of the root system, is a challenge (though some results have been generated this way, for example via 'shovelomics', a method of excavating, washing and phenotyping crown roots [13,130]).

For this reason, several other root growth methods have been trialled, such as growth on moistened germination paper rolls or pouches [131,132], sand rhizotrons [133] and gel-based systems [134–136], where phenotypic effects can be imaged using flatbed scanners, digital cameras or even lasers [137]. In this issue, Wells *et al.* [138] present novel image acquisition and analysis methods to capture root growth and development. However, these methods are still not an adequate solution, because root architecture is influenced by many soil properties, including mechanical strength and density of the soil, the presence of air pockets in the soil, soil pH and temperature, and a variety of nutrient and biotic-interaction factors, which cannot be easily reproduced in highly artificial growth systems. The challenge of studying roots in the soil therefore remains, because it may be the only way to generate truly meaningful observations.

One strategy has been the use of X-ray computed tomography (CT) [139–141] which has the capability to visualize root architecture *in situ* by collecting many cross-sectional image 'slices' from which a complete three-dimensional root system image can be reconstituted, with no damage to the root system. This technique can be used to study root architecture under many different nutrient, moisture and soil density conditions in a physiologically relevant way. Less extensively, nuclear magnetic resonance (NMR) has been used in a similar way to CT to study root architecture *in situ*; however, CT is the preferred method owing to reservations about paramagnetic interference from ions in iron-rich soils [139]. In both CT and NMR systems, cost is a major drawback.

An alternative approach is mathematical simulation and modelling. From the starting point of programmed 'rules' based on direct phenotypic observations and molecular data, mathematical modelling can prove useful for mapping and integrating root growth and interactions in response to a variety of variables, and has been used to predict root growth patterns under differing conditions, including response to water and nutrient supply [142], various phosphorus concentrations [137], root adaptation to low nitrogen soil under carbon flux modifications [143] and the formation of root cortical aerenchyma in response to soil nutrient status [144], to name just a few. Simulation packages specifically for the study of root architecture have been developed, such as SimRoot [145]. Modelling approaches have been criticized in the past for being over-simplistic and failing to take into account the many complex variables of the soil environment and hydraulics [146]; nevertheless, recent progress in elucidating the biological, chemical and physical processes affecting root growth in soil means that newer models are now better equipped to integrate complex arrays of variables into one mathematical framework than was previously possible [29,147].

7. CONCLUSION

While the past decades have resulted in extensive insight into RSA, mainly in the model plant *Arabidopsis* but also in some (cereal) crops, there are still various aspects of root growth and branching that need to be addressed.

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This issue will provide answers to some outstanding questions, offer new tools to tackle remaining challenges, but also light the way for further studies.

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APPENDIX VII

Small Signaling Peptides in Arabidopsis Development: How Cells Communicate Over a Short Distance

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REVIEW

Small Signaling Peptides in *Arabidopsis* Development: How Cells Communicate Over a Short Distance

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To sustain plants' postembryonic growth and development in a structure of cells fixed in cell walls, a tightly controlled short distance cell–cell communication is required. The focus on phytohormones, such as auxin, has historically overshadowed the importance of small peptide signals, but it is becoming clear that secreted peptide signals are important in cell–cell communication to coordinate and integrate cellular functions. However, of the more than 1000 potential secreted peptides, so far only very few have been functionally characterized or matched to a receptor. Here, we will describe our current knowledge on how small peptide signals can be identified, how they are modified and processed, which roles they play in *Arabidopsis thaliana* development, and through which receptors they act.

INTRODUCTION

Cells within an organism must communicate over both short and long physiological ranges to ensure proper patterning and functional connections. There are several ways to achieve this in plants, including phytohormones, mobile transcription factors, noncoding RNAs, and small signaling peptides (Busch and Benfey, 2010; Van Norman et al., 2011). Most prominently, directional transport of the phytohormone auxin from one cell to the other provides cues for patterning and development (Vanneste and Friml, 2009; Grunewald and Friml, 2010). However, signaling through direct symplastic transport of transcription factors between cells, via plasmodesmata, has also been extensively explored (Busch and Benfey, 2010; Wu and Gallagher, 2011). Major examples of transcription factors that are mobile between cells are TARGET OF MONOPTEROS7 (involved in specification of the hypophysis during embryogenesis) (Schlereth et al., 2010) and SHORT ROOT (involved in the production of cortex and endodermis tissues) (Helariutta et al., 2000; Cui et al., 2007). In addition, microRNAs can also be considered to be intercellular signaling molecules; for example, a gradient of miRNA165/166 is required for specification of root xylem cell fate (Carlsbecker et al., 2010).

While the importance of signaling peptides in cell–cell communication has been recognized in animals for many years, in plants, the focus on nonpeptide, lipophillic phytohormones, such as auxin, has historically overshadowed the importance of signaling peptides (which should not be confused with signal peptide, a presequence directing a protein to the secretory pathway) (Vanneste and Friml, 2009). Nevertheless, in just over a decade, several secreted peptides have been recognized as important in cell–cell communication in plants, coordinating and integrating cellular functions in complex developmental processes (Matsubayashi, 2011a, 2011b). The identification of receptors, such as leucine-rich repeat–receptor-like kinases (LRR-RLKs), further underlines the importance of signaling peptides in plant development (De Smet et al., 2009).

Here, we will describe our current knowledge on the role signaling peptides play in *Arabidopsis thaliana* development and through which receptors they (likely) act. In addition, we will describe how small signaling peptides can be identified and how they are modified and processed.

LOOKING FOR SOMETHING SMALL

Peptide signals are small in size (usually <20 amino acids in the mature form and rarely more than ~120 amino acids as a full-length precursor) and often present in very low (nanomolar range) physiological concentrations, so finding them presents a challenge. Furthermore, microarrays and other tools designed for identification of differentially expressed genes have not been a particularly useful tool in signaling peptide characterization: Small genes are often overlooked or not adequately represented on arrays and are poorly predicted using gene prediction algorithms as they are difficult to distinguish from short, random open reading frames (Olsen et al., 2002; Lease and Walker, 2006). The problem is further compounded due to the low expression levels of signaling peptides. In addition, microarrays are blind to how posttranslational modifications affect protein activity, a drawback that is particularly pertinent due to the crucial role of posttranslational modification processes in the activation of many signaling peptides. By measuring gene expression levels, it cannot be distinguished whether a modified, active form of a signaling peptide or the unmodified, inactive form is prevalent in the relevant sample (Kodadek, 2001).

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The secreted signaling peptides discovered in plants thus far can be considered to fall into two broad groups: small posttranslationally modified peptides and Cys-rich peptides (Matsubayashi, 2012) (Figure 1A). The small posttranslationally modified peptides are a group of secreted signaling peptides characterized by small (<20 amino acid) mature peptides, which are cleaved from a longer translated precursor peptide, with a general paucity of Cys residues, and which are subject to one or more posttranslational modifications. Several small posttranslationally modified peptides have been identified, including CLAVATA3 (CLV3), CLV3/EMBRYO SURROUNDING REGION-RELATED (CLE), TRACHEARY ELEMENT DIFFERENTIATION INHIBITORY FACTOR (TDIF), PHYTOSULFOKINE (PSK), PLANT PEPTIDE CONTAINING SULFATED TYROSINE1 (PSY1), C-TERMINALLY ENCODED PEPTIDE1 (CEP1), INFLORESCENCE DEFICIENT IN ABSCISSION (IDA), and ROOT GROWTH FACTOR (RGF)/GOLVEN (GLV)/CLE-LIKE (CLEL). The Cys-rich peptides have several uniting features despite a generally high amino acid sequence divergence between individual peptides: Though often larger than small posttranslationally modified peptides, they are still small (<160 amino acids), they are cationic, and they have a conserved N-terminal signal peptide. In contrast with small posttranslationally modified peptides, their C-terminal domain is Cys rich, typically containing four to 16 Cys residues. The presence of Cys residues is thought to be required for the formation of disulphide bridges to hold the mature peptide in an active conformation (Pearce et al., 2001a). The Cys-rich peptide family of signaling peptides includes the RAPID ALKALINISATION FACTOR (RALF)/RALF-LIKE (RALFL), EPIDERMAL PATTERNING FACTOR1 (EPF1), EPF2, TAPETUM DETERMINANT1 (TPD1), STOMAGEN/EPF-LIKE9 (EPFL9), and EARLY NODULIN peptides. The discoveries of the currently known plant signaling peptides encompass various and evolving approaches, from the earliest biochemical assay approaches, through genetic screens, to in silico approaches. We discuss these methods below, using appropriate examples.

Biochemical Assays

The first plant signaling peptide, tomato systemin (TomSys), was discovered through observations that extracts from wounded tomato (*Solanum lycopersicum*) leaves, when added in water to young tomato plants, induced production of proteinase inhibitors I and II (Ryan, 1974). Interestingly, the effect could not be generated through substitution of the unknown active ingredient with a variety of plant hormones, including auxin, gibberellic acid, abscisic acid, and ethylene, which were (at the time) the main known candidate factors that could initiate a signaling pathway. Purification of a peptide from tomato leaf extract by HPLC and testing by application of both eluted fractions and a later-generated synthetic peptide to young plants, revealed the identity of the active ingredient as an 18–amino acid polypeptide factor nearly 20 years after its initial discovery (Ryan, 1974; Pearce et al., 1991).

Two signaling peptides with no sequence similarity to TomSys, but with similar functionality, named tobacco systemin I (TobHypSysI) and TobHypSysII ("Hyp" referring to hydroxyprolination of these peptides), were later discovered in wounded tobacco (*Nicotiana tabacum*) leaves using similar methods. However, the method used to identify TobHypSys also incorporated a new assay: the alkalinization assay. This assay was developed following the observation that TomSys causes an alkalinization of suspension culture medium, and it was hypothesized that other active peptides might have a similar effect (Meindl et al., 1998; Schaller and Oecking, 1999; Pearce et al., 2001b). Using this alkalinization assay, crude tobacco leaf extracts were run on HPLC columns, the eluted fractions were added to aliquots of tobacco suspension culture medium, and the pH of the medium measured. Increases in the pH of the media identified two major peaks corresponding to TomHypSysI and TomHypSysII.

This alkalinization assay was also used to identify RALF from tobacco, which alkalinized suspension culture media and induced mitogen-activated protein kinase activity. But, unlike the systemins, RALF was not involved in wound response (Pearce et al., 2001b). RALF-mediated alkalinization of the media occurs due to a receptor-mediated response leading to the inhibition of a membrane-bound ATPase (Meindl et al., 1998; Stratmann et al., 2000).

In a process that echoed the identification of TomSys as the active ingredient from a crude plant extract, PSK was discovered during attempts to identify conditioned media factors. Conditioned media containing extracts prepared from rapidly growing cells in culture had been shown to be necessary for the proliferation of cells in low-density plant cell culture, but it was unclear which unknown substance(s) within the extracts was active in promoting the proliferation. A bioassay based on the mitotic activity of asparagus (*Asparagus officinalis*) mesophyll cells in liquid culture identified an active, growth-promoting compound, purified through chromatography, PSK-α (Matsubayashi and Sakagami, 1996). Following its discovery in asparagus, PSK has been found to be widely conserved in many other plant species, including rice (*Oryza sativa*) and *Arabidopsis*, in which five paralogous *PSK* genes have been identified, designated *PSK1–5*, that are expressed in various tissues (Matsubayashi et al., 1997, 2006).

Likewise, TDIF was discovered during attempts to isolate xylogen, an arabinogalactan protein that mediates cell interaction in vascular development, from *Zinnia elegans* mesophyll cell culture. Tracheary element differentiation in these cells was being inhibited by an unknown extracellular factor, which was subsequently isolated and purified from the conditioned cell medium as a 12–amino acid peptide (Ito et al., 2006). Homology searches revealed that TDIF was identical to the C-terminal 12 amino acids of *Arabidopsis* CLE41 and CLE44 and highly homologous to CLE42 and CLE46. Indeed, dodecapeptides based on the CLE motifs of CLE41 and CLE44 showed strong TDIF activity, in terms of promoting tracheary element differentiation of *Z. elegans* mesophyll cells in culture and in *Arabidopsis* (Ito et al., 2006; Hirakawa et al., 2008; Whitford et al., 2008; Etchells and Turner, 2010).

However, the typically low physiological concentrations of signaling peptides in tissues may evade detection through bioassay methods. Using a different, peptidomics-based approach, taking into account the importance of posttranslational peptide modifications (see below), it is possible to enrich sulfated peptides, present at low concentrations in a complex peptide mixture,

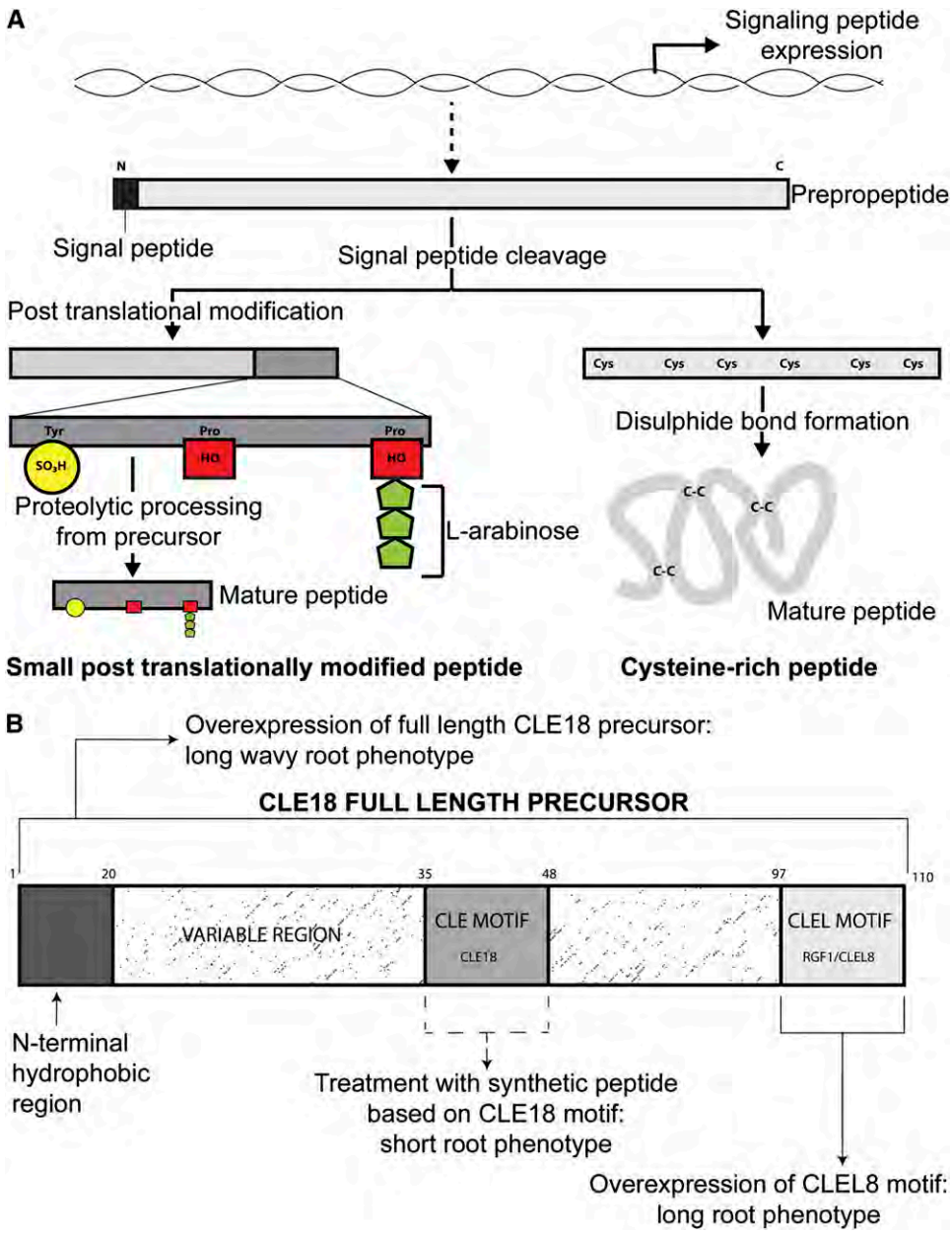

Figure 1. Posttranslational Modifications and Processing of Small Signaling Peptides.

(A) Following entry of the full-length mRNA-encoded prepropeptide to the secretory pathway, the N-terminal hydrophobic signal peptide is cleaved by a signal peptidase. Small posttranslationally modified peptides and Cys-rich peptides then follow different pathways to produce an active peptide. All small posttranslationally modified peptides discovered in plants thus far undergo one or more of three types of post translational modification: Tyr sulfation (yellow), Pro hydroxylation (red), and/or Hyp arabinosylation (green). Proteolytic cleavage of the modified peptide from the precursor sequence completes activation. Cys-rich peptides often do not require proteolytic processing from a precursor, though there are exceptions. Activation of Cys-rich peptides is completed upon formation of disulphide bonds between the conserved Cys residues of the peptide, thus bringing the peptide to an active conformation. Schematic is based on Matsubayashi et al. (2012) with permission.

(B) The CLE18 precursor contains two motifs with sequence similarity to the canonical CLE motif. CLE18 is found within the variable region of the peptide at amino acid residues 35 to 48, rather than at the extreme C terminus, like other currently known CLE motifs. At the C terminus, there is a sequence of 13 amino acids with a CLEL sequence. At the N terminus is a region of hydrophobic amino acids, thought to act as a secretion signal (Meng et al., 2012). Overexpression of the full-length precursor gives a long root phenotype (Strabala et al., 2006); conversely, treatment with a synthetic peptide based on the CLE18 motif causes a short root phenotype (Ito et al., 2006). Overexpression of the *CLEL8/RGF1* motif (derived from the CLE18 precursor) and related *CLELs* (*RGF6/CLEL6*, *RGF5/CLEL7*, and *RGF9/GLV2/CLEL19*), derived from other precursors, also results in a long root phenotype, suggesting that the extreme C-terminal motif represents the sequence of the active peptide (Matsuzaki et al., 2010; Meng et al., 2012; Whitford et al., 2012).

using anion exchangers (Amano et al., 2007). This approach provided a pool of likely candidates for potential signaling peptides, including PSY1, which was found to promote cellular proliferation and expansion in various tissues (Amano et al., 2007).

Genetic Approaches

Forward and reverse genetics approaches have allowed signaling peptide biology to progress further. For example, CLV3 was identified through genetic screening approaches as a putative signaling peptide (Clark et al., 1995; Fletcher et al., 1999). The ethyl methanesulfonate–induced *clv3* mutant has an enlarged shoot apical meristem (SAM) and superfluous floral meristems, suggesting a role in regulating meristematic growth (Clark et al., 1996).

Still, one major issue with peptides is the limited number of (characterized) loss-of-function mutants, since most have no useful, available T-DNA insertions. Partly because small genes, such as potential secreted signaling peptides, are less likely to be directly hit by a T-DNA. To complicate matters further, the functional redundancy of some signaling peptides can mask phenotypes when only one family member is successfully disrupted. Nevertheless, two signaling peptides have been discovered through screening T-DNA insertion mutants, IDA and TPD1. A T-DNA insertion in the promoter of a gene at locus At1G68765 gave rise to a phenotype that failed to undergo floral organ abscission following pollination, which were loss-of-function *ida* mutants (Butenko et al., 2003). Five paralogous genes were also found in *Arabidopsis*, called *IDA-LIKE* (*IDL*). The structure of IDA has not been fully elucidated; however, it is known that the *IDA* gene codes for a 77–amino acid polypeptide (a possible precursor) and, in common with other small signaling peptides, contains a conserved Pro-rich domain at the C terminus. TPD1 was discovered through screens of gene and enhancer trap lines, where one line produced no pollen (Yang et al., 2003).

Based on observations that previously discovered signaling peptides, such as PSK and PSY1, often require Tyr sulfation by a TYROSYLPROTEIN SULFOTRANSFERASE (TPST), it is logical to assume that a *tpst* loss-of-function mutant might display a phenotype arising from a deficiency in active signaling peptides, and this could help identify signaling peptide candidates. The *tpst* loss-of function mutant displays many pleiotropic phenotypes, including severely shortened roots and reduced root meristematic activity with failure of root stem cell maintenance (Komori et al., 2009; Zhou et al., 2010). The *tspt* mutant and a reverse-genetics screen were used to characterize RGF/GLV/CLEL peptides (Matsuzaki et al., 2010).

Bioinformatics Digs Deeper

The identification of several signaling peptide encoding genes shows that in silico methods using bioinformatics tools can be useful for initial identification of small signaling peptides. For example, SignalP is an algorithm that can predict the presence and location of signal peptides and cleavage sites in an amino acid sequence. It has been used as a tool during the discovery and characterization of several secreted peptides, including IDA and CEP1 (Butenko et al., 2003; Ohyama et al., 2009), and was the starting point for the discovery of the RALFL family (Olsen et al., 2002). Some of the results generated from an initial SignalP search of an *Arabidopsis* genome database were compared for homology to known peptides, using various types of BLAST search and ClustalW alignments, leading to discovery of the uncharacterized RALFL gene family in *Arabidopsis* (Olsen et al., 2002). Similarly, computer programs (PHDsec and SOPMA) were used to compare the secondary structure of the identified peptides. Some members of this family had high sequence similarity to RALF peptides, leading to them being designated the RALFL family (Olsen et al., 2002; Haruta and Constabel, 2003). *Arabidopsis* has been shown to have around 40 *RALFL* genes, which are expressed throughout the plant and impact various developmental processes, including seedling development, stem development, and root development (Matos et al., 2008; Srivastava et al., 2009; Bedinger et al., 2010; Mingossi et al., 2010).

CEP1 was identified through an in silico approach by analysis of an *Arabidopsis* genome database, by searching for paralogs to the peptide CLE44 (Ohyama et al., 2009). The reasoning behind this approach was that signaling peptides, such as PSK, PSY1, and CLE, are encoded by several paralogous genes and have many specific features that can be programmed in to computational searches to maximize the quality of the final candidate pool, such as size limitations, presence of an N-terminal signal sequence, and Cys paucity. Tellingly, the candidate pool identified by this search consisted of ~40% of peptides with known or predicted function, such as PSK, PSY precursors, IDA, IDL, and several CLE family peptides. Identification of one uncharacterized gene family led to identification of a 15–amino acid hydroxyprolinated peptide, namely, CEP1 (Ohyama et al., 2009).

This in silico approach was then adopted using similar search parameters as those used earlier to identify CEP1, but including additional search criteria to identify peptides with an Asp-Tyr sulfation motif. From this search, the RGF/GLV/CLEL polypeptide family was found with features matching all the search criteria. Mass spectrometry of peptides derived from plants overexpressing one member identified a 13–amino acid Tyr-sulfated peptide, derived from the C-terminal domain of the full-length cDNA-encoded protein. In parallel, homology searches using the C-terminal sequence of *CLE18* also revealed 10 members of the same RGF/GLV/CLEL family. It has been suggested that the active peptide sequence of RGF1 is derived from the CLE motif of its precursor. Interestingly, the RGF1/CLE18 precursor in fact contains not one, but two functional motifs within its sequence: the RGF1/CLE18 13–amino acid CLE motif, spanning residues 35 to 48 (not the extreme C terminus, as would be expected for a CLE motif), and a second, previously undiscovered motif spanning residues 75 to 87, designated the CLEL motif because of the similarity to the classical CLE motif (Matsuzaki et al., 2010; Meng et al., 2012) (Figure 1B). Homology searches revealed that similar motifs were found in nine other proteins besides the RGF1/CLE18 precursor.

Many of the Cys-rich peptides have been identified through computational analysis of several model plant genomes (Graham

et al., 2004; Silverstein et al., 2005, 2007). For example, the Cys-rich peptide EPF1 was discovered through systematic individual 35S promoter–driven overexpression of 153 genes that were predicted to encode small secreted peptides in *Arabidopsis* followed by phenotypic analysis, and one of the overexpressing lines had reduced stomatal density (Hara et al., 2007). Homology searches for EPF1 identified 10 homologs in *Arabidopsis*: EPF2 and EPFL1 to 9 (Hara et al., 2009; Hunt and Gray, 2009). STOMAGEN/EPFL9 was identified in the same large-scale screen used to identify EPF1 and in an in silico screen to identify genes that are coexpressed with stomatal regulators (Kondo et al., 2010; Sugano et al., 2010).

FROM CODING SEQUENCE TO MATURE, BIOACTIVE SECRETED PEPTIDE

Many signaling peptide genes encode a protein that is longer than the mature, active form of the peptide. Several of them are expressed as ~100–amino acid prepropeptides, which require proteolytic processing by endopeptidases, and cleavage of a signal peptide by signal peptidases, to produce a functional peptide. The small posttranslationally modified peptides and the Cys-rich peptide groups require different types of processing: Both usually undergo removal of signal sequences (by signal peptidases) and removal of extra mRNA-encoded precursor sequence (some Cys-rich peptides do not require this step). Before processing, the small posttranslationally modified peptides undergo enzyme-catalyzed modifications to selected residues of the mature-length peptide. Following processing, Cys-rich peptides are predicted to form disulphide bonds between the conserved Cys residues in their sequence to give the peptides their final active conformation.

Posttranslational Modifications

So far, three types of posttranslational modification, which possibly act to enhance receptor binding, have been observed to occur to signaling peptides in plants: Tyr sulfation, Pro hydroxylation, and Hyp arabinosylation (Matsubayashi, 2011b) (Figure 1A, Table 1). Tyr sulfation is thought to be catalyzed by the plant-specific TPST, a 62-kD type I transmembrane protein localized in the Golgi (Komori et al., 2009). The peptide substrate is known to require, as a minimum, an Asp residue N-terminally adjacent to a Tyr residue (an Asp-Tyr motif), and TPST requires the cosubstrate 3′-phosphoadenosine 5′-phosphosulfate as a sulfate donor (Matsubayashi, 2012). Pro hydroxylation is catalyzed by PROLYL-4-HYDROXYLASE (P4H), a type II Golgi and ER membrane protein of the 2-OXOGLUTARATE-DEPENDANT DIOXYGENASE family (Myllyharju, 2003). Thirteen genes for P4H have been identified in *Arabidopsis*, but no substrate consensus sequence for Pro hydroxylation has been established yet (Matsubayashi, 2012). Following the conversion of Pro to Hyp, a subset of peptides undergoes a further posttranslational modification, Hyp arabinosylation. In a posttranslational modification unique to plants, an *O*-linked L-arabinose chain (triarabinoside) is added to certain Hyp residues within the peptide. The linkage of the triarabinoside chain to the Hyp residue and the linkage of the sugar moieties within the chain to each other are separate reactions and hypothesized to occur in two steps mediated by different enzymes. A Hyp arabinosyltransferase catalyzes a β-linkage between the hydroxyl group of Hyp to an arabinose, and an arabinosyltransferase mediates the β1,2-linkage between the arabinose residues themselves (Matsubayashi, 2012). However, the enzymes responsible for this process in plants are yet to be identified.

CLE family proteins are united by their possession of a conserved 12– to 14–amino acid CLE motif, usually positioned at the extreme C terminus of the full-length translated precursor protein, and a hydrophobic N-terminal signal sequence (Cock and McCormick, 2001; DeYoung and Clark, 2001). Cleavage of the CLE motif from the precursor is necessary for production of the mature 12– to 14–amino acid peptide, which may then undergo further posttranslational modification (Fiers et al., 2005, 2006; Ito et al., 2006; Ni and Clark, 2006; Ohyama et al., 2009; Katsir et al., 2011). The CLE motif contains four conserved Pro residues at positions 4, 7, and 9: Pro-4 and Pro-7 appear to have particular significance as the targets for posttranslational modification (hydroxylation: Pro-4/Pro-7; arabinosylation: Pro-7). The *CLV3* gene is expressed as a protein of 96 amino acids, including an 18 hydrophobic N-terminal amino acid signal peptide to direct the peptide for secretion, a typical feature of CLE peptides. Cleavage occurs at the Arg at the beginning of the CLE motif (Arg-70), and this residue is conserved absolutely through all CLE proteins, including CLV3 (Ni and Clark, 2006; Oelkers et al., 2008). Recent evidence suggests that the cleavage of the CLE motif from the precursor is likely to be due to the action of an as yet unknown Ser protease, based on the ability of the Ser protease inhibitors 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride and bestatin to block the proteolytic processing of the CLE domain (Ni et al., 2011). Following processing, CLV3 undergoes further posttranslational modification. The amino acid sequence of CLV3 contains three Pro residues, of which the first two N-terminal Pro residues are hydroxylated and one Hyp (Hyp-7) then undergoes Hyp arabinosylation. The arabinosylation of Hyp-7 has been shown to enhance binding of CLV3 to the receptor CLV1 (Kondo et al., 2006). TDIF (CLE41/CLE42) is cleaved from a 99– to 112–amino acid protein precursor before proteolytic processing to separate the 12–amino acid CLE domain from the precursor (Ito et al., 2006).

Similar mechanisms may also apply to the RGF/GLV/CLEL family peptides with regards to the processing of the CLE motif from the precursor. However, though it seems likely, it is unknown if CLEL motifs are extracted from the full-length precursor in the same way as the CLE motifs. In addition, it is unclear whether CLEL motif peptides require any posttranslational modification, such as Tyr sulfation, for optimal activity, as both the sulfated and unsulfated forms of the RGF1/CLEL8 peptide have been proposed as the active form in planta (Matsuzaki et al., 2010; Meng et al., 2012). Nevertheless, in agreement with the findings for the RGF1 peptide, Tyr sulfation of synthetic peptides representing the proposed active form of RGF6/GLV1 and RGF4/GLV3 increased their bioactivity by two to three orders of magnitude. This opens the possibility that other precursors might also give rise to multiple functional peptides which are subject to distinct proteolytic processing and posttranslational modifications (Figure 1B).

Table 1. Posttranslational Modifications and Proteolytic Processing of Small Signaling Peptides

Peptide	Precursor Length (Amino Acids)	Mature Length (Amino Acids)	Posttranslational Modifications	Proteolytic Processing Enzyme(s)	Posttranslational Modification Enzyme(s)
CEP1	91	15	Pro hydroxylation (2 residues)	?	Prolyl-4-hydroxylase (P4H)*
CLV3	96	13	Pro hydroxylation (2 residues) Hyp arabinosylation (1 residue)	?	Prolyl-4-hydroxylase (P4H)*
PSK	~80 (77–87 for PSK1-5)	5	Tyr sulfation (2 residues)	Subtilisin-like Ser protease SBT1.1	Tyrosylprotein sulfotransferase
PSY1	75	18	Tyr sulfation (1 residue) Pro hydroxylation (two residues) Hyp arabinosylation (one residue)	?	Tyrosylprotein sulfotransferase Prolyl-4-hydroxylase (P4H)*
RGF1	116 (79–141 for RGF2-9)	13	Tyr sulfation (one residue) Pro hydroxylation (one residue)	?	Tyrosylprotein sulfotransferase Prolyl-4-hydroxylase (P4H)*
TDIF (CLE41, CLE42, CLE44)	88–112	12	Pro hydroxylation (two residues)	?	Prolyl-4-hydroxylase (P4H)*
?	?	?	?	SDD1	?

?, not yet identified; *, likely/predicted.

Amino acid sequencing of purified PSK-α suggested that PSK was subject to posttranslational Tyr *O*-sulfation. The sulfation of two Tyr residues, namely, Tyr-1 and Tyr-3, of the PSK-α peptide was later confirmed through synthesis and subsequent sulfation of the peptide using arylsulfotransferase. Solid-phase peptide synthesis and sulfation of PSK-α and 12 analogs with an adimethylformamide-sulfurtrioxide complex confirmed the active peptide core of PSK as Tyr(SO_3H)-Ile-Tyr(SO_3H)-Thr-Gln. The second peptide identified, PSK-β, was found to be a C-terminal truncated tetrapeptide of PSK-α, which also underwent Tyr sulfation: Tyr(SO_3H)-Ile-Tyr(SO_3H)-Thr (Matsubayashi et al., 1996; Matsubayashi and Sakagami, 1996). Acidic residues surrounding the Tyr residues appear essential for optimal sulfation activity, probably by a class of tyrosylprotein sulfotransferases unique to plants (Hanai et al., 2000). Studies on *PSK1* in rice show that the mature pentapeptide of PSK-α is proteolytically processed from an ~80–amino acid precursor, preprophytosulfokine-α (the exact precursor length varies according to species and PSK paralog). Preprophytosulfokine-α includes a hydrophobic 22–amino acid signal peptide at the N terminus, targeting it for inclusion in secretory pathways (Yang et al., 1999, 2000).

PSY1, which was found to promote cellular proliferation and expansion in various tissues, is expressed as a precursor of 75 amino acids and is proteolytically processed to a mature form of 18 amino acids: In addition to Tyr sulfation, PSY1 also undergoes Pro hydroxylation and subsequent Hyp arabinosylation (Amano et al., 2007; Matsubayashi, 2011a).

Tobacco RALF cDNA encodes a 115–amino acid polypeptide, which includes an N-terminal signal sequence (it is predicted by TargetP that all RALF prepropetides are targeted to the endomembrane system [Bedinger et al., 2010]), and a 49–amino acid C-terminal sequence representing the mature RALF peptide. PreRALF has a conserved dibasic site ~50 amino acids upstream from the C terminus. In animals, precursors to signaling peptides typically require such dibasic motifs to mark the site of mature peptide excision. These are recognition sites for a family of subtilisin/kexin-like preprotein convertases required for processing. However, most plant signaling peptide precursors discovered so far do not appear to require dibasic motifs for proteolytic processing. Thus, the conserved dibasic site found in preRALF is quite unusual. Within the conserved C-terminal sequence are four conserved Cys residues involved in the formation of two disulphide bridges (Pearce et al., 2001b). These disulphide bridges are thought to be essential for the active conformation of the protein and are absolutely required for function: Reduction of RALF with iodoacetamide renders the peptide unable to alkalize the cell culture medium in alkalization assays (Pearce et al., 2001a).

EPF1, EPF2, and EPFL1 to 9 all have a predicted secretory signal sequence and six C-terminal conserved Cys residues (Hara et al., 2009). The *STOMAGEN/EPFL9* gene encodes a 102–amino acid peptide. The N-terminal signal peptide is followed by a predicted cleavage site between residues 31 and 32. Members of the EPF family all contain a long loop between the 4th and 5th conserved Cys: The loop itself has very high sequence diversity between the members of the EPF family, which is thought to explain the functional diversity of the peptides (Kondo et al., 2010). Purification and mass spectrometry (matrix-assisted laser desorption/ionization-time of flight and liquid chromatography–mass spectrometry, respectively) of endogenously derived trypsin and chemotrypsin/lysyl endopeptidase-digested STOMAGEN protein

revealed that the active STOMAGEN peptide is 45 amino acids in length, is processed from a 120–amino acid precursor, and forms three disulphide bonds (Kondo et al., 2010; Sugano et al., 2010). The disulphide bonds are crucial to STOMAGEN function. While STOMAGEN is active at nanomolar (~10 nM) concentrations (a typical active concentration range for signaling peptides), both unfolded STOMAGEN and STOMAGEN where Cys residues were replaced with Ser residues were unable to increase stomatal density even at very high (10 μM) concentrations (Ohki et al., 2011).

Proteolytic Processing

The genes of the small posttranslationally modified peptides and many of the Cys-rich peptides encode a full-length protein that is longer than the length of the active peptide (Figure 1A, Table 1). The active peptide is freed from additional sequence by proteolytic processing to produce the mature-length peptide. The enzymes responsible for processing individual signaling peptide precursors remain largely unknown but are likely to be members of the subtilase group of Ser proteases. Subtilases are found in archaea, bacteria, yeast, and higher eukaryotes, but plants have many more subtilisin-like enzymes than animals: 56 in *Arabidopsis* compared with nine in humans, but very few loss-of-function mutants show obvious visible phenotypes (Rautengarten et al., 2005; Ottmann et al., 2009).

A subtilisin-like Ser protease, SBT1.1, is required for processing of the PSK4 precursor (Srivastava et al., 2008). Overexpression of *SBT5.4* gives a *clv*-like phenotype, though it was not demonstrated to cleave CLV3 in vitro (Liu et al., 2009). Two other members of this family of subtilisin-like enzymes, SITE 1 PROTEASE (S1P) and STOMATAL DENSITY AND DISTRIBUTION (SDD1), have been suggested to be involved in proteolytic processing of peptide signals. In the Cys-rich peptide family, RALF23 has been shown to require S1P for processing. S1P is a Golgi and endoplasmic reticulum–localized Ser protease that requires a dibasic RR motif (typical for animal subtilase substrates) for substrate recognition (Srivastava et al., 2009). SDD1, an extracellular protease, has been suggested to play a role in processing of EPF1 (Berger and Altmann, 2000), though conversely the phenotypes of mutants for *epf1* and *epf2* are more severe when crossed with *sdd1* to create a double mutant. The additive phenotype suggests EPF1 and EPF2 act independently from SDD1 (Hara et al., 2007, 2009; Hunt and Gray, 2009); thus, another enzyme is likely responsible for processing EPF family peptides (Table 1).

SIGNALING PEPTIDES IN *ARABIDOPSIS* DEVELOPMENT

At present, much of our knowledge relies on a few family members that have been investigated in depth, not in the least the CLE family, which has been shown to be associated with development in multiple *Arabidopsis* organs. Already at the first stages of plant life, cell–cell communication mediated by small signaling peptides and receptor kinases plays a key role (Escobar-Restrepo et al., 2007; Okuda et al., 2009; Kessler et al., 2010). Below, we will illustrate the essential involvement of various peptide families in plant organ and tissue development.

Anther and Pollen Development

The anther is a four-lobed structure that develops from three distinct cell layers, known as L1, L2, and L3. The L1 layer generates the epidermis, the L2 layer gives rise to endothecium, middle layer, tapetum, and the microsporocytes, and the L3 layer generates the connective tissues and vasculature within the center of the anther. Microsporocytes, or pollen mother cells, meiotically divide into four daughter cells known as microspores, which will develop into mature pollen granules. During L2 layer formation, the cells divide periclinally to form primary parietal cells. The parietal cells will further divide into secondary parietal cells, which, depending on location, can further divide periclinally into endothecium, middle layer, or tapetum. The tapetal layer is important to pollen development as it provides essential lipids and small molecules that aid in pollen formation and pollen wall generation (Goldberg et al., 1993; Zhao, 2009; Ge et al., 2010).

The *tpd1* mutant shows a complete lack of tapetal layer, zero pollen granules, and extra microsporocyte cells within the anther, since tapetal cell precursors develop into microsporocytes instead of tapetal cells (Yang et al., 2003). This suggests that the microsporocyte stage of development occurs before the tapetal development stage and that the TPD1 peptide may act somewhere between the two, initiating tapetal cell differentiation (Ge et al., 2010).

Recently, a number of CLE peptides have also been shown to be expressed during anther development. *CLE1* has been located in the tapetum of the anther, as well as within the pollen granules. *CLE11*, *CLE12*, and *CLE13* are all found within the pollen granules, during various stages of pollen development, while *CLE25* is expressed only during early anther development (Jun et al., 2010). However, their role in anther and pollen development remains to be demonstrated.

Embryo

The female gametophyte receives two sperm cells from a pollen tube: One sperm cell fertilizes the egg, which will develop into the embryo, and the other leads to endosperm formation, which are cells that regulate and nourish the embryo during development. The embryo, encased in the protective seed coat, will undergo a series of stereotypical divisions to form a miniature seedling (De Smet et al., 2010; Lau et al., 2012).

Several *CLE* peptides recently have been shown to be expressed during embryogenesis; specifically, CLE8 has been directly linked to embryo and endosperm proliferation and differentiation (Fiers et al., 2004; Jun et al., 2010; Fiume et al., 2011; Fiume and Fletcher, 2012). The *cle8* knockout mutants and *CLE8* artificial microRNA lines do not display any obvious vegetative aberrant phenotypes, but small, defective, underdeveloped, and misshaped seeds often occur within mature siliques. This correlated with deviations in early embryo cell division patterns and in endosperm proliferation and differentiation. Transgenic lines overexpressing *CLE8* show a slight increase in embryo length and overall size, suggesting a positive correlation between the CLE8 peptide and seed size. CLE8 has been shown to act upstream of WUSCHEL-LIKE HOMEOBOX8

(WOX8), evidenced by severely reduced *WOX8* expression in the *cle8* mutant. Conversely, overexpression of *CLE8* shows a marked increase in *WOX8* expression, suggesting that these two proteins act synergistically in suspensor and endosperm development (Fiume and Fletcher, 2012).

SAM

The leaves, stems, and floral parts of plants develop from the SAM, a conserved stem cell niche that resides at the top of the apical-basil axis of the plant. It is a tightly organized and regulated structure separated in three zones: the peripheral zone (PZ), the central zone, and the rib zone. The central zone consists of a highly conserved stem cell population, residing directly above the organizing center (OC), whose daughter cells are displaced into the PZ. It is the cells located in the PZ that generate the aerial tissues and organs of the plant (Fletcher and Meyerowitz, 2000; Stahl and Simon, 2005) (Figure 2A).

In shoot development, the peptide ligand CLV3 is a major regulator of the stem cell niche (Fletcher et al., 1999; Kondo et al., 2006) (Figure 2A). The *clv3* mutant results in overproliferation of the SAM stem cell population, to sizes often 1000 times larger than that of wild-type SAMs, resulting in a club-like shaped stem (*clava* meaning club) (Koornneef et al., 1983; Clark et al., 1995). By contrast, overexpression of *CLV3* eliminates the stem cell niche completely, terminating the SAM. These *CLV3* overexpression plants cease to initiate organs after the emergence of the first leaves, and, in a subset of the lines, flowers formed without the ability to generate inner whorls with stamens or carpels (Brand et al., 2000). CLV3 is shown to be involved in a negative feedback signaling pathway with the stem cell promoting homeodomain transcription factor protein WUSCHEL (WUS) (Mayer et al., 1998; Fletcher et al., 1999; Schoof et al., 2000; Nimchuk et al., 2011). *CLV3* is expressed in stem cells at the meristem apex, and the CLV3 peptide is thought to diffuse down toward the OC to restrict the expression of *WUS*, thereby regulating and inhibiting the expansion of stem cells in the SAM (Kinoshita et al., 2010; Nimchuk et al., 2011; Yadav et al., 2011). The expression of *WUS* defines the boundaries of the OC and surrounding stem cell niche and actively promotes the proliferation and division of stem cells in the apical cell layers (Buechel et al., 2010). Furthermore, WUS migrates between cells, toward the *CLV3* expressing cell layers, where WUS directly binds to genomic regions of *CLV3*, activating its transcription in the central zone at the meristem apex (Yadav et al., 2011). This negative feedback loop between WUS and CLV3 is well established (Figure 2A): *clv3* mutants overproduce stem cells, resulting in an enlarged SAM, whereas *wus* mutants eliminate stem cell populations and result in SAM termination (Laux et al., 1996; Nimchuk et al., 2011). By contrast, *CLV3* overexpression results in a *wus*-like phenotype and *WUS* overexpression results in a *clv3*-like phenotype (Kinoshita et al., 2010; Nimchuk et al., 2011). Thus, the homeostatic model shows that as stem cells decrease in number, *CLV3* expression is lowered, which raises *WUS* expression, inducing stem cell divisions. If stem cells become too abundant, *CLV3* expression increases, lowering WUS levels and thus lowering the rate of stem cell divisions (Brand et al., 2000; Schoof et al., 2000; Müller et al., 2006; Yadav et al., 2011).

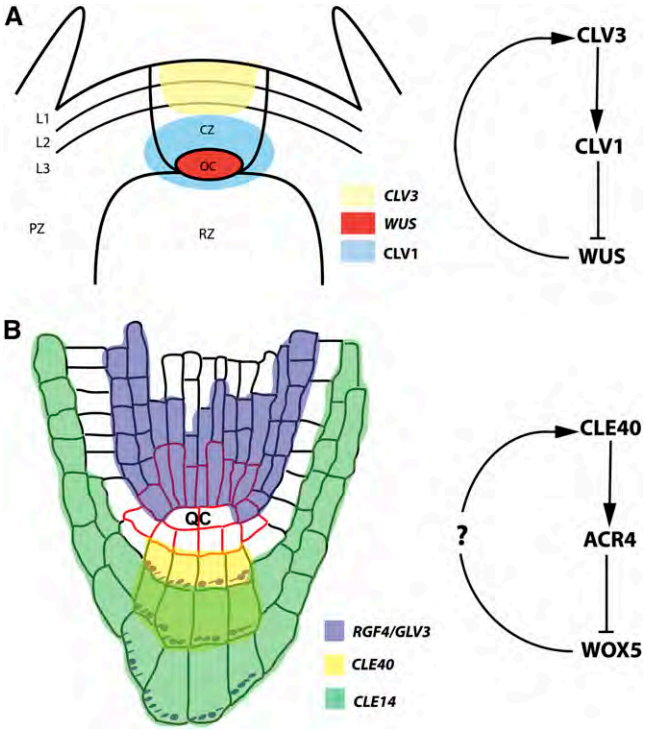

Figure 2. SAM and RAM.

(A) *CLV3* (yellow) is expressed in the central zone (CZ) of the SAM and inhibits expression of *WUS* (red) in the OC. WUS itself promotes *CLV3* expression. The CLV1 receptor is expressed below layers one and two of the SAM (L1 and L2). On the right is the proposed CLV3-CLV1-WUS signaling module. PZ, peripheral zone; RZ, rib zone.

(B) Several peptides, such as *CLE14*, *CLE40*, and *RGF4/GLV3*, are expressed in the RAM in overlapping domains to control cell identity and meristematic activity. On the right is the proposed CLE40-ACR4-WOX5 signaling module. At present, there is no evidence for WOX5 controlling *CLE40* expression (?). Red outline, stem cells and QC; purple, starch granules.

In addition to CLV3, there are over 30 CLE-related peptides containing the CLE motif, of which, CLE16, CLE17, and CLE27 have also been identified in and around the SAM (Lenhard and Laux, 2003; Sharma et al., 2003; Fiers et al., 2005; Jun et al., 2010). With the known redundancy of CLE peptides, there is a strong possibility that these CLE peptides diffuse from the SAM periphery and have significant functional roles within SAM development (Brand et al., 2000; Schoof et al., 2000; Jun et al., 2010). For example, it has been shown that many CLE motif–containing peptides can replace CLV3 function in the SAM (Ni and Clark, 2006). Overexpression of *CLE2*, *3*, *4*, *5*, *6*, *7*, *9*, *10*, *11*, and *13* all results in phenotypes similar to overexpression of *CLV3* or *wus* lines (Brand et al., 2000; Hobe et al., 2003; Strabala et al., 2006). With the majority of *CLV3*, *CLE9*, *CLE10*, *CLE11*, and *CLE13* overexpression lines dying significantly earlier than the wild type, it appears that these, possibly redundant, CLE peptides are integral to normal SAM development (Strabala et al., 2006). Possibly though, the CLE peptides may in fact be activating the endogenous CLV3 receptors, mimicking *CLV3* overexpression.

Also, during axillary bud formation, CLE41, CLE44, and CLE42 play an important role through regulating meristematic activity as demonstrated through application of synthetic peptides and overexpression studies (Yaginuma et al., 2011).

Lastly, EPFL peptides have been shown to play a role in inflorescence growth. While the single knockout mutants *epfl4/challah-like2* (*cll2*), *epfl5/cll1*, and *epfl6/chal* show no distinguishable phenotype from the wild type, *epfl4 epfl6* and *chal cll1 cll2* mutants show a strong compacted inflorescence with clustered flowers (Abrash et al., 2011; Uchida et al., 2012).

Primary Root Development

The underground parts of the plant, the main root, lateral roots, and root hairs, all of which play a role in water and nutrient uptake (De Smet et al., 2012; Smith and De Smet, 2012), are derived from cells originating in the root apical meristem (RAM). The RAM consists of stem cells surrounding the quiescent center (QC), which transmits developmental cues to the neighboring stem cells. Stem cells can generate further stem cells, in a nondifferentiated state, as well as daughter cells that differentiate into the respective cell layers. The formation of the columella represents an elegant example, where columella stem cells give rise to two daughter cells of which the lower one differentiates in the columella (containing starch granules) (van den Berg et al., 1998) (Figure 2B).

Several signaling peptides are expressed in the root tip, and several of these have been assigned a role in primary root development. Among the peptides involved in developmental processes in the RAM, the CLE and the RGF/GLV/CLEL families of peptides are the most prominent (Casamitjana-Martínez et al., 2003; Hobe et al., 2003; Stahl et al., 2009; Matsuzaki et al., 2010; Meng et al., 2012; Whitford et al., 2012) (Figure 2B).

Communication between QC and distal columella (stem) cells in the RAM has been elucidated as a signal transduction pathway between WOX5 and CLE40 (Figure 2B). *CLE40* is expressed in the inflorescence apices, leaves, and roots of the plant but has only been observed at functionally significant levels within the root tip. Overexpression of *CLE40* results in stunted primary root growth shortly after germination and these lack stem cells surrounding the QC but instead consists of large, differentiated cells. The *cle40* mutant shows a significant enlarged root phenotype due to rapid and aberrant division of columella initial cells. This overproliferation of initial cells is connected to an expansion of the *WOX5* domain, suggesting that CLE40 has a role in negatively regulating *WOX5*, supporting its proposed role in controlling or inhibiting the signals transmitted from the QC to the surrounding stem cells (Hobe et al., 2003; Stahl et al., 2009).

Various other CLE family peptides have been shown to be expressed in the RAM: *CLE12*, *CLE14*, *CLE16*, *CLE17*, *CLE22*, and *CLE27* (Jun et al., 2010; Meng et al., 2010). For example, *CLE14* (root cap and root hairs), *CLE16* (root cap and root elongation zone), and *CLE17* (root meristematic zone) are expressed in specific tissues of the root, suggesting specific roles. While *cle19* mutants give no phenotype, when overexpressed or applied exogenously, CLE14, CLE19, and CLE20 can irreversibly induce differentiation of stem cells rather than division and elongation, resulting in early consumption of the RAM and causing a strong short root phenotype (Casamitjana-Martínez et al., 2003; Fiers et al., 2004, 2005; Jun et al., 2010; Meng et al., 2010). Overexpression of *CLE9*, *CLE10*, *CLE11*, or *CLE13* results in severe inhibition of primary root elongation, while *CLE18*, *CLE25*, and *CLE26* overexpression leads to enhanced root elongation (Strabala et al., 2006). How CLE family peptides, with highly similar homologies, can have antagonistic roles in root elongation has yet to be elucidated, but similar antagonistic roles of related signaling peptides have also been observed during stomatal development (see below) (Kondo et al., 2008). Interestingly, the phytohormone cytokinin was shown to be involved in processing or transmitting CLE peptide signals, thus regulating the effects of CLE14 and CLE20 in planta. While exogenous application of CLE14 and CLE20 results in a strong short root phenotype, the combination with cytokinin largely rescued the reduction in primary root length (Meng and Feldman, 2010).

Two other signaling peptides expressed in roots, PSK and PSY1, are also involved in root elongation (Amano et al., 2007; Matsuzaki et al., 2010). These peptides can restore cell elongation activity in the elongation differentiation zone of roots in *tpst* but cannot restore meristem activity to that of the wild type, as evidenced by additional QC cells and aberrant columella cells (Matsuzaki et al., 2010). To restore the meristem size of *tpst* to a comparable level to that of the wild type, application of RGF1/GLV11/CLEL8 is required, suppressing formation of additional QC cells and restoring columella stem cell numbers. *RGF1/GLV11/CLEL8* is expressed in the QC and columella stem cells (with *RGF2* and *RGF3* expression in the innermost layers of the columella) and the protein diffuses into surrounding cell types forming a concentration gradient (Matsuzaki et al., 2010). While a single *rgf1* mutant did not show a significant phenotype, the *rgf1 rgf2 rgf3* triple mutant exhibits a similar phenotype as that of *tpst*, being a short root phenotype with a reduction in meristematic cells. Exogenous application of RGF1/GLV11/CLEL8 to *rgf1 rgf2 rgf3* rescued cell numbers to that of the wild type, suggesting a strong functional redundancy between these peptides. The overexpression of *RGF1/GLV11/CLEL8* shows the opposite phenotype, with seedlings possessing enlarged meristems, due to additional columella cells (Matsuzaki et al., 2010). Thus, the redundant, sulfated RGF/GLV/CLEL family of peptides is clearly inherent to the maintenance of the stem cell population within the root and appears to act on PLETHORA (PLT) transcription factors (Matsuzaki et al., 2010). *PLTs* are expressed in response to auxin and, in concert with PIN FORMED (PIN) proteins, act on auxin signaling required for root stem cell specification (Aida et al., 2004; Galinha et al., 2007). Interestingly, RGF/GLV/CLEL peptides also have an effect on auxin-dependent gravitropic response within roots, resulting in a waving phenotype, when overexpressed or exogenously applied. Specifically, RGF1/GLV11/CLEL8 interacts with the PIN2 protein, namely, accumulating the PIN2 proteins at the plasma membrane, through an unknown process, thereby affecting auxin transport (Meng et al., 2012; Whitford et al., 2012).

The small 15–amino acid hydroxyprolinated peptide CEP1 affects root growth but it is as yet unknown through what pathway. Both overexpression of *CEP1* and exogenous application of synthetic CEP1 results in reduced primary root growth.

Detailed analyses revealed that there are fewer cells in the RAM when *CEP1* is overexpressed, with a reduced cell size in the mature region of the root tip. No reduction in QC cells was observed, however, suggesting a role in cell division potential and efficacy, without interfering with QC stem cell capabilities. Strikingly, no *CEP1* expression was observed in the RAM, suggesting a nonspecific effect of generally increasing CEP1 levels (Ohyama et al., 2008).

The RALF peptide also reversibly inhibits *Arabidopsis* root growth and root hair initiation when seedlings are cultured in synthetic RALF-containing media (Pearce et al., 2001b; Bedinger et al., 2010). Overexpression of *RALF23* produces slow-growing seedlings, with roots that have a reduced capacity to acidify the rhizosphere, which may have developmental implications, resulting in a semidwarf phenotype (Srivastava et al., 2009). Exogenous application of RALF1 also causes a significant rise in cytoplasmic Ca^{2+} concentration, suggesting a functional role in Ca^{2+} modulation and signal transduction (Haruta et al., 2008).

Finally, members of the IDA family are also expressed in the RAM, such as columella cells; however, a functional role for the peptide in RAM management has yet to be elucidated (Stenvik et al., 2006).

Lateral Root Development

Lateral roots develop to aid in nutrient mining and water uptake and develop from a subset of pericycle cells at the xylem poles in the root stele. These pericycle cells and their daughter cells undergo a series of anticlinal and periclinal cell divisions generating a lateral root primordium (De Smet et al., 2008; De Smet and Beeckman, 2011; De Smet, 2012).

While the role of phytohormones during lateral root initiation has been highlighted extensively, only recently has the RGF/GLV/CLEL family of peptides been shown to significantly delay lateral root development when overexpressed (Péret et al., 2009; De Smet, 2012; Meng et al., 2012). Specifically, overexpression of *CLEL6* and *CLEL7* results in inhibition of pericycle division in the second round of pericycle cell divisions, arresting the initiation of lateral roots. Interestingly, analyses on *CLEL*-overexpressing plants in conjunction with auxin-containing media have shown that the CLEL peptides actually act in an auxin-independent manner (Meng et al., 2012). In addition, the *CLE20* peptide is also expressed in lateral roots, suggesting a possible dual role of the peptide in RAM maintenance and lateral root development (Meng and Feldman, 2010). Finally, *CEP1* is expressed in lateral root primordia, with expression of *CEP1* peaking during lateral root emergence and decreasing significantly after the lateral roots have elongated, suggesting that CEP1 might be involved in lateral root elongation, similar to its effect on the primary root (Ohyama et al., 2008).

Vascular Development

Within the roots and shoot of the plant lies the vasculature, the cells that are responsible for transporting water, minerals, nutrients, hormones, and even small signaling peptides throughout the organism. The vascular system in plants consists fundamentally of two functional cell types with vastly different functions: xylem and phloem (Sieburth and Deyholos, 2006) (Figure 3). Among the many hormone, transcription factor, and microRNA signals that are required for plant vasculature development, signaling peptides, especially the CLE family, have also become a prominent player (Figure 3) (Fiers et al., 2007; Elo et al., 2009; Hirakawa et al., 2011; Kondo et al., 2011). TDIF (CLE41/44) is shown to promote the proliferation of procambial cells in leaf and hypocotyl vasculature development, while it also inhibits the differentiation of procambial cells into tracheary elements (Ito et al., 2006; Whitford et al., 2008). Overexpression of *CLE41* results in a strong stunted and bushy phenotype, with disrupted secondary and mature xylem bundles within the hypocotyls (Whitford et al., 2008), while *cle41* and *cle44* mutants show a reduction in the number of procambial cells (Hirakawa et al., 2010). Specifically, overexpression of *CLE41* results in a wide range of cellular orientations and divisions in contrast with the highly orientated cell divisions, disrupting the localized pattern between procambium and cambium, resulting in overproliferation of vascular bundles and increased vascular root tissue (Hirakawa et al., 2008; Etchells and Turner, 2010). CLE10 plays a role in xylem formation as *CLE10* overexpression results in significant inhibition of the early stages of protoxylem vessel formation, which, in roots, appears to occur through activation of cytokinin signaling (Kondo et al., 2011). CLE10 has the ability to inhibit protoxylem differentiation in the root, by the repression of *ARABIDOPSIS RESPONSE REGULATOR* (*ARR*) genes, *ARR5* and *ARR6*, which are known to negatively regulate cytokinin signaling, allowing suppression of procambium differentiation into protoxylem vessels (Hirakawa et al., 2011). Finally, *CLE19* overexpression results in the xylem network to fail at making viable connections between elements (Fiers et al., 2007).

Stomatal Development

Stomata, which are composed of two guard cells, are the organs responsible for the vital gas exchange required to perform respiration. Stomata in *Arabidopsis* are usually separated by at least one nonstomatal cell to optimize spatial arrangement between the photosynthetic epidermal surface area and the gas exchange organs. The developmental processes behind stomatal differentiation rely heavily on asymmetric cell division derived from positional cues from one leaf cell to the next, but also environmental cues play a role in leaf stomatal density. The guard cells are derived from multipotent epidermal stem cells, the meristemoid mother cells (MMCs), which divide asymmetrically to provide two daughter cells of different sizes. The smaller triangular shaped cells (meristemoids) have stem cell–like capabilities and generally engage in further asymmetric division. The larger daughter cells, while capable of further asymmetric divisions, generally terminally differentiate into guard mother cells (GMCs). The GMCs divide symmetrically to yield the two guard cells, surrounding a pore, to become the stomata (Nadeau, 2009; Serna, 2009; Dong and Bergmann, 2010; Rowe and Bergmann, 2010) (Figure 4).

Various members of the EPF family of Cys-rich small peptides, specifically, EPF1, EPF2, EPF-LIKE6 (EPFL6)/CHALLAH (CHAL), and EPFL9/STOMAGEN, are involved in regulating

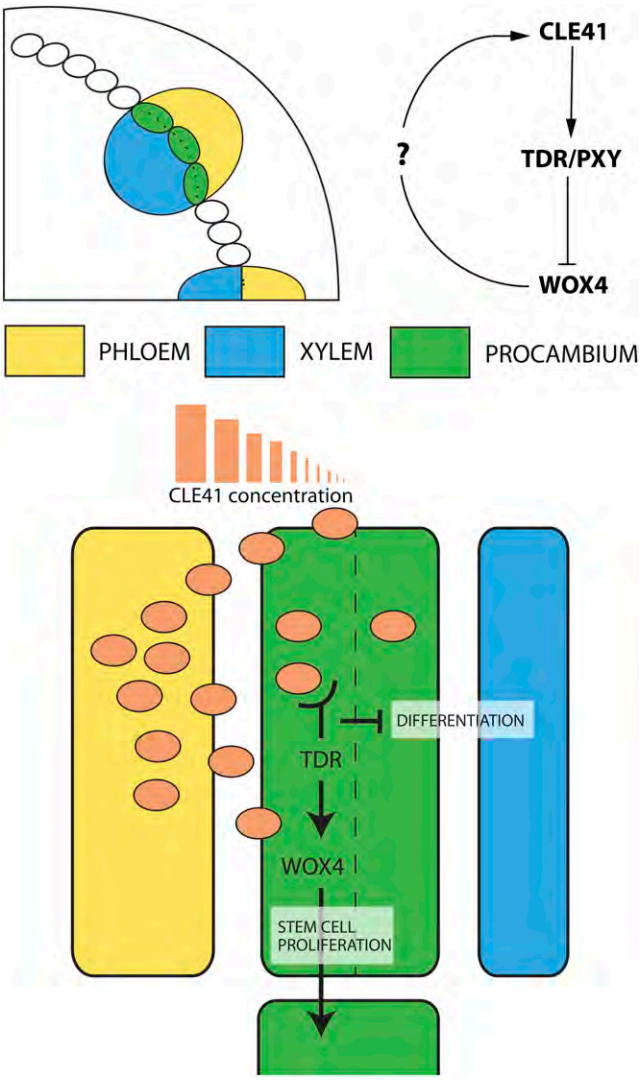

Figure 3. Vascular Differentiation.

Schematic of a stem section highlighting the vascular bundles (top left). Phloem cells (yellow) generate and secrete CLE41 peptides (peach) into procambial cells (green) where they are bound by membrane-localized TDR/PXY. TDR/PXY inhibits differentiation of procambium into xylem cells (blue) and upregulates the expression of the transcription factor *WOX4*, initiating stem cell proliferation (green). As CLE41 concentration lowers (toward plane of division: dashed line), procambial cells (green) can differentiate into xylem cells (blue) without inhibition from TDR. On the top right is the proposed CLE41-TDR/PXY-WOX4 signaling module. At present, there is no evidence for WOX4 controlling *CLE41* expression (?).

stomatal density and positioning (Figure 4) (Hara et al., 2007; Abrash and Bergmann, 2010; Kondo et al., 2010; Rowe and Bergmann, 2010; Sugano et al., 2010; Shimada et al., 2011). The overexpression of *EPF1* or *EPF2* results in reduced stomatal density, but loss-of-function mutants indicated they have differing actions (Hunt and Gray, 2009; Kondo et al., 2010). In *epf2* mutants, asymmetrical cell divisions occur more frequently than the wild type and stomatal densities significantly increase, while *epf1* mutants form random stomatal division patterns, which often leads to stomata in contact with each other (Hara et al., 2007). Promoter swapping experiments between the related EPF1 and EPF2 suggest that *EPF2* is expressed earlier in development than *EPF1*, as EPF2 has the ability to partially rescue stomatal clustering in *epf1* with expression of *pEPF1*:*EPF2* but not *pEPF2*:*EPF1* (Hunt and Gray, 2009; Rowe and Bergmann, 2010). This shows that EPF1 efficacy in stomatal patterning is somehow related to *EPF2* expression (Rowe and Bergmann, 2010). Indeed, transcriptomics analysis has shown higher levels of *EPF2* expression in juvenile leaves, prior to *EPF1* expression (Hunt and Gray, 2009). EPF2 may act early in development to control differentiation in meristemoids, controlling asymmetric cell division and proliferation rates, while EPF1 may affect the directionality and spacing of the developing stomata (Hunt and Gray, 2009; Rowe and Bergmann, 2010; Shimada et al., 2011; J.S. Lee et al., 2012). This theory is strengthened by the expression patterns, with *EPF2* expressed in mother meristemoid cells and early meristemoids, while *EPF1* is expressed in GMCs and young guard cells, showing a clear temporal-spatial division between the two peptides (Shimada et al., 2011) (Figure 4).

EPFL9/STOMAGEN is produced in the mesophyll cells of the plant and positively regulates stomatal development in the epidermis (Hunt et al., 2010; Sugano et al., 2010) (Figure 4). Overexpression of *STOMAGEN* greatly increases stomatal density to a degree where guard cells are often surrounded by

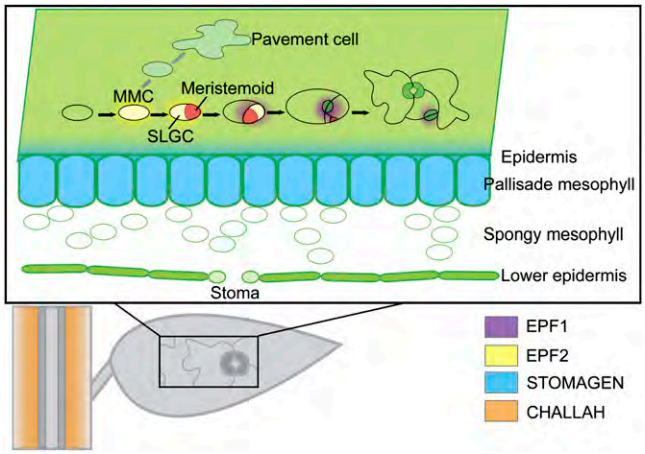

Figure 4. Stomatal Development.

Stomatal development begins when protodermal cells differentiate into MMCs. Protodermal cells that do not differentiate into MMCs instead differentiate into epidermal pavement cells. The MMC then divides asymmetrically to form a small triangular meristemoid cell (red) and a larger type of daughter cell, a stomatal lineage ground cell (SLGC). At these early stages of stomatal development, cells secrete EPF2 (yellow); after these stages, there is a switch to *EPF1* expression (purple). Meristemoids often further divide to amplify the number of cells in the stomatal lineage, before finally differentiating into GMCs. GMCs then divide symmetrically to form two guard cells surrounding a pore, completing stomatal development. Once guard cell maturation is complete, EPF1 secretion is terminated. STOMAGEN (light blue) is expressed in the mesophyll and diffuses to the epidermis, promoting stomatal development. CHAL (orange) is secreted by cells surrounding the vascular bundle and diffuses to the epidermis, restricting stomatal development.

other stomatal precursor cells (Hunt et al., 2010; Sugano et al., 2010). Conversely, knockout mutants show a significant decrease in stomatal density (Hunt et al., 2010; Sugano et al., 2010; Shimada et al., 2011). Lastly, EPFL6/CHAL negatively regulates stomatal development when overexpressed (Shimada et al., 2011; Uchida et al., 2012). Paralagous to *EPFL6/CHAL* are *EPFL4* (*CHAL-LIKE2*) and *EPFL5* (*CHAL-LIKE1*), together known as CHAL family (CHALf) ligands (Abrash et al., 2011). Nevertheless, single *epfl6*, *epfl4*, and *epfl5* mutants as well as *epfl4 epfl5 epfl6* triple mutants show no difference in stomatal density nor positioning compared with the wild type (Abrash et al., 2011; Uchida et al., 2012). *EPFL6/CHAL* expression is seen in the hypocotyl and stem of the plant, unlike *EPF1* and *EPF2*, which are expressed in the leaf, showing that there is an organ-specific role of these peptides (Shimada et al., 2011; Uchida et al., 2012).

Abscission

Abscission, whereby entire organs are shed from the plant, involves the organized and programmed cell separation of the middle lamella in plant cells. The middle lamella is the area of the cell wall that is shared between two neighboring cells, solidifying and holding the cells together. Abscission during development is essential for optimal plant growth when specific cells and organs are no longer necessary or functional (Stenvik et al., 2006, 2008).

The majority of signaling molecules known to play a role in abscission are plant hormones (Jinn et al., 2000). However, the small signaling peptide IDA has been shown to stimulate abscission through promotion of cell separation or through inhibiting cellular repair processes (Stenvik et al., 2006, 2008). *IDA*, together with *IDL* genes, belongs to a family of six genes and *IDA* is predominantly expressed during floral abscission. The *ida* knockout mutants lack the ability to abscise floral organs, with plants fully retaining the petals, sepals, and filaments indefinitely. Furthermore, overexpression of *IDA* and *IDL* shows premature floral abscission, with overproliferation of abscission zone cells; however, it varies in intensity between the members of the family. Using *IDA* upstream and downstream *cis*-regulatory elements to drive *IDL* expression in *ida* knockout mutants, it was shown that only IDL1 had the capability to fully rescue and replace native IDA activity, whereas, IDL2 to IDL5 had very little effect. IDA and IDL proteins have an extended PIP motif (EPIP), which has been shown as the active part of the peptide used in abscission. *Arabidopsis* plants subjected to synthetic IDA EPIP peptides showed phenotypes similar to *IDA* overexpression lines, with abscission occurring prematurely, whereas *ida* knockout lines subjected to IDA EPIP showed a partial rescue of phenotype. Due to the activity of the EPIP motif, it is further hypothesized that IDA and IDL peptides are cleaved, through a similar process as CLV3, where the active EPIP site becomes available to downstream effectors, thereby initiating floral abscission (Stenvik et al., 2008).

SIGNALING PEPTIDES: IDENTIFYING THE CORRESPONDING RECEPTOR

While plant genomes encode >600 putative RLKs and >1000 potential secreted peptides, so far only very few could be shown to form pairs (Torii, 2004; Butenko et al., 2009). Nevertheless, unequivocal identification of the ligand is crucial to fully understand receptor kinase–mediated signaling pathways, as was recently demonstrated for a number of ligand-receptor pairs (Hara et al., 2007; Hirakawa et al., 2008; Jia et al., 2008; Ogawa et al., 2008; Etchells and Turner, 2010; Kinoshita et al., 2010; J.S. Lee et al., 2012; Shinohara et al., 2012; Uchida et al., 2012). Unfortunately, identification of ligand-receptor pairs is technically very challenging and requires strategies that combine transcriptional analyses at cellular resolution, microscopy characterization of loss- and gain-of-function plants, easily to monitor readouts (as, for example, monitoring luciferase activity in protoplasts transiently expressing signaling components [Albert et al., 2010; Mueller et al., 2012a]), and genetic interaction studies to identify candidate pairs, followed by biochemical assays to demonstrate direct physical interactions. In this section, we will describe some of the known peptide ligand-receptor kinase pairs and comment on some likely candidates to others.

Pollen and Anthers

EXCESS MICROSPOROCYTES1 (EMS1)/EXTRA SPOROGENOUS CELLS encodes an LRR-RLK that is localized to the cell surface and is needed for tapetal cell development (Ge et al., 2010). The *ems1* mutant displays a lack of tapetal cell development and an excess production of microsporocytes (Albrecht et al., 2005). The nearly identical phenotypic similarities between *tpd1*, *ems1*, and *tpd1 ems1* mutants suggest a strong link between receptor and putative ligand during tapetal cell and pollen development (Yang et al., 2003; Albrecht et al., 2005). Through yeast two-hybrid and in planta coimmunoprecipitation assays, it has been shown that TPD1 directly interacts with EMS1 in vitro and in vivo and that TPD1 activates phosphorylation of the EMS1 kinase domain (Jia et al., 2008). As TPD1 signaling depends on functional EMS1, these data clearly establish a peptide ligand-receptor kinase pair (Jia et al., 2008). SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE1 (SERK1) and SERK2 are also proposed receptors for the TPD1 ligand, as *serk1 serk2* also results in a lack of tapetal layer, with extra meiotic cells, but biochemical evidence is lacking at present (Albrecht et al., 2005; Ge et al., 2010).

The BARELY ANY MERISTEM1 (BAM1) and BAM2 receptors have also been shown to play a role in anther and pollen development (Hord et al., 2006; Deyoung and Clark, 2008). Since the BAM receptors have been shown to directly bind with CLE family peptides, it is possible that the BAM1 and BAM2 perceive *CLE1*, *CLE11*, *CLE12*, *CLE13*, and/or *CLE25* anther and pollen expressed peptides (Hord et al., 2006; Jun et al., 2010; Shinohara et al., 2012).

Embryo

Little is known about the targets of the CLE8 signaling peptide involved in embryonic development. Several RLKs have already been identified as integral to normal embryo and seed development (Luo et al., 2005; Nodine et al., 2011; Fiume and Fletcher, 2012; Shinohara et al., 2012). As there is often a strong similarity between binding affinities within CLE family peptides, it is likely that the target for CLE8 signaling is an LRR-RLK.

SAM

CLV3 is proposed to interact with four major membrane-associated protein receptors: CLV1, CLV2-CORYNE (CRN), TOAD2/RPK2 (Clark et al., 1997; Jeong et al., 1999; Müller et al., 2008), and, more recently, FLS2 (Lee et al., 2011). The *CLV3* and the LRR-RLK *CLV1* expression domains significantly overlap, and through competitive binding assays, CLV3 has been shown to directly bind to the CLV1 protein receptor (Fletcher and Meyerowitz, 2000; Ogawa et al., 2008; Bleckmann et al., 2010; Nimchuk et al., 2011). CLV2 has been shown to associate with the CRN protein to form a receptor-like protein complex, and CRN requires CLV2 to localize to the plasma membrane (Bleckmann et al., 2010). The CLV2-CRN complex is thought to associate with or be involved in the CLV3 signaling pathway. However, as no direct interaction between CLV3 and the CLV2 extracellular domain has been shown, the CLV2-CRN complex is seen as a secondary pathway through which apical meristem development is regulated (Nimchuk et al., 2011). The interaction with RPK2 is not well characterized: single *rpk2* and *clv1 clv2 rpk2* triple mutant lines display phenotypes highly similar to *clv3* mutants, suggesting a functional role for RPK2 in CLV3 perception and repressing *WUS* expression in the meristem (Kinoshita et al., 2010). Finally, CLV3 was reported to interact with the FLS2 receptor (Lee et al., 2011; H. Lee et al., 2012), which is known to recognize the unrelated bacterial flagellin-derived peptide flg22 and activate plant innate immune responses (Gómez-Gómez and Boller, 2000). The CLV3 interaction with FLS2 in the meristem might play a role in protection of the meristem from bacterial pathogen infection (Lee et al., 2011). However, as other researchers have been unable to confirm binding of CLV3 to FLS2 under a variety of conditions (Mueller et al., 2012a, 2012b; Segonzac et al., 2012), it appears that more definitive results are necessary to support this interaction and its importance in vivo. Therefore, at present, the binding of CLV3 by FLS2 and a potential role in immune responses in the meristem remains an open question.

Another well-established receptor in floral stem cell maintenance is the BAM family of receptors: *BAM1* to *BAM3* genes encode a RLK with homology to CLV1 (DeYoung et al., 2006). Their homology and location in the SAM would suggest a functional similarity; however, this appears not to be the case. Single *bam1*, *bam2*, and *bam3* mutants do not produce obvious phenotypes, but double and triple mutants produce striking arrested development of meristematic tissues, the opposite phenotype to *clv1*. The lack of phenotype with *bam* single mutants suggests a functional overlap between BAM1, BAM2, and BAM3 in the SAM. Interestingly, CLV1 was sufficient to replace BAM1 and BAM2 functionality in *bam1 bam2*, fully rescuing the phenotype to the wild type. Conversely, BAM1 and BAM2 can partially rescue the *clv1* phenotype when expressed in the meristem (DeYoung et al., 2006). It is currently unknown what role BAMs play in the CLV3-CLV1 signaling pathway. BAM1 has been shown to bind with a high degree of affinity to numerous CLE peptides (CLE8 to CLE14), which are expressed during organ-specific developmental processes (Jun et al., 2010; Shinohara et al., 2012). Since *CLV1* and *BAM* expression profiles do not overlap, it has been suggested that BAMs, located in the periphery of the meristem, may sequester CLE (or similar) peptides from entering the meristem, thereby influencing *WUS* expression (Deyoung and Clark, 2008; Butenko et al., 2009; Wang and Fiers, 2010).

In addition to the above receptors, TDIF RECEPTOR (TDR) appears to be a major receptor for CLE42 during axillary bud formation, but other receptors likely play a role as the *tdr* mutation did not completely suppress the CLE42 effect (Yaginuma et al., 2011).

Finally, the RLK ERECTA (ER) is generally associated with stomatal development but has been recently shown to be involved in inflorescence development (Uchida et al., 2012). Single *er* mutants show a strong compacted inflorescence phenotype, very similar to *epfl4 epfl6* double mutants. Knockout experiments have shown a negligible role of *ERECTA-LIKE1* (*ERL1*) and *ERL2* during inflorescence development. Transcriptional analysis has shown that *EPFL4* and *EPFL6* are predominantly expressed in endodermal tissue and, through coimmunoprecipitation assays, have been shown to interact directly with ER in neighboring phloem tissue following their diffusion through cell layers (Uchida et al., 2012). Interestingly, both auxin-regulated genes, such as *ARGOS* that is required for meristematic competence of aboveground organs, and gibberellin metabolic genes were differentially regulated in *er* and *epfl4 epfl6* (Uchida et al., 2012). This suggests that potential interactions between phytohormones and signaling peptides participate in non-cell-autonomous control of inflorescence development.

RAM

For the RAM, it has been suggested that CLE40 acts as a secreted peptide ligand to the RLK *ARABIDOPSIS* CRINKLY4 (ACR4) (Stahl et al., 2009). *ACR4* is expressed in the columella stem cells, in close proximity of *CLE40* (De Smet et al., 2008; Stahl et al., 2009), and *cle40* and *acr4* mutants have similar phenotypes, namely, an expansion in the *WOX5* domain and additional layers of columella stem cells (De Smet et al., 2008; Stahl et al., 2009). As *CLE40* is expressed in differentiated columella cells, the expression overlaps with an area of weak *ACR4* expression (Stahl and Simon, 2009). This area of weak *ACR4* expression has been shown to be upregulated by CLE40 treatment. ACR4 is thought to act as a dynamic buffer when CLE40 levels are too high. Increased ACR4 levels will sequester CLE40, allowing the expression of *WOX5*. WOX5 in turn expands and protects the quiescent center, counteracting the effects of CLE40 (Stahl and Simon, 2009). Alternatively, CLE40 could activate ACR4 signaling, but this remains to be demonstrated biochemically. Intriguingly, this CLE40-ACR4-WOX5 signaling pathway in the RAM is highly similar to the CLV3-CLV1-WUS signaling pathway in the SAM (Figure 2).

The peptides CLE14, CLE19, and CLE20 also appear to act along the CLV2-CRN receptor pathway in the root tip, a pathway that is prevalent in the development of the SAM. The *cle14*, *cle19*, and *cle20* knockout mutants show a strong short root phenotype that is highly similar to the short root phenotype observed in *clv2* and *crn* mutants. Thus, the CLV2-CRN receptor complex is not only vital to SAM stem cell maintenance through CLV3 signaling, but is also inherent in regulating

the RAM through CLE14, CLE19, and CLE20 signaling (Meng and Feldman, 2010).

Lateral Root Initiation

Auxin plays an important role at various stages of lateral root development (De Smet, 2012). However, ACR4 was also shown to be key in the initiation and development of lateral roots (De Smet et al., 2008). ACR4 has been shown to be required for specifying the fate of neighboring cells during pericycle cell division in lateral root initiation and restricting the division of neighboring pericycle cells, thereby controlling the pattern of cell division (De Smet et al., 2008). ACR4 likely acts as signal receiver for one, or many, small signaling peptides generated in or surrounding the lateral root initiation site, such as RGF/GLV/CLEL peptides acting during lateral root initiation (Meng et al., 2012). However, the majority of known receptors for CLE peptides are LRR-RLKs (e.g., CLV1, TDR/PHLOEM INTERCALATED WITH XYLEM [PXY], and BAM), and the active domain of ACR4 has been shown to consist of extracellular crinkly repeats, resulting in a critical difference in receptor structure compared with LRR-RLKs (Gifford et al., 2005), suggesting that other, yet unidentified, signaling peptides and/or receptor kinases could be involved.

Vasculature

The receptor for TDIF has been identified as the LRR-RLK TDR/PXY (Fisher and Turner, 2007; Hirakawa et al., 2008; Etchells and Turner, 2010). TDR/PXY was identified through selecting 56 *LRR RLK* genes predicted to be expressed in procambium cells (Hirakawa et al., 2008). From this gene set, three T-DNA insertion lines in the same gene were found to be insensitive to TDIF, and this gene, previously described as PXY, was designated as TDR (Fisher and Turner, 2007; Hirakawa et al., 2008). *TDR*/*PXY* is primarily expressed in procambium cells, while *TDIF* is expressed in adjacent phloem cells, in nonoverlapping expression domains. CLE41 must then be secreted toward the xylem where it is received by TDR/PXY, to transmit the correct positional information (Etchells and Turner, 2010; Hirakawa et al., 2011). The TDIF-TDR/PXY pathway controls the WUS-related HOMEOBOX gene, WOX4, which is required for controlling the fates of the vasculature cell fates (Suer et al., 2011) (Figure 3). WOX4 is essential for procambial/cambial cell proliferation, as *wox4* mutants show restricted proliferation; however, it is not essential for the inhibition of cambial cells into xylem cells. Thus, two pathways can be distinguished, namely, a TDIF-TDR/PXY-WOX4 pathway, which promotes procambial/cambial stem cell proliferation, and TDIF-unknown factors, which inhibits the differentiation of cambial cells into xylem cells (Hirakawa et al., 2010; Suer et al., 2011). Both TDIF pathways, however, show crosstalk between xylem and phloem cells, as the TDIF derived from phloem is then responsible for the maintenance of the procambial/cambial cells via their differentiation and proliferation capabilities (Hirakawa et al., 2011).

Finally, the signaling peptide CLE10 is thought to act through the CLV2 receptor. *CLE10* expression normally inhibits protoxylem formation in roots; however, in *clv2* knockout lines, protoxylem formation is no longer inhibited, suggesting a link between the CLE10 signaling peptide and the CLV2 receptor (Kondo et al., 2011).

Stomata

Several receptor-like proteins and RLKs, such as TOO MANY MOUTHS (TMM), ER, and ERL are involved in stomatal development (Yang and Sack, 1995; Shpak et al., 2005). Through coimmunoprecipitation assays of tagged proteins in *Nicotiana benthamiana*, it has been shown that EPF1 and EPF2 strongly bind to ER and ERL1 receptors and that EPF2 also binds to TMM (J.S. Lee et al., 2012). Since TMM lacks an intracellular domain, this suggests that signal transduction occurring through TMM requires the interaction with a protein with a functional intracellular domain (Shpak et al., 2005). Indeed, TMM has been shown to interact directly with the ER receptor, thereby influencing EPF signaling through signal modulation in a putative ER-TMM complex (J.S. Lee et al., 2012). STOMAGEN/EPFL9, the EPFL peptide with opposite function to EPF2, has also been shown to require TMM to function (Shimada et al., 2011). These two signals acting antagonistically on the TMM receptor may in fact be a novel regulatory system, as this is the first time two antagonistic peptides have been shown to work through the same receptor in a plant system (Shimada et al., 2011). CHALf peptides have also been shown to act through the TMM receptor (Abrash et al., 2011). In a *tmm* background, *CHALf* overexpression leads to a striking reduction in stomatal density, suggesting that TMM binds CHALf to buffer signaling and block transmission into stomatal lineage cells. *CHALf* overexpression phenotypes are also mediated by ER family mutations, suggesting an additional pathway through which CHALf signaling is moderated (Abrash et al., 2011).

Finally, BAM receptors have also been identified in aerial organs during stomatal development and bind with high affinity to peptides like CLE9. As *CLE9* is expressed during stomatal development, it is likely that BAMs are functionally involved in stomatal development (Jun et al., 2010; Shinohara et al., 2012).

Abscission

IDA and IDL peptides are thought to act as the peptide ligand to the LRR-RLK HAESA (HAE) and to a family of HAESA-LIKE (HSL) receptor kinases, which have been shown to be expressed in floral abscission zones. While the *hae* and *hsl2* mutants were indistinguishable from wild-type plants, the *hae hsl2* double mutants completely lacked floral abscission. The highly similar expression patterns of *HAE* and *HSL2*, along with the double mutant results, suggest a functional redundancy within this family of receptors. Interestingly, *hae hsl2* plants overexpressing *IDA* showed all the characteristics of the double mutant phenotype and none of the *IDA* overexpression phenotype. In addition, exposure of *hae hsl2* to the synthetic IDA EPIP-C, IDA EPIP, and IDL1 EPIP peptides did not induce floral abscission. Taken together, this suggests that IDA and HAE-HSL2 may act along the same pathway or may in fact be ligand-receptor pairs (Stenvik et al., 2008). IDA possibly regulates floral organ abscission by acting as a ligand in HAE-HSL2–mediated suppression of BP/KNAT1, which

are repressors of the abscission-promoting transcription factors KNAT2 and KNAT6 (Shi et al., 2011).

CONCLUSION AND FUTURE PERSPECTIVES

In the past decade, our knowledge of small peptide signals has increased enormously, not only with respect to their identification through biochemical assays or in silico predictions but, more importantly, also at the level of their functional characterization. However, both genetic and bioassay approaches have drawbacks since many signaling peptides are subject to functional redundancy, hindering the genetic approach, and the typically low physiological concentrations of peptide hormones in tissues may evade detection through bioassay methods. Nonetheless, an increased focus on obtaining knockout mutants, for example, through TILLING, will be essential to elucidate the specific role(s) of peptide signals in developmental processes. Loss-of-function data are especially important since exogenous application of peptides and broad overexpression often results in nonspecific or unrelated phenotypes.

Many of the enzymes involved in posttranslational modification of peptide signals, and virtually all of the enzymes involved in proteolytic processing, remain unknown (Table 1). Given their importance for correct activation of many known small peptide signals, the identification of the genes and/or gene families that encode these enzymes could prove important not only for a more complete understanding of known peptide signals but also give a useful starting point for discovering new peptide signals through genetic methods.

Furthermore, a major gap in our knowledge is that of the enormous number of possible peptide ligand-receptor (kinase) pairs, so far only very few have been shown to form pairs (Butenko et al., 2009). While peptide signals can be matched to their corresponding receptor through a variety of methods, eventually a biochemical approach is required to demonstrate physical interaction. To achieve this, methods need to be applied to investigate these interactions on a larger scale and tools to study the in vivo dynamics of peptide ligand−receptor and receptor−receptor interactions need to be developed. It has been shown on numerous occasions that one RLK can bind several signaling molecules within a peptide family, showing a strong functional redundancy between peptide signals. Conversely, it has been shown that some receptors may functionally replace one another, when expressed in non-native expression profiles. Understanding why and how these proteins can act interchangeably will help to elucidate further peptide signaling pathways whose ligand or receptor pairs are currently unknown. Such an approach will likely further support the observations that there is not a single peptide-receptor pair but that multiple possible interactions exist. Identifying peptide ligand-receptor (kinase) pairs will furthermore provide insight in the (distinct) roles of the numerous peptides in overlapping expression domains. However, the debate on the CLV3-FLS2 pair (Lee et al., 2011; H. Lee et al., 2012; Mueller et al., 2012a, 2012b; Segonzac et al., 2012) illustrates the complexities of peptide signaling and the difficulties of working with these small peptides. In general, conclusions need to take into account experimental setup, differential effects of posttranslational modifications, sensitivity of the receptor for the ligand, and even inadvertent contamination of synthetic peptides.

As mentioned above, knowledge of crosstalk between phytohormones and signaling peptides is only just emerging, with a focus on auxin and cytokinin. For example, RGF/GLV/CLEL peptides influence auxin transport during root and hypocotyl gravitropism by modulating PIN2 trafficking dynamics, ensuring that the asymmetrical auxin gradient required for the gravitropic response is maintained. Additionally, *GLV1* and *GLV2* expression is rapidly induced by auxin, providing an example of an auxin signaling input modulating an auxin transport output via a signaling peptide, thus controlling the initial trigger (Whitford et al., 2012). In addition, CLE peptides are regulated through cytokinin signaling during root and vasculature development (Meng and Feldman, 2010). The exact nature of this crosstalk remains to be elucidated.

In conclusion, small signaling peptides play an essential role in plant growth and development, but we have only revealed the tip of the iceberg with respect to their signaling potential, including the interaction with receptors and downstream changes.

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AUTHOR CONTRIBUTIONS

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Small Signaling Peptides in *Arabidopsis* Development: How Cells Communicate Over a Short Distance

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APPENDIX VIII

The CEP family in land plants: evolutionary analyses, expression studies, and role in Arabidopsis shoot development

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RESEARCH PAPER

The CEP family in land plants: evolutionary analyses, expression studies, and role in *Arabidopsis* shoot development

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Abstract

In *Arabidopsis*, more than 1000 putative small signalling peptides have been predicted, but very few have been functionally characterized. One class of small post-translationally modified signalling peptides is the C-TERMINALLY ENCODED PEPTIDE (CEP) family, of which one member has been shown to be involved in regulating root architecture. This work applied a bioinformatics approach to identify more members of the CEP family. It identified 10 additional members and revealed that this family only emerged in flowering plants and was absent from extant members of more primitive plants. The data suggest that the CEP proteins form two subgroups according to the CEP domain. This study further provides an overview of specific *CEP* expression patterns that offers a comprehensive framework to study the role of the CEP signalling peptides in plant development. For example, expression patterns point to a role in above-ground tissues which was corroborated by the analysis of transgenic lines with perturbed *CEP* levels. These results form the basis for further exploration of the mechanisms underlying this family of peptides and suggest their putative roles in distinct developmental events of higher plants.

Key words: *Arabidopsis*, small signalling peptides, phylogeny, evolutionary analyses, *CEP* expression, shoot development.

Introduction

In the last 20 years, the importance of small signalling peptides in plant cell-to-cell communication has become increasingly clear, with several families of small signalling peptides known at present to play vital roles in plant growth and development (Butenko *et al.*, 2009; Matsubayashi, 2011; Murphy *et al.*, 2012; Czyzewicz *et al.*, 2013). The majority of small signalling peptides falls into one of two broad groups: the cysteine-rich peptides, which are characterized by a typical mature peptide length of <160 amino acids with a cysteine-rich C-terminal domain; and the small post-translationally modified peptides, which are expressed as longer precursor proteins before undergoing post-translational modifications and subsequent cleavage to form an active, mature peptide <20 amino acids in length (Matsubayashi, 2011; Murphy *et al.*, 2012). Three types of post-translational modification—tyrosine sulfation, proline hydroxylation, and

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hydroxyprolinearabinosylation—are known to occur in small post-translationally modified peptides, and these modifications appear to be crucial for optimal peptide bioactivity (Matsubayashi, 2011; Shinohara and Matsubayashi, 2013).

Identification of small signalling peptides in plants can be challenging due to the small size of their encoding genes (Murphy *et al.*, 2012). An *in silico* approach, specifically designed to identify genes encoding the hallmarks of small peptide signals, led to the discovery of the C-TERMINALLY ENCODED PEPTIDE (CEP) family of putative small post-translationally modified peptides (Ohyama *et al.*, 2008). In this initial study, five small genes encoding peptides of 82–126 amino acids in length were identified. The five different members of the CEP family display considerable sequence diversity in the majority of the expressed protein, with the exception of a conserved domain at the C-terminus, which represents the mature, active peptide following proteolytic cleavage from the expressed precursor. This was confirmed using mass spectrometry on *CEP1* (At1g47485) overexpression lines, further revealing that the mature product of 15 amino acids contains two hydroxyprolinated residues (Ohyama *et al.*, 2008). Initial analyses showed that overexpression of *CEP1* arrests root growth through repression of meristematic cell division and expansion, while no effects were observed in the quiescent centre and adjacent stem cells (Ohyama *et al.*, 2008).

Apart from these observations restricted to one member of the CEP family, hardly anything is known about the other members and their potential importance for plant growth and development. Therefore, this study further explored this family of small signalling peptides. First, the phylogenetic results showed that the CEP family contains more than five members and is conserved throughout higher land plants. Second, analysis of the expression of CEP family members revealed distinct patterns throughout plant development. Third, altering the expression levels of CEP family members resulted in dramatic growth and developmental phenotypes in aboveground plant parts.

Materials and methods

Plant growth

For analyses of aboveground parts, plants were grown on Levington M3 compost (Everris, Ipswich UK) in a glasshouse at 20–22 °C under long-day conditions (16/8 light/dark). Measurements were taken after 3 weeks of growth, immediately post-bolt. For rosette area quantification and leaf series analysis, plants were grown horizontally on square Petri plates (12 cm × 12 cm, Greiner Labortechnik) containing 50 ml solid half-strength MS growth medium (per litre: 2.15 g MS salts, 0.1 g *myo*-inositol, 0.5 g MES, 10 g plant tissue culture agar; pH adjusted to 5.7 with KOH) in a growth room at 22 °C under continuous light. Measurements were taken at 18 d after germination. For *GUS* expression analyses, seedlings were grown at 22 °C under continuous light (110 µE m^{-2} s^{-1} photosynthetically active radiation, supplied by cool-white fluorescent tungsten tubes, Osram) on square Petri plates containing 50 ml solid half-strength MS growth medium supplemented with 1% sucrose (per litre: 2.15 g MS salts, 0.1 g *myo*-inositol, 0.5 g MES, 10 g sucrose, 8 g plant tissue culture agar; pH adjusted to 5.7 with KOH), and flowering plants were grown in a greenhouse at 21 °C under long-day conditions.

Sequence identification and conserved motif analysis of CEP proteins

New members of the CEP family in *Arabidopsis thaliana* were identified using the 15-amino-acid mature region from known CEP peptides as input sequences for a TBLASTN search in all six open reading frames of the complete *A. thaliana* genome nucleotide sequence (http://blast.ncbi.nlm.nih.gov/Blast.cgi), and CEP assignment and naming was aligned with the results from Delay *et al.* (2013). The position on the genome of each hit was determined (using the SeqViewer browser from TAIR, http://tairvm09.tacc.utexas.edu/servlets/sv) and was screened in all six possible open reading frames (using the translate tool from ExPASy; http://web.expasy.org/translate/) for a peptide of approximately 75–250 amino acids (Supplementary Dataset S1, available at *JXB* online). If these proteins contained an N-terminal signal peptide (SignalP4.1; http://www.cbs.dtu.dk/services/SignalP; with standard settings, except for a D-cutoff of 0.45) and a 'CEP-like' sequence in the C-terminal region of the peptide, they were classified as CEP peptides. To identify potential members of the CEP gene family in the plant lineage, *A. thaliana* CEP family members were used as a query in BLAST searches against Phytozome version 9.0 (http://www.phytozome.net/search.php), which contains the most up-to-date list of genomes (13 December 2012). The BLAST analyses using full-length protein sequences were performed using standard settings (except for an E threshold of 10). The program MEME (Bailey *et al.*, 2009) (http://meme.nbcr.net/meme/cgi-bin/meme.cgi) was used to identify motifs in the candidate CEP protein sequences. MEME was run with the following parameters: number of repetitions = any, maximum number of motifs = 5, and with optimum motif widths constrained to between 6 and 50 residues. Weblogo (http://weblogo.berkeley.edu/logo.cgi) was used with standard settings to represent the consensus CEP domains.

Data mining analyses

For environmental and hormonal effects on CEP genes, the transcriptome meta-analysis tool Genevestigator (Hruz *et al.*, 2008) was used, with a significance level of <0.05. For cell, tissue, and organ *CEP* expression data, the eFP browser (http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi) was used with standard settings.

Alignment and phylogenetic analysis

Phylogenetic analyses of the CEP proteins and CEP domains based on amino acid sequences were carried out using UPGMA methods in the CLC Main Workbench version 6.8.1 (www.clcbio.com). Support for each node was tested with 10 000 bootstrap replicates.

CEP *constructs*

Gateway cloning was used for every construct. Entry clones containing the *CEP* promoter sequences (*CEP1*, 1997 bp; *CEP2*, 1400 bp; *CEP3*, 1560 bp; *CEP4*, 2000 bp; *CEP5*, 900 bp) were created by cloning PCR-fragments into the pDONRP4P1R vector. The *pCEPx::GUS* constructs were created by cloning the promoter fragment into the pEX-K7SNFm14GW destination vector. An entry clone containing the genomic coding sequence of CEP5 (318 bp) was created by cloning the PCR-fragment into the pDONR221 vector. The *p35S::CEP5* construct was created by cloning this genomic coding sequence in the pK7GW2 destination vector. The *CEP5* RNAi construct was created using the pAGRIKOLA constructs (Hilson *et al.*, 2004). Constructs were transformed in Col-0 using floral dip (Clough and Bent, 1998).

GUS *expression*

For the GUS assays, plants were put overnight in 90% acetone, then transferred to a GUS-solution [1 mM X-Glc, 0.5% (v/v) dimethylformamide (DMF), 0.5% (v/v) Triton X-100, 1 mM EDTA (pH 8), 0.5 mM potassium ferricyanide ($K_3Fe(CN)_6$), 0.5% potassium

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ferrocyanide ($K_4Fe(CN)_6$), 500 mM phosphate buffer (pH 7)] and incubated at 37 °C for GUS staining, and finally washed in 500 mM phosphate buffer (pH 7). For microscopic analysis, samples were cleared with 90% lactic acid or as described in Malamy and Benfey (1997). Samples were analysed by differential interference contrast microscopy (Olympus BX53) and a stereomicroscope (Leica MZ16).

RNA extraction, cDNA synthesis, and qRT-PCR analysis

Arabidopsis RNA was isolated from 20 pooled seedlings at 7 d after germination using a Plant RNeasy Kit (Qiagen, Germany) according to the manufacturer's instructions. cDNA was subsequently prepared from a minimum of 250 ng RNA (determined by UV spectrophotometry) using a SuperScript II reverse transcriptase kit and Oligo(dT)12–18 primers (Invitrogen, USA), according to the manufacturer's instructions. Quantitative real-time PCR (qRT-PCR) was performed in a 384-well white dish format using a LightCycler 480 (Roche Applied Science, USA) with 40 PCR amplification cycles using SYBR Green I fluorescent dye (Quanta Biosciences, USA) and primers for *CEP5* (5′-CCATGGACGAACCCTAAAAG-3′ and 5′-TGCCATCATCGTCTTGCTAT-3′) and *ACTIN* (5′-CTGGA GGTTTTGAGGCTGGTAT-3′ and 5′-CCAAGGGTGAAAGCAA GAAGA-3′). Expression was determined from a minimum of three biological replicates, each with three technical repeats, and normalized against ACTIN.

Results and discussion

Extended CEP family in A. thaliana

Originally, the CEP family was described as containing five members in *A. thaliana* (Ohyama *et al.*, 2008). In addition to these five, the current work and Delay *et al.* (2013) identified 10 additional members. We used BLAST searches and filtered for proteins with an N-terminal signal peptide sequence, a C-terminal 'CEP-like' sequence, and a total length of 75–250 amino acids (Table 1). The genes are located on different chromosomes, but not chromosome 4, and *CEP3* and *CEP11* and *CEP5*, *CEP6*, *CEP7*, and *CEP8* are organized in tandem repeats (Supplementary Fig. S1). The phylogenetic relationships between the CEP family proteins are shown in Fig. 1A.

In agreement with the five original CEP family members, this study predicted the presence and location of putative signal peptide cleavage sites in all CEP pre-propeptides using SignalP4.1 (Fig. 1B and Supplementary Dataset S1). Although SignalP4.1 does not always predict this, it is likely that the cleavage occurs at a conserved arginine at the N-terminus (Supplementary Dataset S1).

Previously, a C-terminal conserved domain was identified in the original five members of the CEP family (Ohyama *et al.*, 2008). To identify the presence and distribution of this domain in all CEP family members, pattern analyses were performed on the full-length *A. thaliana* CEP proteins using MEME (with optimized settings following iterative analyses) (Bailey and Elkan, 1994). Indeed, a motif that is similar to the active CEP1 peptide sequence (Ohyama *et al.*, 2008) is present across all CEP protein sequences (Fig. 1B and Supplementary Dataset S1), and therefore this was called the CEP domain. Interestingly, in several instances the CEP domain occurs multiple times within one pre-propeptide and not exclusively at the C-terminus (Fig. 1B and Supplementary Dataset S2).

This work then used all the CEP domains to build a phylogenetic tree, which revealed two main branches within the CEP family (Fig. 2A). Based on these phylogenetic relationships, the CEP family was divided into two groups: group I, CEP1–CEP12; and group II, CEP13–CEP15 (Fig. 2A). The amino acid sequences for the CEP domains for groups I and II were separately aligned, which resulted in a consensus sequence for these two groups (Fig. 2B). For both groups, the C-terminal part of the CEP domain (SPGV/IGH) showed high amino acid similarity (Fig. 2B). The CEP domain in group I contains three prolines, while in group II it contains two prolines. This is important as LC-MS/MS analysis of CEP1 revealed hydroxylation of some of the prolines within the CEP domain (Ohyama *et al.*, 2008). This hydroxyprolination likely affects bioactivity and hydrophilic nature of the CEP peptides. In future, it will be important to assess to what extent these *in silico* results are supported by biological validation.

Table 1. *The 15 members of the* CEP *family*

Gene	Locus	Publication
CEP1	At1g47485	Ohyama *et al.* (2008)
CEP2	At1g59835	Ohyama *et al.* (2008)
CEP3	At2g23440	Ohyama *et al.* (2008)
CEP4	At2g35612	Ohyama *et al.* (2008)
CEP5	At5g66815	Ohyama *et al.* (2008)
CEP6	At5g66816	This study and Delay *et al.* (2013)
CEP7	Between At5g66817–At5g66820[a]	This study and Delay *et al.* (2013)
CEP8	Between At5g66817–At5g66820[a]	This study and Delay *et al.* (2013)
CEP9	At3g50610	This study and Delay *et al.* (2013)
CEP10	Between At1g36040–At1g36050[a]	This study and Delay *et al.* (2013)
CEP11	Between At2g23440–At2g23450[a]	This study and Delay *et al.* (2013)
CEP12	At1g31670[b]	This study and Delay *et al.* (2013)
CEP13	At1g16950	This study and Delay *et al.* (2013)
CEP14	At1g29290	This study and Delay *et al.* (2013)
CEP15	At2g40530	This study and Delay *et al.* (2013)

[a], not listed on TAIR; [b], likely incorrectly annotated on TAIR.

CEP family is evolutionarily conserved in monocot and eudicot plants

Notwithstanding the fact that CEP pre-proproteins are short and the CEP domain is only 15 amino acids (AAs) long (Figs. 1 and 2), BLAST with *A. thaliana* full-length CEP proteins and 15-amino-acid CEP domain sequences and phylogenetic analyses were used to identify CEP family genes within the supergroup Plantae (data not shown). This revealed that CEP peptides are present in eudicots and monocots, but absent in lower land plants (*Selaginella moellendorffii* and *Physcomitrella patens*) and in the green algae for which a genome sequence was available (Fig. 3).

CEP family members display limited transcriptional control by hormones and nutrients in Arabidopsis

Whilst small signalling peptides are often not well represented on available micro-arrays (Murphy *et al.*, 2012), *in*

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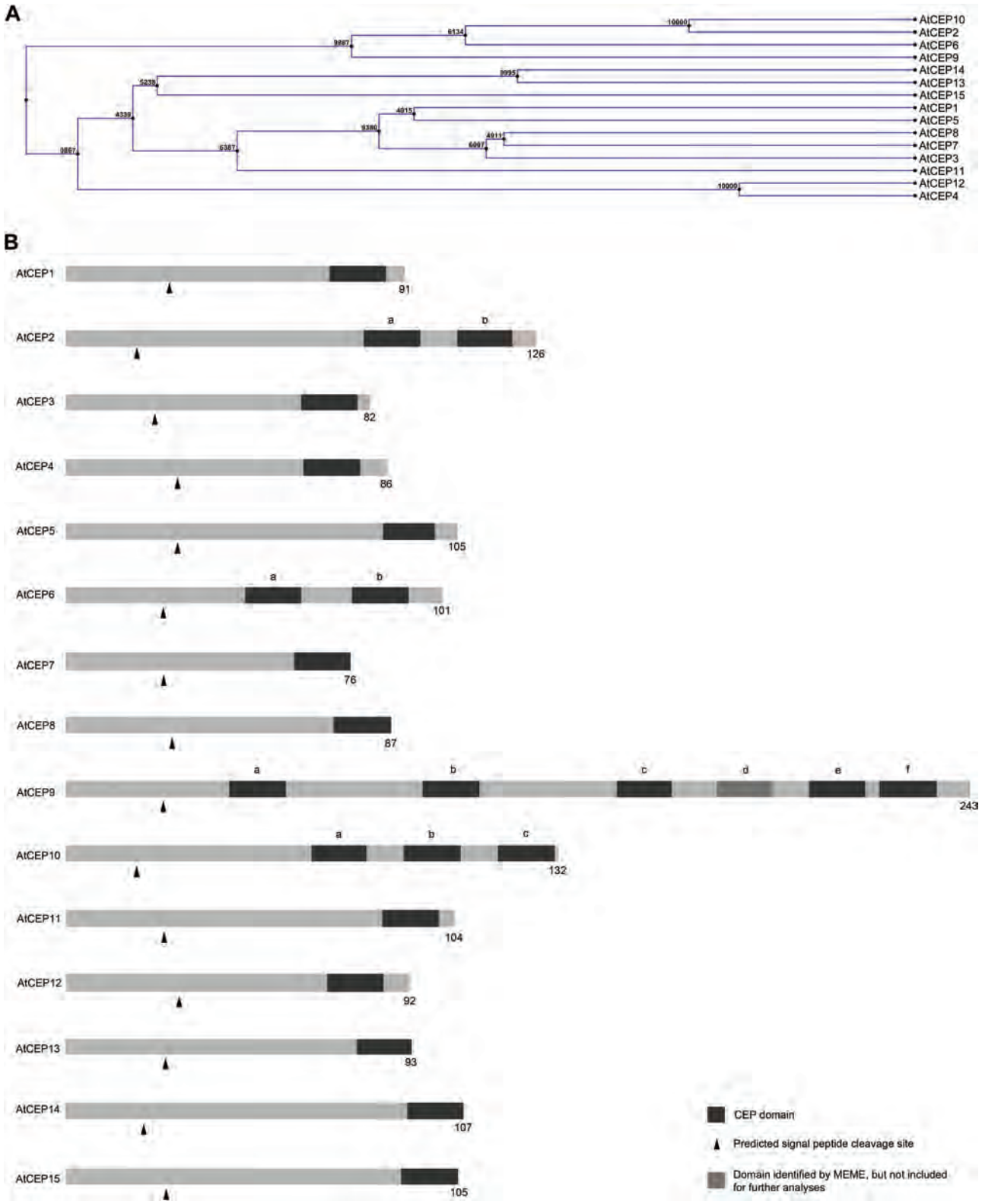

Fig. 1. The CEP family in *Arabidopsis*. (A) Phylogenetic tree of *Arabidopsis* CEP family members (based on full length protein sequences). Bootstrap values based on 10 000 replications are shown at branch nodes. (B) Schematic representation of *Arabidopsis* CEP family members. Numbers are number of amino acids). Dark grey, CEP domain; light grey, predicted CEP domain by MEME but with deviating sequence and not included for further analyses; arrowhead, predicted signal peptide cleavage site.

silico expression data for seven out of 15 *CEP*s were available. To examine, *CEP* family gene expression changes under several hormonal and nutritional stimuli, this study used the transcriptome meta-analysis tool Genevestigator (Hruz *et al.*, 2008) (Table 2). Both *CEP5* and *CEP15* were downregulated by salicylic acid treatment, gibberellic acid induced expression

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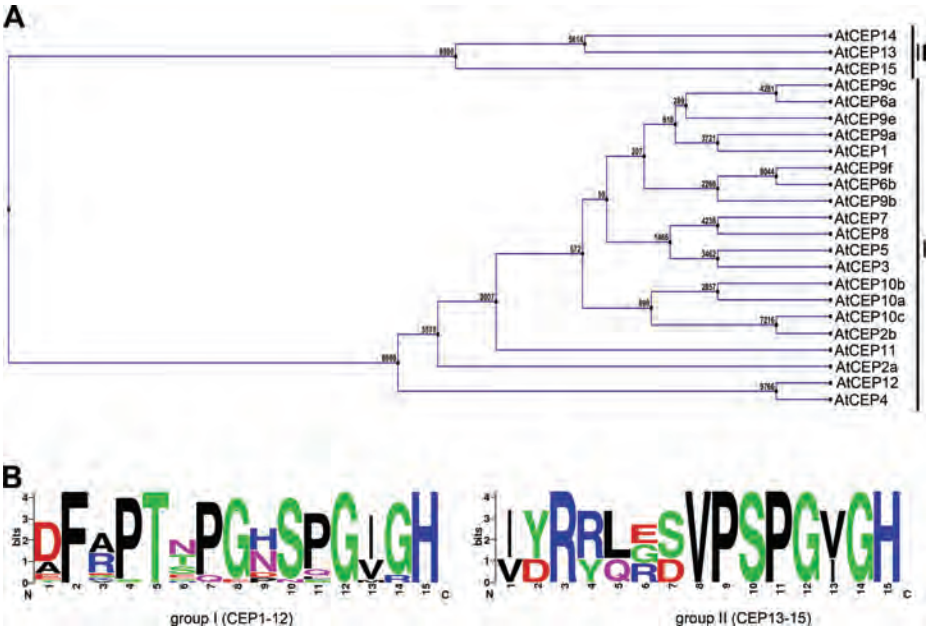

Fig. 2. The CEP family groups into two groups based on CEP domain. (A) Phylogenetic tree based on CEP domain sequences (see Fig. 1B, dark grey). Groups I and II are indicated. Bootstrap values are indicated on the tree. (B) Weblogo representation of group I and group II CEP domains.

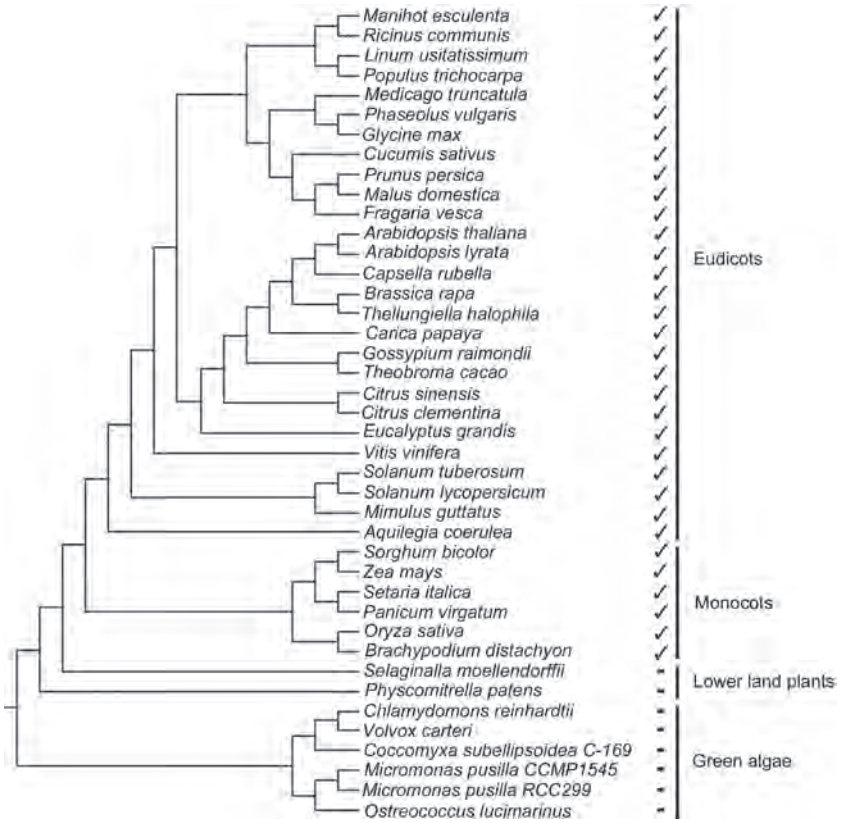

Fig. 3. Evolutionary analysis of the CEP family. Phylogenetic tree indicating the presence (√) or absence (–) of *CEP* family members in the indicated species.

of *CEP5* and *CEP13*, abscisic acid decreased expression of *CEP15*, whilst jasmonic acid increased expression of *CEP12*. Contrastingly, auxin (IAA) had opposite effects on the expression of some CEP family genes, namely an increase in expression of *CEP1* and *CEP3* but a decrease in *CEP5* and *CEP9* expression. Similarly, brassinolide reduced expression of *CEP5* and induced *CEP15* expression. Other hormones, such as ethylene, strigolactone, and cytokinin, did not have a significant effect on *CEP* expression. With respect to nutrients, a high nitrogen level downregulated *CEP3*, *CEP5*, and *CEP13*,

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Table 2. *The effect of hormones and nutrients on* CEP *expression*

All data used were generated on AffymetrixGeneChip ATH1 22K platform. +, Increased expression following stimulus; –, reduced expression; =, no significant effect on expression (defined as $P \geq 0.05$ and/or a <1.5 fold change in expression levels compared with control); ND, *CEP* genes and/or stimuli for which no expression data are currently available in Genevestigator. ABA, abscisic acid; BR, brassinosteroids; GA, gibberellic acid; JA, jasmonic acid; SA, salicylic acid; Strigo, strigolactone; Cyto, cytokinin. Hormones and nutrients in *CEP2*, *CEP4*, *CEP6*, *CEP7*, *CEP8*, *CEP10*, *CEP11*, and *CEP14* are all ND.

Gene	Hormones									Nutrients		
	ABA	Ethylene[a]	BR[b]	GA	Auxin[c]	JA	SA	Strigo	Cyto[d]	N	P	K
CEP1	=	ND	ND	ND	+	ND	=	ND	ND	+	=	=
CEP3	=	=	=	=	+	=	=	ND	=	–	ND	–
CEP5	=	=	–	+	–	ND	–	ND	=	–	+	=
CEP9	=	=	=	=	–	=	=	=	=	+	=	+
CEP12	ND	ND	ND	ND	ND	+	ND	ND	ND	ND	ND	ND
CEP13	=	=	=	+	=	ND	=	ND	=	–	=	=
CEP15	–	=	+	=	=	=	–	=	=	=	=	=

[a–d]Measurements were taken using: [a]ACC (1-aminocyclopropane-1-carboxylic acid); [b]brassinolide; [c]indole-3-acetic acid, 1-naphthaleneacetic acid, and 2,4-dichlorophenoxyacetic acid; [d]zeatin.

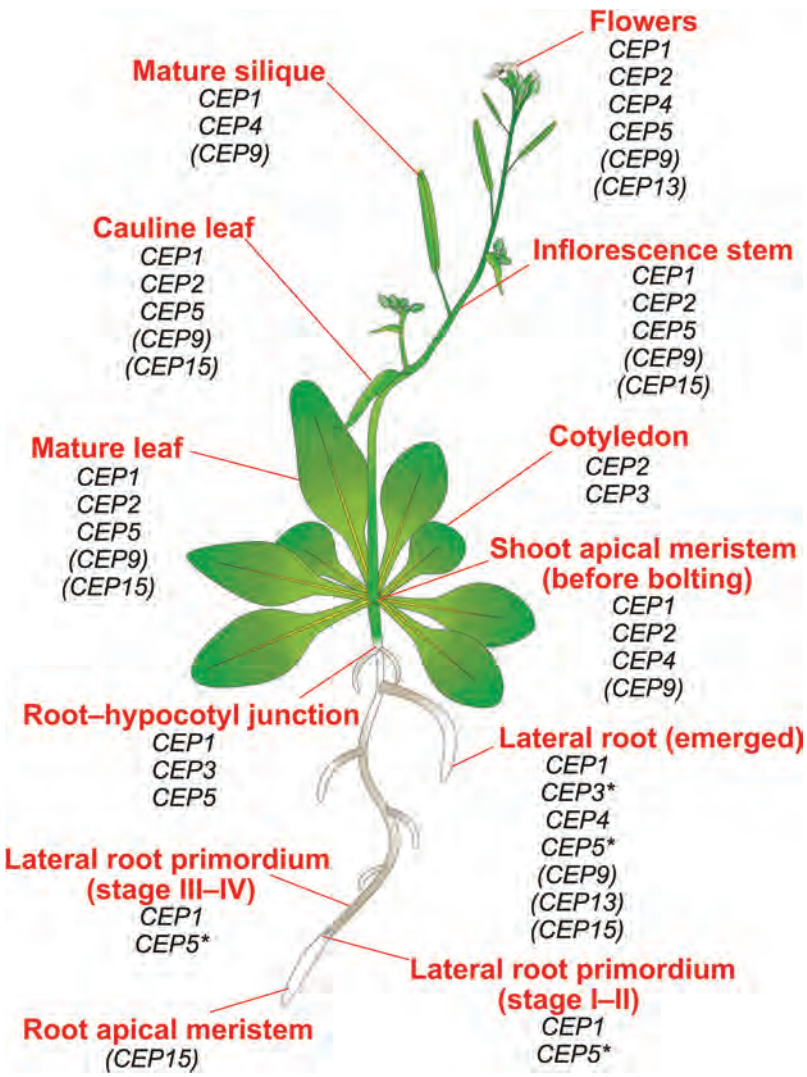

Fig. 4. Expression of *CEP* genes throughout the *Arabidopsis* plant, based on data from *in planta* (*GUS* expression; see Figs. 5–8) and *in silico* (eFP Browser; in parentheses) studies. *In silico* patterns were not included in the figure if there was a discrepancy with the *GUS* expression data. *, Associated with vasculature of primary root at these stages.

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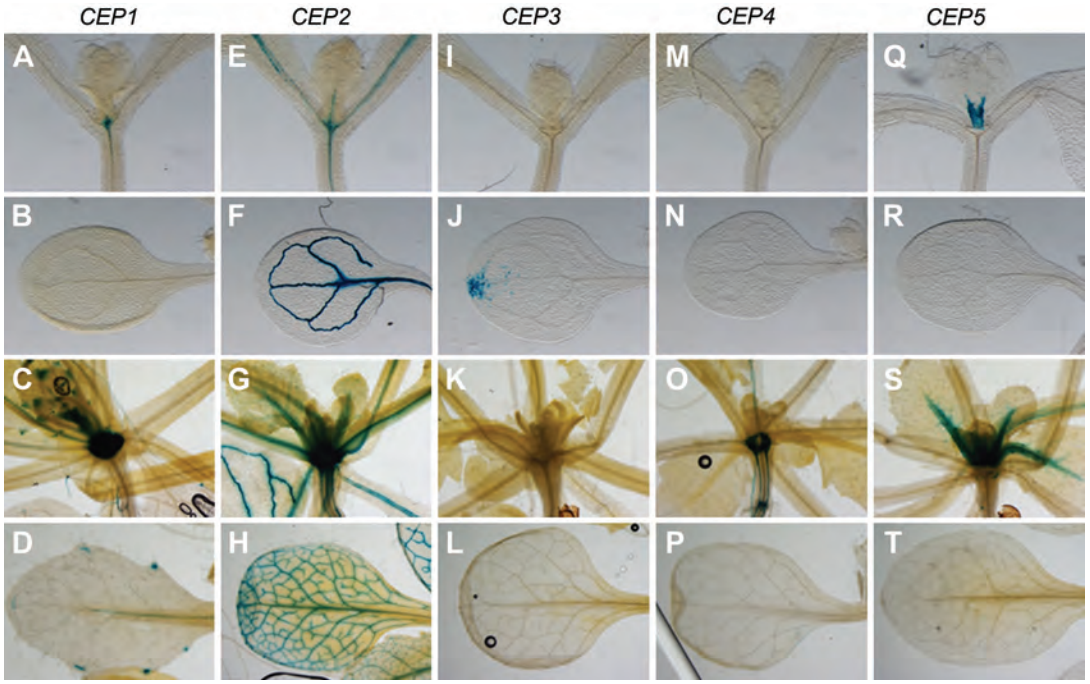

Fig. 5. *CEP* expression in the vegetative shoot. (A–D) *pCEP1::GUS* reporter line, (E–H) *pCEP2::GUS* reporter line, (I–L) *pCEP3::GUS* reporter line, (M–P) *pCEP4::GUS* reporter line, (Q–T) *pCEP5::GUS* reporter line. (A, E, I, M, Q) Shoot apical meristem region of 5-d-old seedling. (B, F, J, N, R) Cotyledon of 5-d-old seedling. (C, G, K, O, S) Shoot apical meristem region of 2-week-old seedling. (D, H, L, P, T) Leaf of 2-week-old seedling.

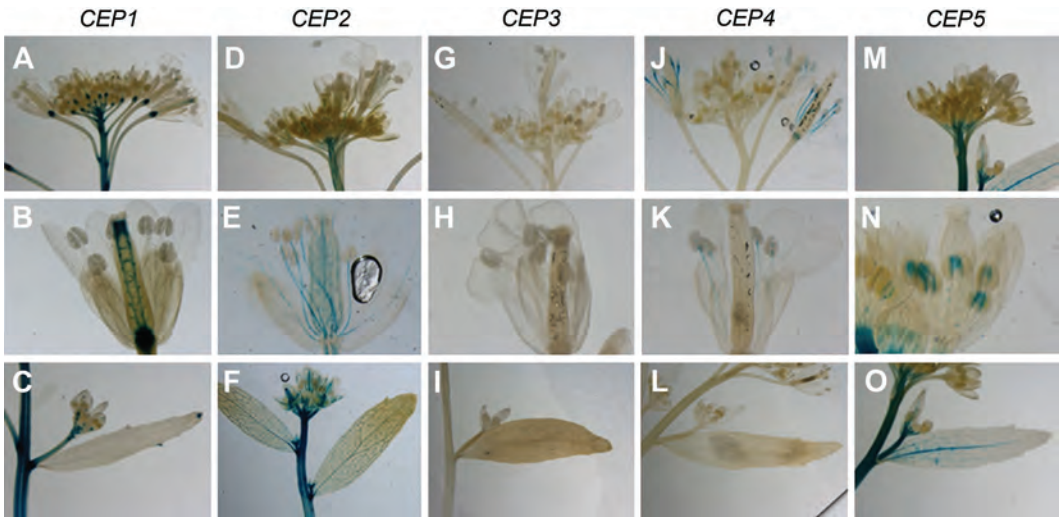

Fig. 6. *CEP* expression in the inflorescence. (A–C) *pCEP1::GUS* reporter line, (D–F) *pCEP2::GUS* reporter line, (G–I) *pCEP3::GUS* reporter line, (J–L) *pCEP4::GUS* reporter line, (M–O) *pCEP5::GUS* reporter line. (A, D, G, J, M) *CEP* expression in the apical part of inflorescence. (B, E, H, K, N) *CEP* expression during flower development. (C, F, I, L, O) *CEP* expression in a cauline leaf.

and upregulated *CEP1* and *CEP9*. Phosphorus upregulated expression of *CEP5*. In addition, potassium downregulated *CEP3* expression and upregulated *CEP9* expression.

CEP *family members display distinct expression patterns during* Arabidopsis *development*

To gain further insight in the expression patterns of the *CEP* family during development, the *CEP* expression data were first compiled and visualized from online repositories, namely eFP browser (Winter *et al.*, 2007) and Genevestigator v3 (Hruz *et al.*, 2008). These *in silico* expression patterns suggested that CEP peptides are expressed throughout the plant (Fig. 4; Supplementary Tables S1 and S2). *CEP1*, *CEP3*, and *CEP9* were expressed in the shoot apical meristem of the vegetative shoot, and *CEP3* was also expressed in the shoot apical meristem of the inflorescence stem. Only *CEP1* and *CEP15* were expressed in the primary root apical meristem, making these

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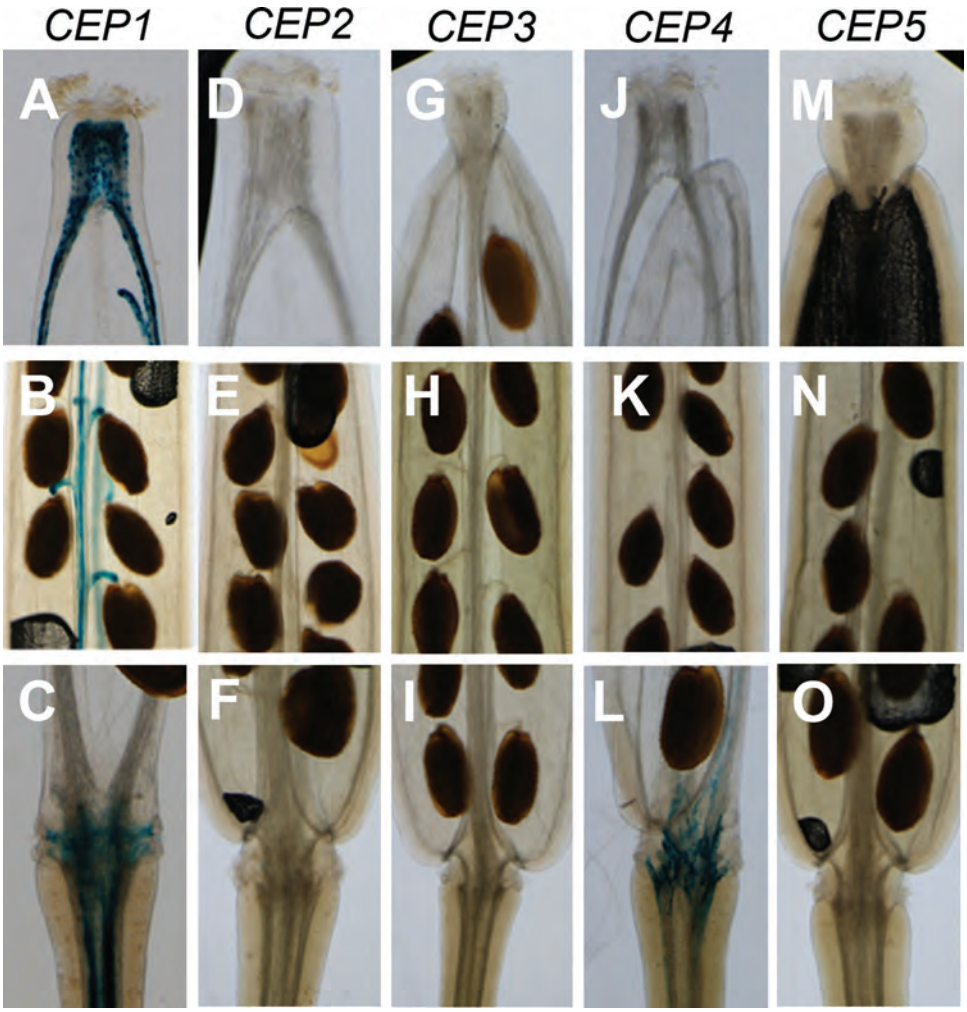

Fig. 7. *CEP* expression in a mature silique. (A–C) *pCEP1::GUS* reporter line, (D–F) *pCEP2::GUS* reporter line, (G–I) *pCEP3::GUS* reporter line, (J–L) *pCEP4::GUS* reporter line, (M–O) *pCEP5::GUS* reporter line. (A, D, G, J, M) *CEP* expression in the tip of a mature silique. (B, E, H, K, N) *CEP* expression in middle part of mature silique. (C, F, I, L, O) *CEP* expression at the base of a mature silique.

likely candidates for controlling root apical meristem maintenance. *CEP3*, *CEP9*, *CEP13*, and *CEP15* were expressed in cotyledons and/or leaves. During flower development, *CEP1*, *CEP3*, *CEP9*, and *CEP13* were expressed. During lateral root development, *CEP1*, *CEP3*, *CEP5*, *CEP9*, *CEP13*, and *CEP15* were expressed.

To further explore *CEP* expression patterns *in planta*, this study selected the five CEPs from group I that were also identified by Ohyama *et al.* (2008), generated *promoter::GUS* reporter lines, and characterized the reported lines throughout plant development (Figs. 4–8).

In the shoot apical meristem of the vegetative shoot of a 5-d-old seedling, only *CEP1* and *CEP2* were expressed (Fig. 5A and E), while in a 2-week-old plant, *CEP4* was also expressed (Fig. 5C, G, and O). In cotyledons and leaves, the expression domains of the CEP peptides were remarkably distinct and restricted to specific regions. In the cotyledon of 5-d-old seedlings, *CEP2* was expressed in the leaf veins and *CEP3* was expressed only in the tip of the cotyledons (Fig. 5F and J). In the leaves of 2-week-old plants, *CEP1* was expressed in the small dentations at the leaf margin and *CEP2* was expressed in the leaf veins (Fig. 5D and H). Both *CEP2* and *CEP5* are expressed in the leaf petioles (Fig. 5Q and S).

In the cauline leaves of the inflorescence, a similar expression pattern for *CEP1*, *CEP2*, and *CEP5* as in the mature vegetative leaves was observed (Fig. 6C, F, and O). In the shoot apical meristem of the inflorescence shoot, *CEP1*, *CEP2*, and *CEP5* were expressed (Fig. 6A, D, and M). During flower development, *CEP1* and *CEP2* were expressed in the gynoecium (Fig. 6B). In the androecium, both *CEP2* and *CEP4* were expressed in the filaments (Fig. 6E and K) and *CEP5* was expressed in the anthers (Fig. 6N).

In maturing siliques, *CEP1* and *CEP4* were expressed (Fig. 7A, B, C, and L), and both were expressed in the abscission zone (Fig. 7C and L). None of the five investigated CEP peptides showed a signal in the root apical meristem of the primary root (Supplementary Fig. S2). During adventitious root development at the root–hypocotyl junction, expression of *CEP1*, *CEP3*, *CEP4*, and *CEP5* was observed (Supplementary Fig. S3).

During lateral root development *CEP1*, *CEP3*, and *CEP5* were expressed at various stages (Fig. 8). For example, *CEP1* was expressed in the inner layer of the stage II primordium (Fig. 8B), and later in the central core of the developing

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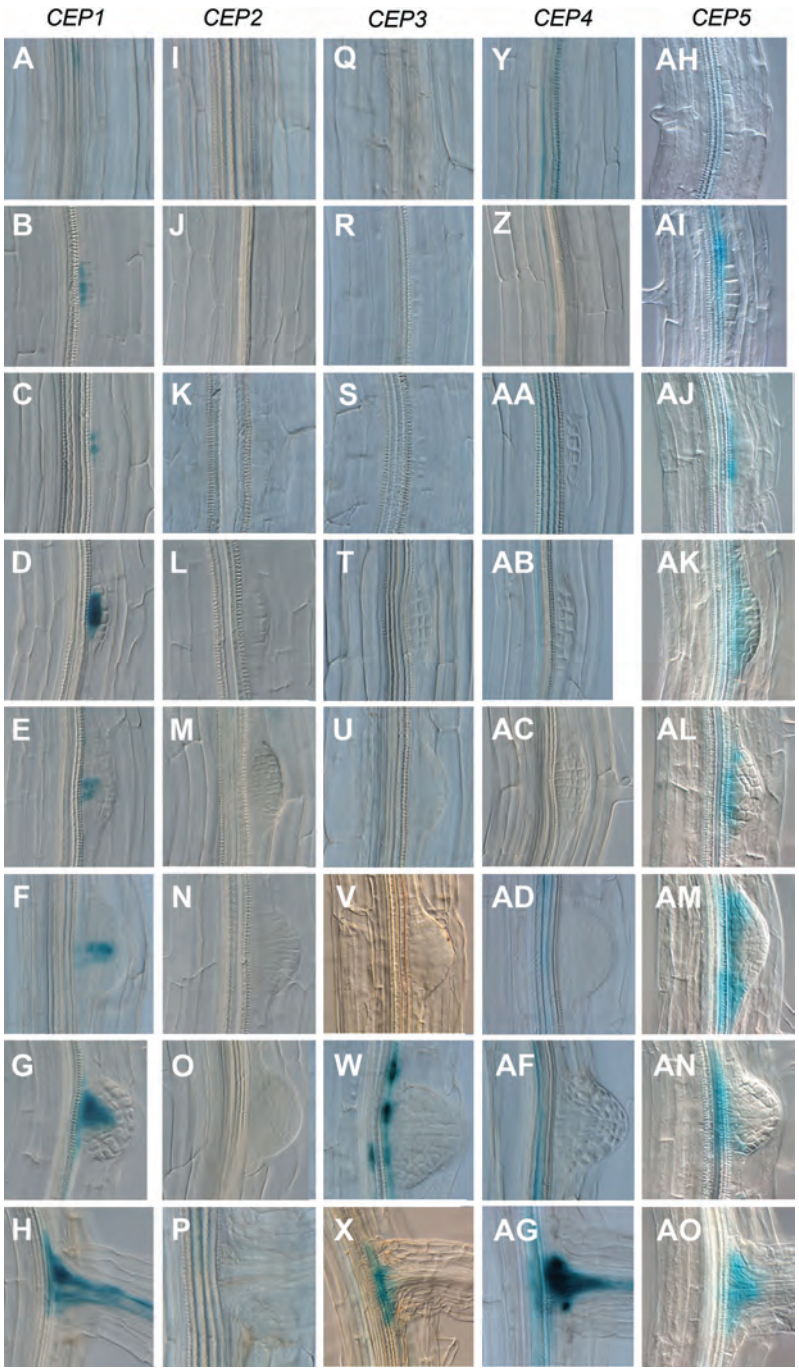

Fig. 8. *CEP* expression during lateral root development. (A–H) *pCEP1::GUS* reporter line, (I–P) *pCEP2::GUS* reporter line, (Q–X) *pCEP3::GUS* reporter line, (Y–AG) *pCEP4::GUS* reporter line, (AH–AO) *pCEP5::GUS* reporter line. Various stages of lateral root development, from initiation (asymmetric cell division) (A, I, Q, Y, AH) to an emerged lateral root (H, P, X, AG, AO), are shown.

lateral root primordium, coinciding with the (future) vasculature (Fig. 8E–H). Both *CEP3* and *CEP5* were expressed at the base of the lateral roots, where *CEP5* seemed to be expressed from an earlier time point in development compared to *CEP3* (Fig. 8W, X, and AH–AO). *CEP4* was also expressed at the base and in the vasculature of the emerged lateral root (Fig. 8AG).

The combination of *in planta* and *in silico* expression patterns showed that *CEP* genes are expressed throughout plant development (Fig. 4). This study observed some variation between *in planta* and *in silico* data (Supplementary Table S1), which could be due to experimental conditions (e.g. responsiveness of *CEPs* to external and/or environmental stimuli). It is also interesting to note that the *CEP* expression patterns were often associated with the vasculature during a specific developmental process. At present, there is no functional evidence for any evolutionary reason behind these vasculature-associated expression patterns. However, CEPs are absent in the more primitive groups of the green lineage, such as green algae, the non-vascular land plant *P. patens*, and *S. moellendorffii*, which is a representative of the earliest vascular plants that has a simple protostele (phloem surrounding the xylem).

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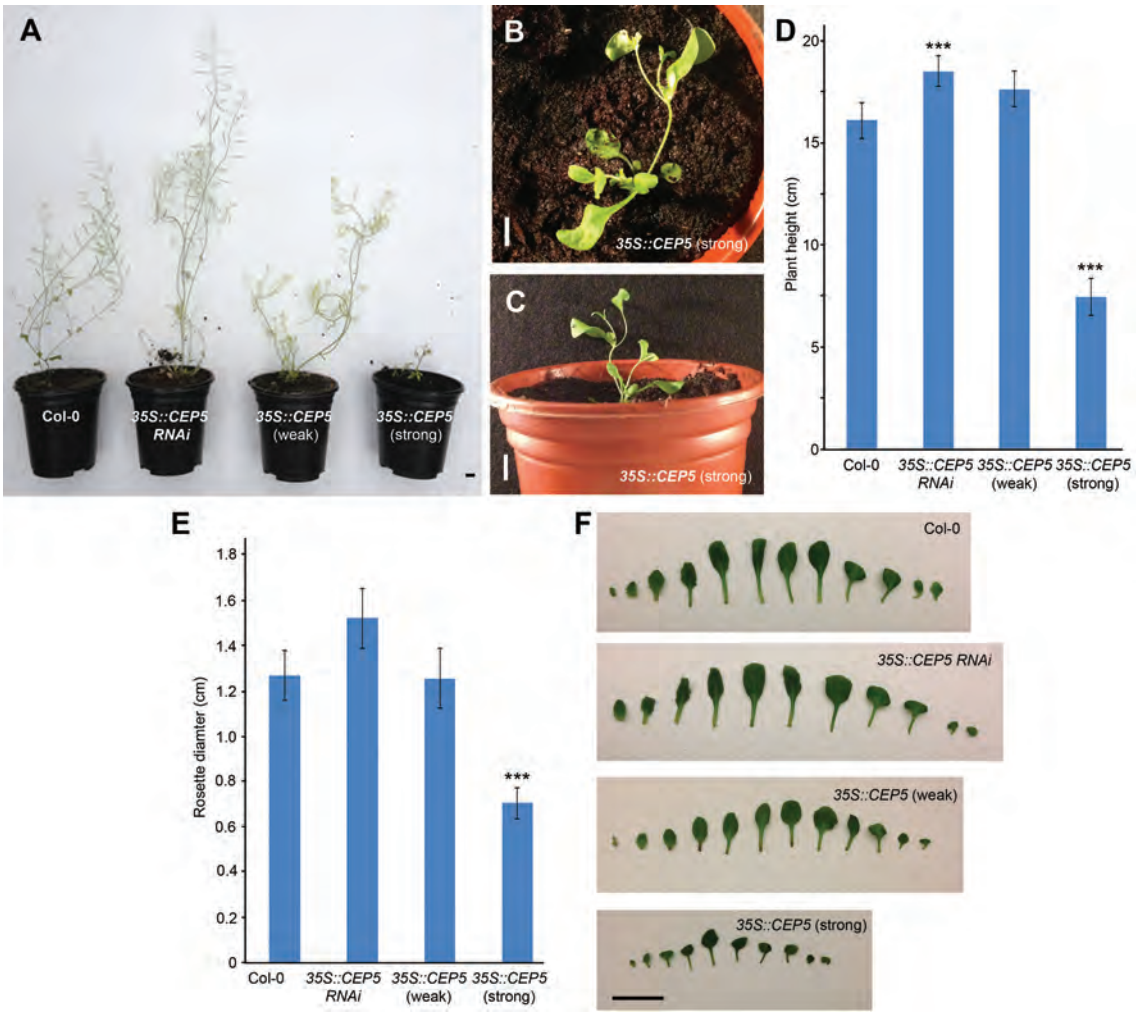

Fig. 9. Characterization of aboveground growth in lines with perturbed *CEP5* expression levels. (A) Representative shoot of Col-0 (background line), 35S::CEP5 RNAi knockdown line, and two overexpression lines [35S::CEP5 (weak) and 35S::CEP5 (strong)] after 3 weeks of growth post-bolting. (B and C) Severely stunted growth of 35S::CEP5 (strong) plants after 3 weeks of growth post-bolting. (D) Quantification of the plant height. (E) Rosette area 18 d after germination. (F) Representative leaf series of Col-0 (background line), 35S::CEP5 RNAi, 35S::CEP5 (weak), and 35S::CEP5 (strong). Data in D and E are means±standard errors of at least 20 plants. ***, Student's *t*-test with a *P*-value <0.05. Bars, 1 cm.

Therefore, one explanation could be that the appearance of CEPs coincides with the formation of more complex vascular tissues, such as actinosteles and eusteles in which the vascular tissue becomes more fragmented in separate bundles.

CEP5 is involved in aboveground growth

Since a role for CEP1 in root growth and development had been described previously (Ohyama *et al.*, 2008), this work focused on the effects of perturbing *CEP* levels on aboveground parts to determine if CEPs play a role in shoot development. Overexpression and knockdown lines were generated and analysed for *CEP5*, which is expressed in the shoot (Fig. 4). Multiple lines with a range of *CEP5* expression levels and displaying similar phenotypes were generated (data not shown), but this work selected representative knockdown and overexpression lines. The obtained phenotypes for knockdown or overexpression lines (see Fig. 9) were correlated with reduced or increased *CEP5* expression levels, respectively (Supplementary Fig. S4).

RNAi knockdown plants for *CEP5* after 3 weeks of growth post-bolting demonstrated a slight increase in plant height compared to Col-0 (Fig. 9A and D). In contrast, a strong overexpression line for *CEP5* was extremely stunted and displayed a loss of shoot gravitropic response (Fig. 9A–D). A weaker *CEP5* overexpression line did not display the same dramatic phenotype but appeared mildly defected in stem gravitropism (Fig. 9A). The rosette diameter was significantly reduced in the strong *CEP5* overexpression line, compared to the control (Fig. 9E). In addition, strong *CEP5* overexpression resulted in smaller, often curled, leaves (Fig. 9F). However, given the *CEP5* expression pattern, the leaf size phenotype might be due to non-specific effects and might reflect a role for another, highly similar, CEP peptide.

Conclusion

In conclusion, in *A. thaliana*, more than 1000 small signalling peptides have been predicted, but very few have been

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functionally characterized (Lease and Walker, 2006; Butenko *et al.*, 2009; Matsubayashi, 2011; Murphy *et al.*, 2012; Czyzewicz *et al.*, 2013). One class of small post translationally modified signalling peptides is the CEP family (Ohyama *et al.*, 2008). One member of this family has already been shown to be involved in regulating root architecture (Ohyama *et al.*, 2008). Here, a bioinformatics approach was applied to identify more members of this family and to reveal that this family only emerged from higher land plants onward. The data further suggest that the CEP proteins form two subgroups according to their CEP domain. The specific *CEP* expression patterns offer a comprehensive framework to study the role of the CEP signalling peptides in plant development and hint to a possible role in cell communication mechanisms in the more complex vasculature of flowering plants. Expression patterns and perturbing levels of CEP family peptides pointed to a role in aboveground tissues, such as leaf and flower development. These results form the basis for further exploration of the mechanisms underlying this family of peptides and suggest that this family of small signalling peptides has a distinct role associated with developmental events associated with higher plants.

Supplementary material

Supplementary data are available at *JXB* online.

Supplementary Dataset S1. *Arabidopsis* CEP proteins.

Supplementary Dataset S2. CEP domains predicted by MEME.

Supplementary Fig. S1. Position of CEP family members on the five *Arabidopsis* chromosomes.

Supplementary Fig. S2. Expression patterns of *CEP1–CEP5* in the root apical meristem.

Supplementary Fig. S3. *CEP1–CEP5* expression in the root–hypocotyl junction.

Supplementary Fig. S4. *CEP5* expression levels in 35S::CEP5RNAi and 35S::CEP5 lines

Supplementary Table S1. Comparison of *in silico* (eFP browser) and *in planta* (*pCEPx::GUS*) *CEP* expression.

Supplementary Table S2. Genevestigator data on *CEP* expression.

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APPENDIX IX

A secreted peptide acts on BIN2-mediated phosphorylation of ARFs to potentiate auxin response during lateral root development

Hyunwoo Cho, Hojin Ryu, Sangchul Rho, Kristine Hill, Stephanie Smith, Dominique Audenaert, Joonghyuk Park, Soeun Han, Tom Beeckman, Malcolm J. Bennett, Daehee Hwang, Ive De Smet and Ildoo Hwang.

2014. Nature Cell Biology **16:** 66–78.

nature cell biology

A secreted peptide acts on BIN2-mediated phosphorylation of ARFs to potentiate auxin response during lateral root development

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The phytohormone auxin is a key developmental signal in plants. So far, only auxin perception has been described to trigger the release of transcription factors termed AUXIN RESPONSE FACTORs (ARFs) from their AUXIN/INDOLE-3-ACETIC ACID (AUX/IAA) repressor proteins. Here, we show that phosphorylation of ARF7 and ARF19 by BRASSINOSTEROID-INSENSITIVE2 (BIN2) can also potentiate auxin signalling output during lateral root organogenesis. BIN2-mediated phosphorylation of ARF7 and ARF19 suppresses their interaction with AUX/IAAs, and subsequently enhances the transcriptional activity to their target genes *LATERAL ORGAN BOUNDARIES-DOMAIN16* (*LBD16*) and *LBD29*. In this context, BIN2 is under the control of the TRACHEARY ELEMENT DIFFERENTIATION INHIBITORY FACTOR (TDIF)–TDIF RECEPTOR (TDR) module. TDIF-initiated TDR signalling directly acts on BIN2-mediated ARF phosphorylation, leading to the regulation of auxin signalling during lateral root development. In summary, this study delineates a TDIF–TDR–BIN2 signalling cascade that controls regulation of ARF and AUX/IAA interaction independent of auxin perception during lateral root development.

Spatial and temporal regulation of auxin signalling is essential for diverse developmental processes in plants[1], and the nuclear auxin response mechanism has been well characterized[2,3]. In brief, auxin maxima formation through its graded distribution promotes the interaction between its co-receptors, TRANSPORT INHIBITOR1 (TIR1) or AUXIN-SIGNALLING F-BOX PROTEIN (AFB) F-box proteins and AUX/IAA transcriptional repressors, which triggers degradation of AUX/IAAs by E3 ubiquitin-ligase $SCF^{TIR1/AFBs}$ complexes[4]. At low auxin levels, AUX/IAA repressors are stable and interact with ARF transcription factors, and recruit co-repressor complexes leading to suppression of auxin-responsive genes[1,5]. At higher auxin levels, degradation of AUX/IAAs allows free ARFs to initiate transcription of auxin-responsive genes[2,3]. In *Arabidopsis*, interactions between 6 TIR1/AFB auxin receptors, 23 ARF proteins, and 29 AUX/IAA repressors are associated with diverse auxin responses during plant growth and development[1]. Distinct expression patterns of these central components, and remarkable differences in binding affinities between TIR1/AFBs and AUX/IAAs, could potentially result in a broad range of auxin signalling outputs[6]. Whereas the molecular basis of auxin perception and subsequent proteolysis of AUX/IAAs has been elucidated, the regulatory mechanism underlying how ARFs determine auxin signalling output and dissociate from the repressor complex is still unclear.

One important, well-characterized developmental process that is dependent on the tight regulation of ARFs and AUX/IAAs is lateral root (LR) initiation, patterning and emergence[7–9]. The auxin response module composed of ARF7 and ARF19 along with the target genes *LBD16* and *LBD29* controls LR organogenesis through auxin-dependent degradation of AUX/IAAs, such as SOLITARY ROOT (SLR)/IAA14 and MASSUGU 2 (MSG2)/IAA19 (refs 10,11).

GLYCOGEN SYNTHASE KINASE3 (GSK3) is a key regulator of diverse developmental processes in animals[12]. In *Arabidopsis*, distinct expression patterns of *GSK3*-like genes have been observed in various developing organs[13,14]. However, molecular links connecting these

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genes to development are largely unknown. The plant GSK3 BIN2 (also known as ULTRACURVATA1, UCU1, and DWARF12, DWF12) is a critical regulator in brassinosteroid (BR) signal transduction and stomata development[15,16]. BIN2 kinase targets YODA (YDA) and SPEECHLESS (SPCH) in stomata development[15,17], and negatively regulates BR signalling by phosphorylation-dependent inactivation of two BR-related key transcriptional regulators, *bri1* EMS SUPPRESSOR1 (BES1) and its homologue BRASSINAZOLE RESISTANT1 (BZR1; refs 18–20). The negative action of BIN2 in BR signalling is directly relieved by a phosphatase BSU1 (ref. 21). In addition to its action in BR signalling, BIN2 has been implicated in the regulation of auxin signalling[22,23]. A gain-of-function allele of BIN2, *ucu1*, showed auxin hypersensitivity in root elongation[22], and BIN2 seemed to attenuate DNA-binding affinity of ARF2, a repressor of auxin signalling[23]. However, it is largely unknown how BIN2 action is integrated into auxin signalling pathways during plant growth and development.

Here, we show that the TDIF–TDR–BIN2 signalling module directly regulates auxin signalling output during LR organogenesis by controlling the transcriptional activity of ARF7 and ARF19.

RESULTS

BIN2 positively regulates LR development

As BIN2 has been implicated in the regulation of auxin signalling[22,23], we investigated its role in auxin-mediated LR development. We examined the LR phenotype of gain-of-function *BIN2* mutants *dwf12-1D-gof* and *bin2-1-gof*, which exhibited a brassinosteroid (BR)-insensitive short root[24,25], and we observed an increased LR density compared with wild-type plants (Fig. 1a–c). In contrast, loss-of-function *BIN2* mutants *bin2-3-lof* (ref. 13) and *bin2-3 bil1 bil2* (ref. 26) exhibited a reduced LR density compared with wild-type plants (Fig. 1a,b). To eliminate the possibility that the LR phenotypes of *dwf12-1D-gof* and *bin2-1-gof* are due to their relatively short root length, the BR-insensitive mutant *bri1-116* was also examined. Indeed, although *bri1-116* exhibited a shorter root phenotype, this was not associated with increased LR density compared with wild-type plants (Fig. 1d). Furthermore, the *bin2-3 bil1 bil2* mutant phenotype was recapitulated in seedlings treated with bikinin, a GSK3-specific inhibitor[27] (Fig. 1a,b).

The involvement of BIN2 in root branching was further supported by examination of the *pBIN2–GUS* reporter line[18]. *BIN2* expression was detected in xylem pole pericycle cells, epidermal cells and cortex in the basal meristem, but restricted to the vasculature in the elongation zone (Fig. 1e and Supplementary Fig. 1a). During LR development, *BIN2* expression was observed in LR initiation sites and in the basal part of the dome-shaped LR primordium (Fig. 1e and Supplementary Fig. 1a).

To evaluate the physiological basis of the BR signalling pathway in LR development, we examined the role of the BR receptor BRI1, the BR biosynthesis enzyme DWARF4 (DWF4), and the downstream components BRI1 SUPPRESSOR 1 (BSU1), BES1 and BZR1. Interestingly, *DWF4*- (ref. 28) and *BRI1*- (ref. 29) overexpressing plants exhibited a higher density of LR primordia than wild-type plants, whereas *BSU1*-overexpressing[30] and gain-of function *bes1-D* (ref. 20) and *bzr1-1D* (ref. 19) plants did not (Fig. 2a). Furthermore, BR treatment further increased LR development in *bin2-1-gof*, but not in the *bri1-116* mutant, and *BIN2* overexpression increased LR development in the loss-of-function *bri1-5* mutant (Fig. 2b–d). These results indicate that BIN2 positively regulates LR initiation, and that BR action in LR development is bifurcated upstream of BSU1, an inhibitor of BIN2.

BIN2 enhances auxin response during LR development through ARF7 and ARF19

The *BIN2* expression patterns during LR development and in the vasculature overlapped with several well-known auxin-related transcriptional regulators for LR development, including *ARF5*, *ARF7*, *ARF19, IAA1, IAA3, IAA12, IAA14, IAA18* and *IAA19* (Supplementary Fig. 1b,c)[10,11,31,32]. Thus, we investigated whether BIN2 is connected to auxin signalling during LR development. First, we found that *dwf12-1D-gof* was hypersensitive to auxin with respect to primary root growth, whereas *bin2-3-lof* was less sensitive compared with wild-type plants (Fig. 3a). Next, after auxin depletion by NPA treatment, the auxin-responsive reporter *pDR5–GUS* was more rapidly and strongly activated by auxin in *bin2-1-gof* than in wild-type plants, particularly in the pericycle (Fig. 3b and Supplementary Fig. 2a). Moreover, expression of *LBD16* and *LBD29*, which are marker genes for LR initiation that function directly downstream of ARF7 and ARF19, was higher in *bin2-1-gof* and lower in *bri1-116* compared with wild-type plants (Supplementary Fig. 2b). To further confirm the *in planta* molecular action of BIN2 during auxin-mediated LR development, auxin sensitivity of *bin2-1-gof, dwf12-1D-gof* and *bin2-3 bil1 bil2* loss-of-function mutants was evaluated with respect to LR formation and expression of *LBD*s (Supplementary Fig. 2c,d). Compared with wild-type plants, in the presence of 1 μM auxin, *dwf12-1D-gof* mutants exhibited a 50% increase in LR density, whereas *bin2-3 bil1 bil2* triple mutants showed a 20% lower LR density (Fig. 3c). Compared with wild-type plants, *dwf12-1D-gof* consistently exhibited hypersensitivity to exogenous auxin in a time-dependent manner, as determined by the expression of *LBD16* and *LBD29*. However, *LBD16* and *LBD29* expression was reduced in the *bin2-3 bil1 bil2* mutant in the presence of exogenous auxin (Supplementary Fig. 2d). Consistently, bikinin treatment reduced *LBD* expression and auxin-mediated LR development in a dosage-dependent manner (Supplementary Fig. 2e and Fig. 3d). Taken together, these results suggested that BIN2 functions to increase auxin-responsive gene expression. Consistent with this mechanism, co-expression of wild-type BIN2 and the auxin-responsive reporter *pGH3–LUC* significantly induced reporter expression in a transient protoplast system[33] in a dose-dependent manner (Fig. 3e and Supplementary Fig. 2f). In addition, co-expression of a gain-of-function $BIN2^{E263K}$ (*dwf12-1D*) activated the *GH3* reporter more strongly than BIN2, and synergistically enhanced the reporter activity in the presence of auxin (Fig. 3e).

We then examined the ability of other *Arabidopsis* group II GSK3 proteins to regulate auxin signalling using the *pGH3–LUC* reporter system. Whereas BIN2-LIKE1 (BIL1) increased *GH3* promoter activity to the same degree as BIN2, BIL2 did not significantly enhance *GH3* expression (Supplementary Fig. 3a). Consistent with a positive regulatory function for BIL1, the *bil1* null mutant showed 10% fewer emerged LRs than wild-type plants (Supplementary Fig. 3b,c). As expected, both *bil1 bin2-3* and *bin2-3 bil1 bil2* loss-of-function mutants exhibited a similar reduction of approximately 20% in LR primordia density (Supplementary Fig. 3c). Taken together, these results indicate

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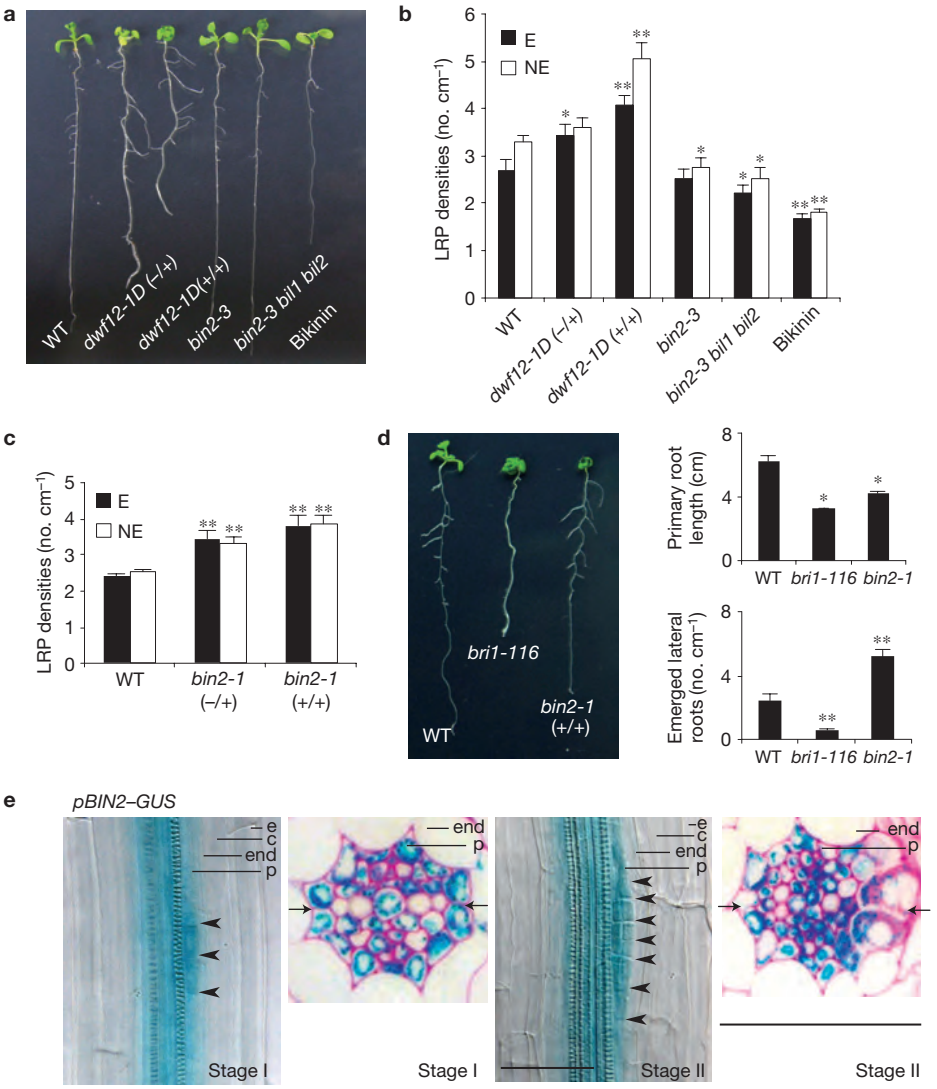

Figure 1 BIN2 positively regulates LR development. (**a**,**b**) BIN2 kinase activity is essential for proper LR development. Representative twelve-day-old wild-type (WT; Ws-2), *dwf12-1D-gof* (heterozygote (−/+) and homozygote (+/+)), and *bin2-3-lof*, *bin2-3 bil1 bil2* mutant and bikinin-treated seedlings are shown (**a**). LR primordium (LRP) densities. E, emerged LRs; NE, non-emerged LRP ($n = 15$ plants; **b**). (**c**) LRP densities of 12-day-old seedlings of wild-type (Col-0), *bin2-1-gof* heterozygote (−/+) and homozygote (+/+) mutant plants ($n = 12$ plants). (**d**) The LR development phenotype of 12-day-old seedlings of wild-type (Col-0), *bri1-116* and *bin2-1-gof* (left-panel). The primary root lengths and densities of emerged LRs in wild-type (Col-0), *bri1-116,* and *bin2-1-gof* (+/+; right panel; $n = 12$ plants). (**e**) *pBIN2–GUS* expression limited to LRP and vascular tissues at stage I and II (left panel, black arrowheads indicate dividing pericycle cells) and transverse sections of *pBIN2–GUS* seedlings through the LRP (right panel, black arrows indicate xylem pole pericycle cells). e, epidermis; c, cortex; end, endodermis; p, pericycle. Scale bars, 50 μm. Asterisks indicate statistically significant differences as compared with wild-type at $^{*}P < 0.05$ and $^{**}P < 0.01$. Error bars indicate s.e.m. For details on the statistical analysis, see the Methods.

that BIN2 and BIL1 function redundantly to stimulate auxin signalling during LR development.

To genetically dissect BIN2 action in auxin-mediated LR formation, *bin2-1-gof* was crossed with various auxin signalling defective mutants. For this purpose, the following alleles were used: *arf7-1* and *arf19-1* (loss-of-function alleles of *ARF7* and *ARF19*, respectively[10,31]), *tir1-1* (a loss-of-function allele of the auxin receptor *TIR1*; ref. 34), and *msg2-1* (a gain-of-function allele of *IAA19* encoding an auxin-insensitive, constitutively stable repressor protein[11]). The *bin2-1-gof* mutant slightly restored the *arf7-1* LR phenotype, which was possibly due to the presence of the functional ARF7 homologue, ARF19 (Fig. 3f and Supplementary Fig. 3d,e). Indeed, *bin2-1-gof* failed to rescue the *arf7-1 arf19-1* double-mutant lateral rootless phenotype (Fig. 3f). To exclude the possibility that alterations in TIR1-mediated degradation of AUX/IAAs caused the observed lack of suppression, *bin2-1*, *msg2-1 bin2-1-gof* and *tir1-1 bin2-1-gof* double mutants were analysed. *bin2-1-gof* rescued *msg2-1* and *tir1-1* LR phenotypes, as evidenced by the significant increase in emerged LR density (Fig. 3f,g). These results suggested that BIN2 functions either downstream of TIR1 or in parallel with TIR1-mediated auxin signalling during LR development. In addition, BIN2-mediated activation of LR development required ARF7 and ARF19. This prompted the hypothesis that BIN2 may relieve ARF7 and ARF19 from their repressor complex, thus leading to their activation irrespective of TIR1 activity during LR organogenesis.

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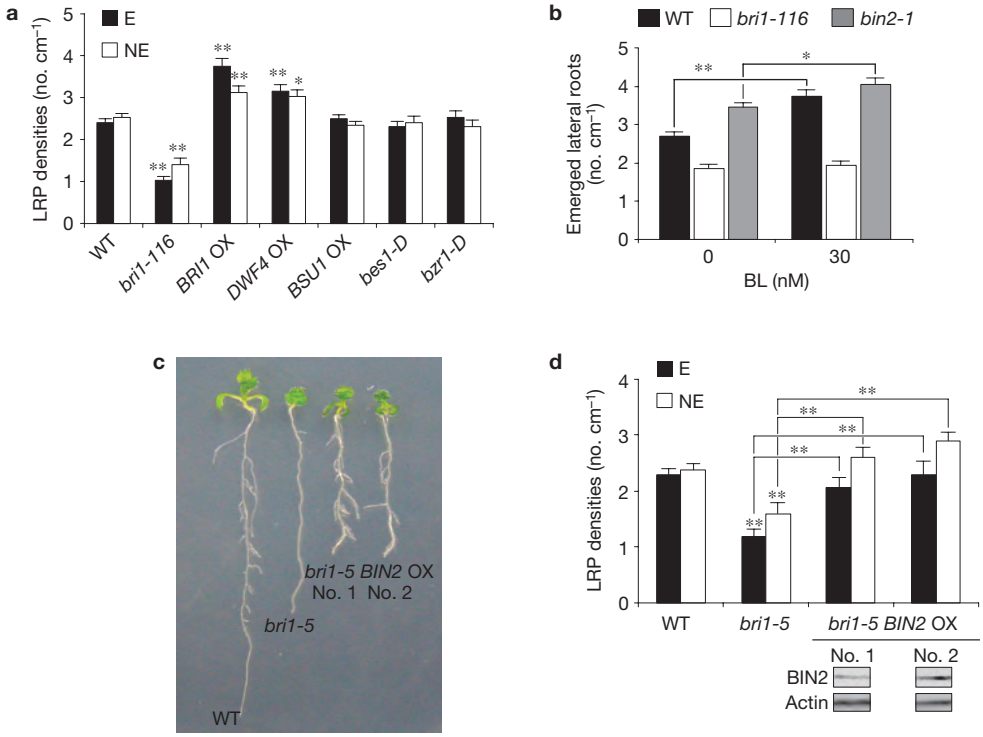

Figure 2 BR plays a minor role in BIN2-mediated LR development. (**a**) LRP densities of 12-day-old seedlings of wild-type (Col-0), *bri1-116*, *BRI1* overexpressing (OX), *DWF4* OX, *BSU1* OX, *bes1-D* (*pBES1–bes1-D–HA*) and *bzr1-D* mutants ($n = 15$ plants). (**b**) Different BL responses of *bri1-116* and *bin2-1-gof* (+/+) in LR development. Treatment: BL for 5 days ($n = 20$ plants). (**c**) The LR development phenotype of 12-day-old seedlings of wild-type (Ws-2), *bri1-5,* and *bri1-5 BIN2* OX. (**d**) LRP densities of 12-day-old seedlings of wild-type (Ws-2), *bri1-5,* and *bri1-5 BIN2* OX. BIN2–HA proteins in the transgenic plants are presented ($n = 12$ plants; lower). Error bars indicate s.e.m. ($^{*}P < 0.05$, $^{**}P < 0.01$ by Student's *t*-test). For details on the statistical analysis, see the Methods. A full scan image of the western blot is shown in Supplementary Fig. 8.

BIN2 directly phosphorylates ARF7 and ARF19

To elucidate the molecular mechanism of BIN2 action on auxin signal transduction, we examined whether BIN2 directly interacts with ARF or AUX/IAA proteins. In an *in vitro* pulldown assay with GST–BIN2, HA-tagged ARF7 was pulled down (Fig. 4a). Specifically, the QSL-rich carboxy-terminal domain, including the middle region and AUX/IAA dimerization domain (d2 ARF7), but not the B3 DNA-binding domain (d1 ARF7), directly interacted with BIN2 (Fig. 4b). In contrast, AUX/IAA proteins did not interact with BIN2 (Supplementary Fig. 4).

We next investigated whether ARF7 was phosphorylated by BIN2. BIN2, BIN2^{E263K} and BIL1 increased the intensity of high-molecular-weight bands of ARF7 in a gel shift assay (Fig. 4c). The higher-molecular-weight band shift of ARF7 was abolished by calf intestine alkaline phosphatase (CIAP) treatment (Fig. 4d), indicating that the ARF7 mobility shift was due to phosphorylation by BIN2. Conversely, a kinase-dead loss-of-function mutant BIN2^{K69R} and BIL2 failed to increase the intensity of the phosphorylated ARF7 band (Fig. 4c). In addition, phosphorylated ARF7 was highly enriched in *dwf12-1D-gof* plants (Fig. 4e), and BIN2 interacted with ARF19 and shifted the mobility of ARF19 proteins (Fig. 4f,g). *In vitro* kinase assays revealed that BIN2 induced the phosphorylation in the QSL-rich C-terminal domain of ARF7 (Fig. 4h). Liquid chromatography–tandem mass spectrometry (LC–MS/MS) analysis of the phosphorylated QSL-rich C-terminal domain of ARF7 showed that BIN2-dependent phosphorylation occurred at Ser 698 and Ser 707 (Fig. 4i). Taken together, these results suggest that BIN2 directly interacts with and phosphorylates ARF7 and ARF19.

BIN2-mediated phosphorylation induces ARF7 and ARF19 activity by attenuating their interaction with AUX/IAAs

Next, the ability of BIN2 to affect auxin signalling by modulating ARF7 and ARF19 activity through phosphorylation was investigated. BIN2 enhanced *pGH3–LUC* activity, and co-expression of *BIN2* with either *ARF7* or *ARF19* further enhanced reporter expression (Fig. 5a,b). In contrast, the kinase-dead BIN2^{K69R} mutant exerted a dominant-negative effect, reducing ARF7-induced activation of the reporter by 60% (Fig. 5a). These data indicate that BIN2 enhances ARF7- and ARF19-mediated transcriptional activation.

BIN2-mediated phosphorylation may enhance ARF7 activity by blocking its interaction with AUX/IAA repressors. Thus, we investigated whether phosphorylation by BIN2 affects the association of ARF7 with AUX/IAAs. Among the 29 members of *Arabidopsis* AUX/IAAs, IAA1, IAA3, IAA14, IAA18 and IAA19 are involved in LR initiation[32]. Whereas hypo-phosphorylated ARF7 proteins were precipitated together with GST–IAA1, GST–IAA3, GST–IAA14, GST–IAA18 and GST–IAA19, hyper-phosphorylated ARF7 proteins were only weakly pulled down with these AUX/IAA proteins in the presence of BIN2 (Fig. 5c and Supplementary Fig. 5). However, BIN2^{K69R} did not affect the association of ARF7 with AUX/IAA proteins (Fig. 5c). Furthermore, substitution of both Ser 698 and Ser 707 with alanine (ARF7$^{S698A/S707A}$, ARF7m) could not interfere with the

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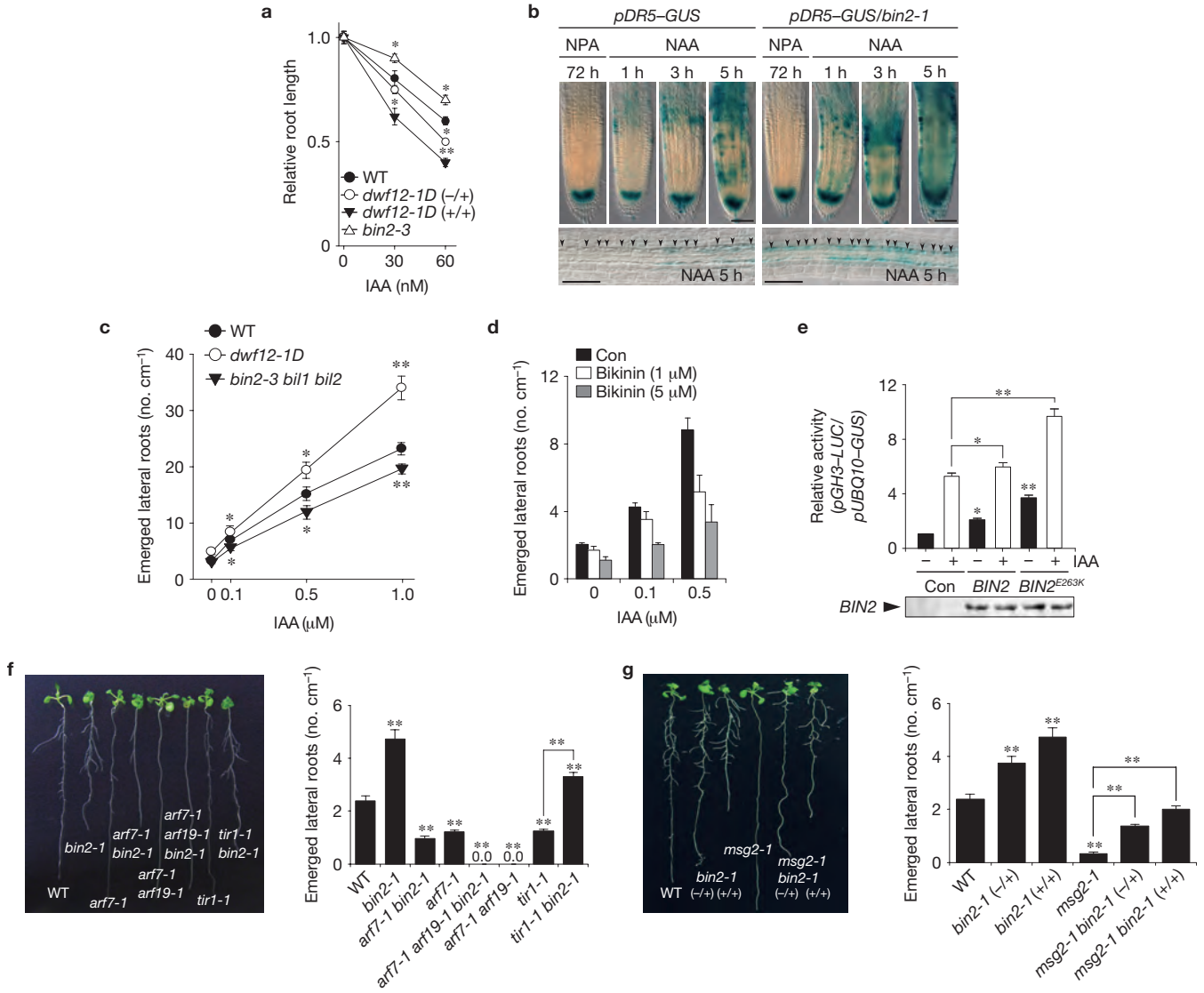

Figure 3 BIN2-mediated signalling is integrated into ARF7/19 during LR development. (**a**) Different auxin responses of *bin2* mutants in root elongation. Relative root length of the 12-day-old *dwf12-1D-gof* (heterozygote (−/+) and homozygote (+/+)) and *bin2-3-lof* compared with wild-type. Treatment: IAA for 5 days ($n = 12$ plants). (**b**) The *bin2-1-gof* mutation increases auxin sensitivity in *pDR5–GUS* activation. The indicated plants were grown for 72 h in the presence of 10 μM NPA and then transferred to 1 μM NAA for 1, 3 and 5 h. The arrowheads indicate dividing pericycle cells. Scale bars, 50 μm. (**c**) BIN2 increases auxin sensitivity in LR formation. The densities of emerged LRs in 11-day-old seedlings of wild-type (Ws-2), *dwf12-1D-gof* homozygote (+/+), and *bin2-3 bil1 bil2* mutant. Treatment: IAA for 4 days ($n = 12$ plants). (**d**) Bikinin treatment inhibits auxin-mediated LR development. The densities of emerged LRs were measured in the presence of the indicated concentrations of auxin and bikinin. ($n = 15$ plants). (**e**) BIN2 and auxin synergistically enhance the activity of the GH3 promoter. Protoplasts were co-transfected with pUBQ10–GUS (internal control), *pGH3–LUC* and the indicated effector constructs, and treated with 1 μM IAA for 3 h. BIN2 protein levels were detected using anti-HA antibody ($n = 4$, 20,000 cells per $n = 1$). A full scan image of the western blot is shown in Supplementary Fig. 8. For statistics source data, see Supplementary Table 2. (**f**) *bin2-1-gof* homozygote mutation suppresses the LR defects of *tir1-1*, but not *arf7-1*, and *arf7-1 arf19-1*. The densities of the emerged LRs of 12-day-old seedlings of indicated genotypes are presented ($n = 16$ plants). (**g**) *bin2-1-gof* partially suppresses the defective LR phenotype of *msg2-1*. The densities of the emerged LRs of 12-day-old seedlings of indicated genotypes are presented ($n = 15$ plants). Error bars indicate s.e.m. ($^{*}P < 0.05$, $^{**}P < 0.01$ by Student's *t*-test). For details of the statistical analysis, see the Methods.

interaction with AUX/IAA proteins in the absence and presence of BIN2 (Fig. 5c). We then examined whether phosphorylation affects ARF7-mediated auxin response. ARF7m itself was able to activate *pGH3–LUC* as much as wild-type ARF7, and its repressor IAA19 inhibited the activity of the reporter (Fig. 5d). Interestingly, IAA19 suppression of the reporter activity was compromised by BIN2 together with ARF7, but not with ARF7m. Consistently, overexpression of *ARF7* in *arf7-1* (referred to as *ARF7 arf7-1*) restored the LR defects of *arf7-1* to wild–type levels, but *ARF7m* overexpression in three independent *ARF7* transgenic plants with similar protein levels (referred to as *ARF7m arf7-1*) only partially recovered the LR defects of *arf7-1* (Fig. 5e). Furthermore, *bin2-1-gof* increased the LR formation of *ARF7 arf7-1* transgenic plants, but not *ARF7m arf7-1* plants (Fig. 5f). We therefore concluded that BIN2-mediated phosphorylation of ARF7 attenuates the interaction of ARF7 with AUX/IAAs and leads to the activation of auxin signalling. The ability of BIN2-mediated phosphorylation to

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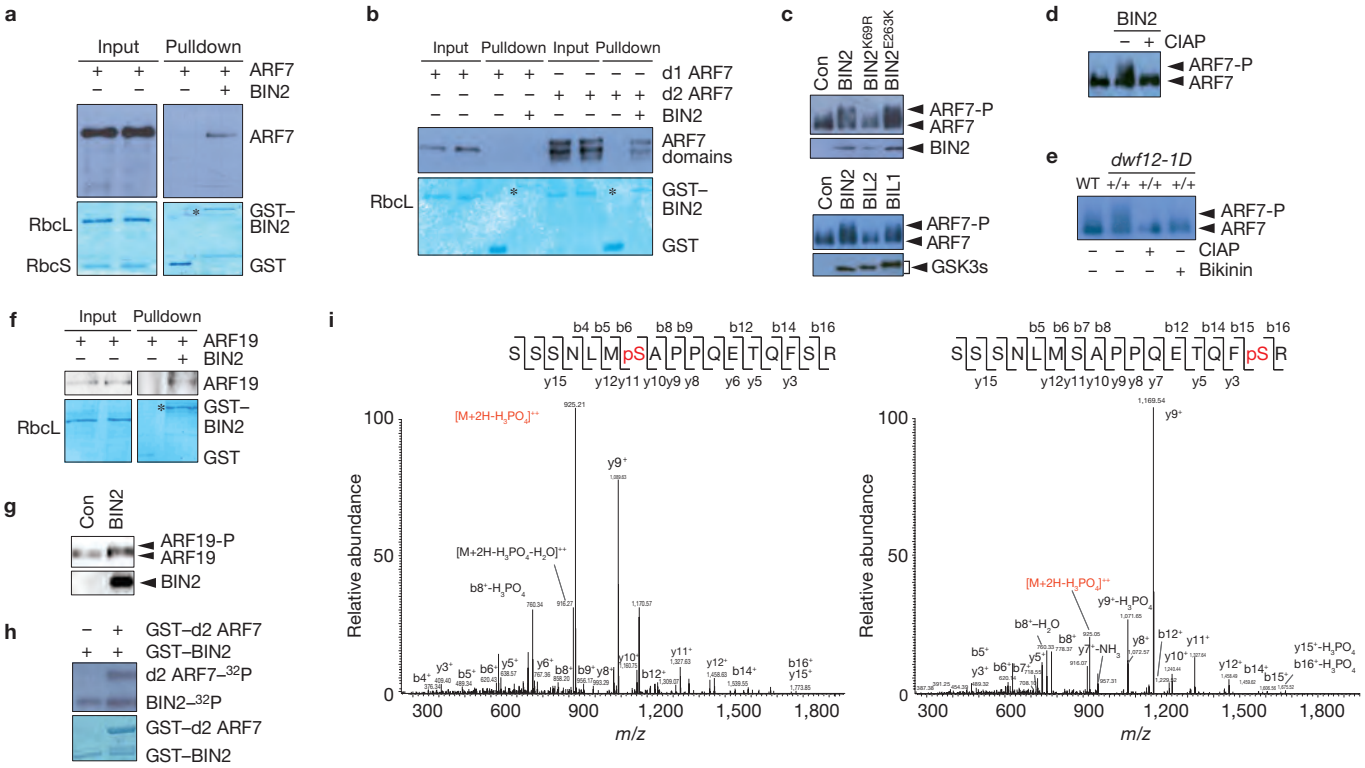

Figure 4 BIN2 directly phosphorylates ARF7. (**a**) BIN2 interacts with ARF7. Protoplast lysates overexpressing *ARF7–HA* were incubated with purified GST–BIN2 proteins and pulled down with glutathione Sepharose 4B. GST–BIN2-bound proteins were visualized with anti-HA HRP antibody. An asterisk indicates GST–BIN2 proteins. (**b**) BIN2 interacts with the QSL-rich C-terminal domain of ARF7 (d2 ARF7). Protoplast lysates overexpressing the HA-tagged B3 DNA-binding domain of *ARF7* (*d1 ARF7*) or QSL-rich C-terminal domain (*d2 ARF7*) were incubated with GST–BIN2 or GST proteins and pulled down with glutathione Sepharose 4B. Precipitated proteins were visualized by anti-HA antibody. (**c**) BIN2 kinase activity is required for ARF7 phosphorylation. HA-tagged ARF7 was co-transfected into protoplasts with *BIN2–HA*, *BIN2*^{E263K}*–HA*, *BIN2*^{K69R}*–HA* or *BIL1–HA*, *BIL2–HA*. After 6 h of transfection, the indicated proteins were visualized with anti-HA antibody. (**d**) The BIN2-induced mobility shift was abolished by CIAP treatment. (**e**) ARF7 is phosphorylated by BIN2 *in vivo*. The phosphorylation status of ARF7 proteins in wild-type, *dwf12-1D-gof* plants harbouring *35S–ARF7–HA* in the presence or absence of 5 μM bikinin. The BIN2-induced mobility shift was abolished by CIAP or bikinin treatment. (**f**) BIN2 interacts with ARF19 *in vitro*. Protoplast lysates overexpressing *ARF19–HA* were incubated with GST–BIN2 proteins and pulled down with glutathione Sepharose 4B. GST–BIN2-bound proteins were visualized with anti-HA HRP antibody. An asterisk indicates GST–BIN2 proteins. (**g**) BIN2 phosphorylates ARF19 proteins. *ARF19–HA* was co-transfected into protoplasts with *BIN2–HA*. ARF19 and BIN2 proteins were visualized with anti-HA antibody. (**h**) BIN2 directly phosphorylates ARF7. GST-tagged BIN2 and the QSL-rich C-terminal domain of ARF7 (d2 ARF7) were used in an *in vitro* kinase assay. The autoradiogram shows protein phosphorylation (upper panel). The protein levels were determined by Coomassie blue staining (lower panel). (**i**) MS/MS spectra for two major phosphopeptides of GST–d2 ARF7 showing *in vitro* BIN2 phosphorylation sites of ARF7 at Ser 698 (left panel) and Ser 707 (right panel). GST–d2 ARF7 was incubated with GST–BIN2 and ATP for 12 h. The proteins were digested by trypsin and analysed by LC–MS/MS. Full scan images of western blots are shown in Supplementary Fig. 8.

alter the interaction between ARF7 and IAA19 was further supported by the rescue of *msg2-1* by *bin2-1-gof* (Fig. 3g).

We next investigated whether AUX/IAAs dissociated from phosphorylated ARFs could be more rapidly degraded through the TIR1 pathway. To test this possibility, the effect of ARF7 phosphorylation on the relative amount of interacting proteins in TIR1–ARF7–IAA19 complexes was tested. Co-expression of *BIN2* attenuated the interaction of IAA19 with ARF7, and BIN2 increased the interaction of IAA19 with TIR1 (Fig. 6a and Supplementary Fig. 6a). We then examined the protein level of IAA19 in wild-type or *bin2-1-gof* plants. The proteolysis of IAA19 proteins occurred more rapidly in *bin2-1-gof* than in wild-type plants, but bikinin treatment effectively inhibited the IAA19 protein degradation (Fig. 6b). Consistently, the fluorescence of DII–VENUS, which has no dimerization domain with ARFs, was not significantly affected by bikinin treatment (Supplementary Fig. 6b). These results suggested that BIN2-mediated phosphorylation of ARF7 diminishes its interaction with AUX/IAA proteins and subsequently enlarges the pool of free ARF7 proteins by facilitating degradation of their repressor AUX/IAAs.

We next examined whether BIN2-mediated phosphorylation of ARF7 and ARF19 affects their binding to target promoters using a chromatin immunoprecipitation assay. Whereas DNA binding of ARF7 and ARF19 to the promoters of *LBD16* and *LBD29* was increased in the presence of auxin, bikinin treatment suppressed the auxin-induced enhancement of DNA binding (Fig. 6c,e). Furthermore, the *dwf12-1D-gof* enhanced DNA binding of ARF7 to their target promoters (Fig. 6d). The *bin2-1-gof* further increased auxin-induced enhancement of ARF19 binding, but bikinin treatment compromised the enhancement of ARF19 binding in *bin2-1-gof*, to the *LBD29* promoter (Fig. 6f). Together, these results indicated that phosphorylation of ARFs could increase the pool of active ARFs by attenuating the interaction with AUX/IAAs and enhance their DNA-binding capacity.

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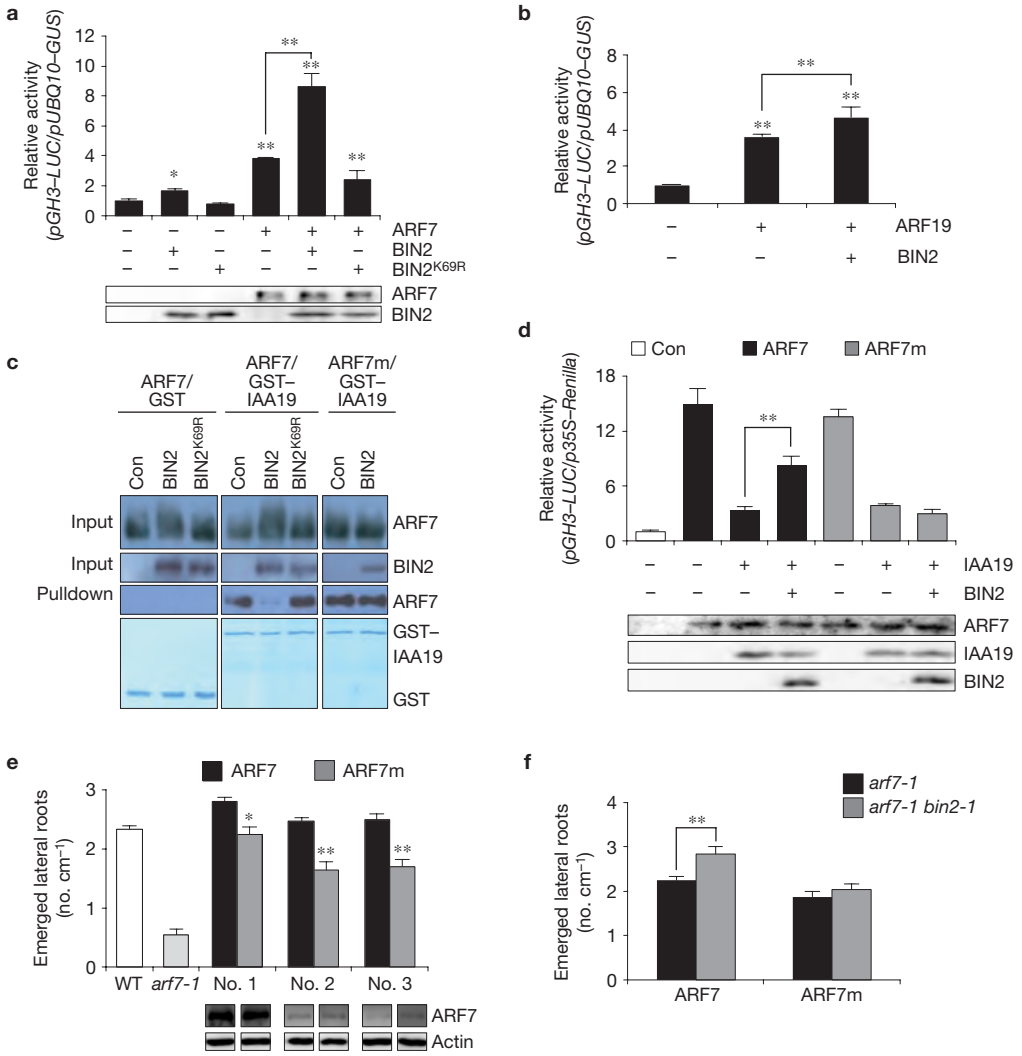

Figure 5 BIN2 attenuates the interaction of ARF7 with AUX/IAAs and enhances its transcriptional activity, leading to activation of auxin response. (**a**) BIN2 enhances the transcriptional activity of ARF7. The relative fold induction of luciferase activity was determined ($n = 4$, 20,000 cells per $n = 1$). Protein expression levels are presented (lower). (**b**) BIN2 enhances the transcriptional activity of ARF19. The relative fold induction of luciferase activity was determined ($n = 4$, 20,000 cells per $n = 1$). (**c**) BIN2-mediated phosphorylation of ARF7 represses its interaction with IAA19, but mutation of two phosphorylation sites of ARF7 (ARF7m: ARF7$^{S698A/S707A}$) blocks BIN2-mediated dissociation with IAA19. (**d**) BIN2 could not enhance the transcriptional activity of ARF7m in the presence of IAA19 in protoplasts ($n = 4$, 20,000 cells per $n = 1$). ARF7, IAA19 and BIN2 protein levels were detected using anti-HA antibody. (**e**) The LR defects of *arf7-1* are completely rescued by overexpression of *ARF7*, but partially by *ARF7m* ($n = 25$ plants). ARF7–HA and ARF7m–HA proteins in the transgenic plants are presented (lower). (**f**) The *bin2-1-gof* mutation enhanced ARF7-mediated LR formation, but not ARF7m. The densities of LRs of wild-type, *arf7-1 35S–ARF7–HA (bin2-1-gof)* and *arf7-1 35S–ARF7m–HA* (*bin2-1-gof*) are presented ($n = 42$ plants). Error bars indicate s.e.m. ($^{*}P < 0.05$, $^{**}P < 0.01$ by Student's *t*-test). Full scan images of western blots are shown in Supplementary Fig. 8. For details on the statistical analysis, see the Methods.

TDIF–TDR signalling module affects BIN2 during LR development

Our results suggested that BIN2 action is directly linked to ARF transcription factors, but the limited role of BR-mediated regulation of BIN2 in LR development suggested that other regulatory components could impact on BIN2 activity during this process. To identify other upstream regulators of BIN2-mediated LR development, we screened for BIN2-interacting proteins using a yeast two-hybrid system and identified TDR as a potential interacting partner. TDR is known to function as a receptor of TDIF (CLE41 and CLE44) peptide in vascular bundle development[35]. Yeast two-hybrid assays showed that BIN2 interacts with the kinase domain of TDR (TDR kd; Fig. 7a). Furthermore, we could pulldown full-length TDR proteins with GST-tagged BIN2 (Fig. 7b). This interaction suggested that TDR might be involved in LR development through the regulation of BIN2. To investigate this possibility, we first examined the expression pattern of *pTDR–GUS* in roots. *TDR* was expressed in the vasculature, in pericycle cells and during LR initiation, which overlapped with *BIN2* expression (Figs 1e and 7c). Consistently, a loss-of-function *tdr* mutant exhibited altered LR density compared with wild-type plants (Fig. 7d).

We then investigated whether the TDIF–TDR signalling module plays a role in BIN2-mediated LR development. The LR density of wild-type plants increased following TDIF treatment in a dosage-dependent manner (Fig. 7d). However, inhibition of BIN2 activity by bikinin

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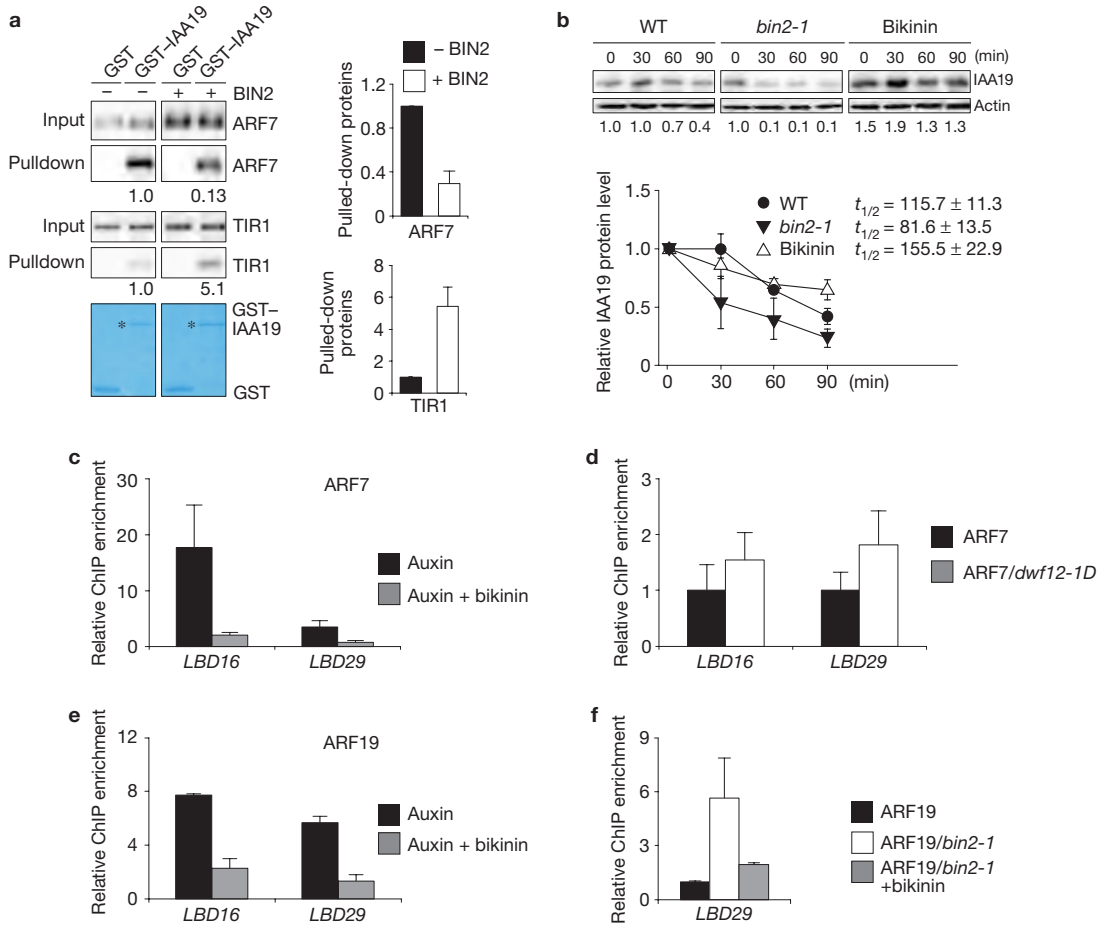

Figure 6 BIN2 increases DNA-binding capacity of ARF7 and ARF19. (**a**) BIN2-mediated phosphorylation of ARF7 results in increased binding of IAA19 with TIR1. Asterisks indicate GST–IAA19 proteins. The levels of GST–IAA19 pulled-down proteins were normalized to those of the input proteins. The normalized intensities of the pulled-down proteins in the absence of BIN2 were set to 1, and the relative protein intensities are presented under the corresponding protein bands (left panel). The average values of the relative band intensities from three independent experiments are plotted (right panel). (**b**) IAA19 proteins are rapidly degraded in *bin2-1-gof* plants but stabilized by bikinin. Wild-type (Col-0) and *bin2-1-gof* homozygote plants expressing *35S–IAA19–HA* were grown in the presence of 5 μM bikinin or mock control, and incubated for the indicated times with 100 μM cycloheximide. The levels of IAA19 proteins were determined with anti-HA antibody (upper panel). The average value of IAA19 band intensities and half-life of IAA19 proteins from three independent experiments was calculated (lower panel). ($n = 3$, 10 plants per $n = 1$). For statistics source data, see Supplementary Table 2. (**c**–**f**) BIN2 increases the binding of ARFs to the *LBD16* and *LBD29* promoters. A BIN2 inhibitor, bikinin, represses the binding of ARF7 and ARF19 to the *LBD16* and *LBD29* promoters (**c**,**e**). The *dwf12-1D-gof* and *bin2-1-gof* mutations enhance the DNA binding of ARF7 and ARF19 to the *LBD16* and *LBD29* promoter (**d**,**f**). The DNA binding of ARF7 and ARF19 was determined by quantitative RT–PCR. Error bars indicate s.e.m. ($n = 6$ for ARF7, $n = 4$ for ARF19, 200 plants per $n = 1$). The *LBD29* promoters bound to ARF19 proteins in *bin2-1-gof* were determined with/without 50 μM bikinin in the presence of 1 μM 2.4D. For source data, see Supplementary Table 2. Full scan images of western blots are shown in Supplementary Fig. 8.

treatment or a loss-of-function *tdr* mutation completely suppressed the positive effect of TDIF on LR development (Fig. 7d). TDIF treatment increased the phosphorylation of ARF7 in plants, and its functional receptor, TDR, was required for the phosphorylation. However, bikinin completely blocked TDIF-mediated phosphorylation of ARF7 (Fig. 7e). Furthermore, TDIF increased the LR density of only *arf7-1* mutants expressing wild-type *ARF7*, but not *mARF7* (Fig. 7f). Exogenous TDIF further increased LR formation in *bin2-1-gof* compared with wild-type plants, but *bin2-3-lof* rarely responded to TDIF (Fig. 7g). These results implied that TDIF–TDR signalling could affect the transcriptional activity of ARF7 by BIN2-mediated phosphorylation. To examine this idea, we determined the effects of the TDIF–TDR signalling module on ARF7-mediated activation of *pGH3–LUC*. Interestingly, exogenous TDIF or expression of *TDR* itself did not affect the *pGH3–LUC* activity, but TDIF treatment significantly promoted the reporter activity in the presence of TDR (Fig. 7h). Furthermore, ARF7-mediated reporter activation was enhanced by BIN2, and co-expression of *TDR* with TDIF treatment further increased BIN2-mediated activation of ARF7 activity. These data indicated that the TDIF–TDR module affects BIN2-mediated auxin signalling output during LR organogenesis.

DISCUSSION

Auxin is involved in nearly every aspect of plant development through a relatively simple signalling circuit in which de-repression of ARFs following auxin perception activates a transcriptional response[1]. However, it has been uncertain how auxin could provide a wide range of signalling outputs for plant growth and development. For example, several combinations of auxin co-receptor machineries, especially

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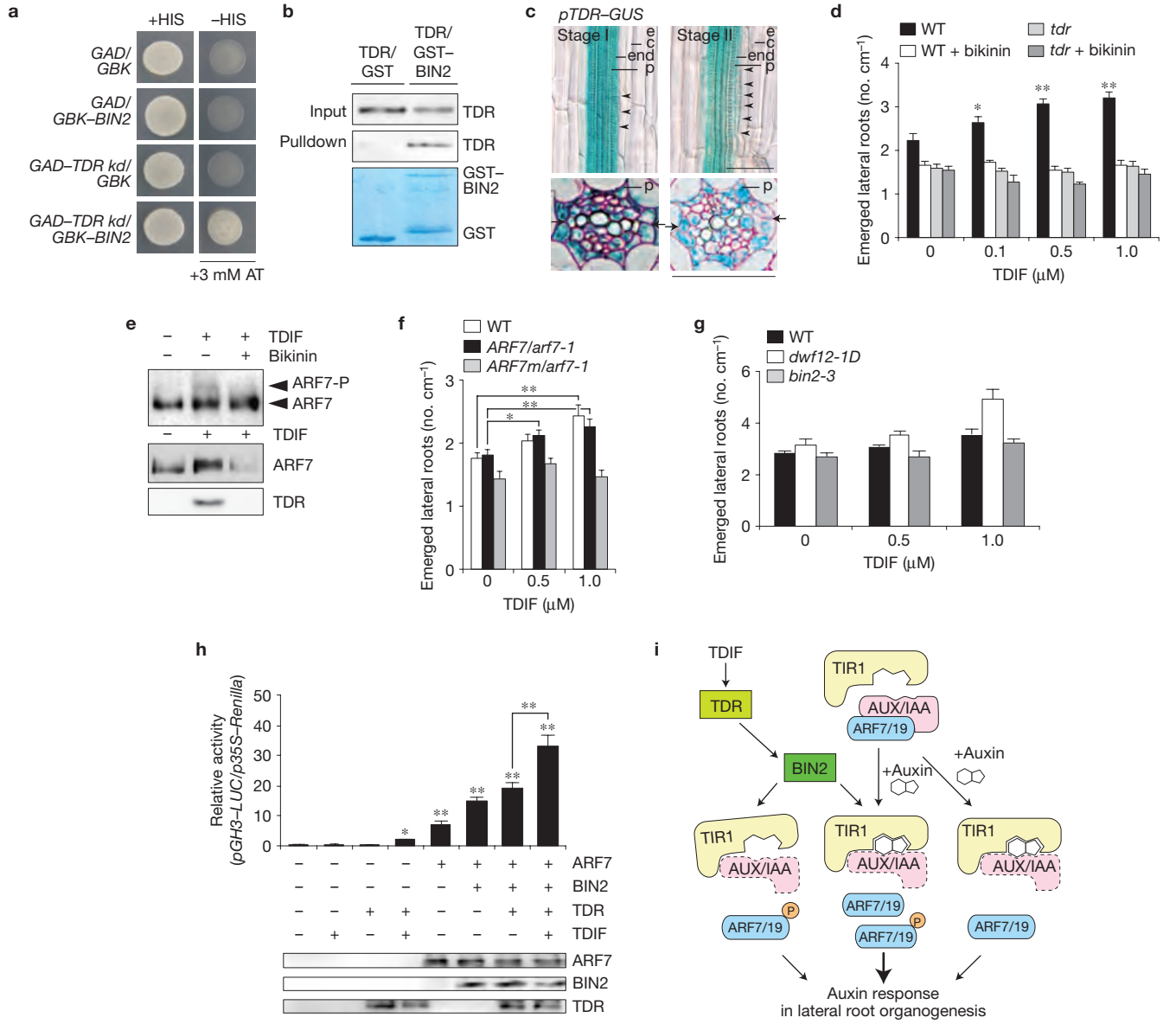

Figure 7 TDIF–TDR module is directly linked to BIN2-mediated auxin signal activation in LR development. (**a**) BIN2 interacts with the TDR kd in a yeast two-hybrid assay. (**b**) BIN2 interacts with TDR. Protoplast lysates overexpressing *TDR–HA* were incubated with purified GST–BIN2 proteins and pulled down with glutathione Sepharose 4B. An asterisk indicates GST–BIN2 proteins. (**c**) *pTDR–GUS* expression limited to LRP and vascular tissues at stage I and II (upper panel, black arrowheads indicate dividing pericycle cells) and transverse sections of *pTDR–GUS* seedlings through the LRP (lower panel, black arrows indicate xylem pole pericycle cells). e, epidermis; c, cortex; end, endodermis; p, pericycle. Scale bars, 50 μm. (**d**) TDR is positively involved in LR development in a GSK3-dependent manner. The densities of emerged LRs of seedlings treated with the indicated concentrations of TDIF with/without 5 μM bikinin ($n = 15$ plants). (**e**) TDIF induces the phosphorylation of ARF7. *35S–ARF7–HA* transgenic plants were treated with 1 μM TDIF in the absence or presence of 5 μM bikinin (upper panel). HA-tagged *ARF7* was co-transfected into protoplasts with or without *TDR–MYC*. After 5 h of transfection, the protoplasts were further incubated with 1 μM TDIF for 1 h (lower panel). (**f**) TDIF increases LR development by BIN2-mediated phosphorylation of ARF7. The densities of the emerged LRs of indicated genotypes are presented ($n = 15$ plants). (**g**) The densities of emerged LRs of wild-type (Ws-2), *dwf12-1D-gof* (+/+) and *bin2-3-lof* treated with the indicated concentrations of TDIF ($n = 15$ plants). (**h**) TDIF–TDR–BIN2 cascade enhances the transcriptional activity of ARF7 in protoplasts ($n = 4$, 20,000 cells per $n = 1$). ARF7, BIN2 and TDR protein levels were detected using anti-HA or anti-MYC antibody. Error bars indicate s.e.m. ($^{*}P < 0.05$, $^{**}P < 0.01$ by Student's *t*-test). (**i**) A model for the TDIF–TDR–BIN2-mediated regulatory module of auxin signal transduction. Full scan images of western blots are shown in Supplementary Fig. 8. For details on the statistical analysis, see the Methods.

between TIR1/AFB receptors and AUX/IAA repressors, could modulate auxin sensitivity[6]. Our study revealed another regulatory module in auxin signalling that involves phosphorylation-dependent regulation of transcriptional activity of ARFs during LR development through the TDIF–TDR–BIN2 signalling cascade.

BIN2 associated with the TDIF–TDR module positively regulates auxin-mediated LR development by phosphorylating ARF7 and ARF19. This alleviates the interaction between these ARFs and AUX/IAAs, facilitates SCF^{TIR1}-mediated degradation of AUX/IAAs, and eventually enhances transcriptional activity of ARFs, possibly by preventing

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the reformation of an ARF–AUX/IAA complex and consequently increasing the pool of free ARF proteins. BIN2 phosphorylates ARF2 *in vitro*[23] and ARF7 *in vivo* (Fig. 4e), but the two identified phosphorylation residues in ARF7 are not conserved in ARF2 and other ARF transcriptional activators, although ARF proteins do harbour many putative GSK3 phosphorylation sites. Interestingly, BIN2-mediated phosphorylation of ARF7 and ARF19 enhances their DNA-binding capacity (Fig. 6), whereas—in contrast—ARF2 phosphorylation reduces its DNA-binding and repressor activity[23]. The regulation of ARF DNA binding by its phosphorylation is likely to be a critical regulatory mechanism for diversity and specificity of auxin signalling during plant growth and development.

BR and auxin interact synergistically during various physiological responses[36]. However, this study revealed that BIN2, a negative regulator in BR signalling, positively regulates auxin signalling during LR development. BRI1, a major BR receptor, is involved in BR-mediated activation of LR development, but the canonical BR signalling output determined by BSU1, a BIN2 repressor, and downstream components BES1 and BZR1 are probably not involved in LR development (Fig. 2). It is plausible that a not-yet identified signalling pathway bifurcated from BRI1-mediated BR signalling enhances auxin transport, leading to stimulation of LR development[37]. Furthermore, BR did not affect BIN2-induced phosphorylation or transcriptional activity of ARF7, but a GSK3 inhibitor suppressed BIN2-mediated activation of auxin signalling (Supplementary Fig. 7). These data demonstrate that BR probably plays a minor role, if any, in the regulation of BIN2 activity during LR development. Importantly, the group II GSK3 proteins seem to have a functional redundancy in BR signalling pathways[26], but only BIN2 and BIL1 showed positive roles in ARF7- and ARF19-mediated LR development (Supplementary Fig. 3), which suggests specificity of GSK3 proteins in diverse developmental processes.

In *Arabidopsis*, cell–cell regulation through small secreted peptides and their receptors is essential for various developmental processes[38]. For example, TDIF–TDR controls xylem differentiation and proliferation of procambium for vascular bundle development[35], and TDIF and its related peptide, CLE42, are also involved in axillary meristem formation and bud outgrowth[39]. Here, we showed that the TDIF–TDR signalling module also regulates LR development through BIN2-mediated control of auxin signalling. Interestingly, *TDIF* is mainly expressed in the phloem and its expression is activated by auxin[40]. This expression pattern suggests that auxin-induced TDIF peptides in the phloem move to the pericycle and stimulate the TDR–BIN2 module, reinforcing auxin signalling for positioning and development of LRs.

Taken together, these events triggered by TDIF–TDR–BIN2 signalling cues could increase the pool of active ARF7 and ARF19, in harmony with auxin-induced degradation of IAA proteins, strengthening the signalling output of auxin during LR organogenesis (Fig. 7i). This peptide-initiated phosphorylation-mediated control of transcriptional activity of ARFs provides a broad spectrum of auxin signalling outputs to specific cells with a graded distribution of auxin during various development processes. Our results also suggest that a TDIF–TDR–GSK3-mediated regulatory module of ARFs is an important node for connecting diverse signalling networks in auxin-driven plant growth and development. □

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Supplementary Information is available in the online version of the paper

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AUTHOR CONTRIBUTIONS

H.C., H.R., S.H., J.P., K.H. and S.S. performed experiments. H.C., H.R., I.D.S. and I.H. designed experiments and analysed the data. D.A. analysed gene expression. S.R. and D.H. performed LC–MS/MS analysis. H.C., H.R., T.B., M.J.B., I.D.S. and I.H. wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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METHODS

Plant materials and growth conditions. *Arabidopsis thaliana* ecotypes Col-0 and Ws-2 were used as wild-type controls and as the genetic backgrounds of the transgenic lines. The wild-type and mutant plants were grown in an environmentally controlled growth room (23 °C, 16-h light/8-h dark). *dwf12-1D, bri1, bzr1-1D* (S. Choe, Seoul National University, Korea), *pDR5–GUS* (H-T. Cho, Seoul National University, Korea), *bin2-3* (J. Chory, Salk Institute, La Jolla, USA), *pARF7–GUS* and *pARF19-GUS* (H. Fukaki, Kobe University, Japan), *tdr-1* (H. Fukuda, Tokyo University, Japan), *bin2-1, pBRI1-BRI1-GFP* (K. H. Nam, Sookmyung Women's University, Korea) and *bin2-3 bil1 bil2* (J. Li, University of Michigan, Ann Arbor, USA) were provided. *tir1-1* (CS3798), *arf7-1* (CS24607) and *arf7-1 arf19-1* (CS24625) were provided by ABRC. The *bil1* null mutant was provided by INRA (FLAG_426B01). All double and triple mutants were obtained by genetic crossing and confirmed by quantitative PCR with reverse transcription (RT–PCR) and DNA sequencing.

Quantitative RT–PCR analysis. Total RNA was isolated using Trizol reagent (Invitrogen). Double-stranded cDNA was synthesized from 1 μg RNA using oligonucleotide dT primers and ImProm-II reverse transcriptase (Promega). Quantitative real-time PCR was performed with gene-specific primers according to the instructions provided for the Light Cycler 2.0 (Roche) and the SYBR Premix Ex Taq system (Takara). To confirm T-DNA insertion knockout lines, semi-quantitative RT–PCR was performed with gene-specific primer sets (primers listed in Supplementary Table 1).

Plasmid constructs and protoplast transient expression assay. The full-length cDNAs of *BIN2*, *BIL1*, *BIL2*, *ARF7, ARF19, TIR1, TDR* and *IAA19* were cloned into plant expression vectors that contained haemagglutinin (HA) and the expression of these genes was driven by the *35S C4PPDK* promoter[31]. All point mutants of *BIN2* (*bin2-1*/*BIN2*^{E263K}, *BIN2*^{K69R}) and ARF7 (ARF7$^{S698/707A}$) were generated using the QuikChange Site-Directed Mutagenesis kit (Stratagene) according to the manufacturer's instructions. For GST-fused recombinant proteins, cDNAs of *BIN2, IAA1, IAA3, IAA14, IAA18* and *IAA19* were cloned into *pGEX 5X-1* (Promega). All cDNAs and the mutations were confirmed by DNA sequencing. For the reporter assay, 2×10^4 protoplasts were transfected with 20 μg of total plasmid DNA composed of different combinations of the reporter (*pGH3–LUC*), effectors (*ARF7–HA*, *ARF7m–HA*, *ARF19–HA*, *BIN2–HA*, *BIN2*^{E263K}*–HA*, *BIN2*^{K69R}*–HA*, *BIL1–HA, TDR–MYC* or *BIL2–HA*), and an internal control (*pUBQ10–GUS* or *p35S–Renilla*). The transfected protoplasts were then incubated at 1×10^4 cells per millilitre with or without 1 μM IAA for 3 h in the presence or absence of 1 μM TDIF. For protein expression and pulldown assays, 4×10^4 protoplasts were transfected with 40 μg of plasmid DNA composed of different combinations and incubated for 6 h at room temperature. The proteins from protoplasts were analysed by 7.5%, or 10% SDS–PAGE and visualized with anti-HA (1:2,000, Roche catalogue no. 12013819001) or anti-MYC (1:1,000, Cell Signaling catalogue no. 2276) antibody. All assays were conducted a minimum of three times and similar results were obtained in all experiments.

Physiological analysis and transgenic plants. For the analysis of LR formation, wild-type (Col-0 or Ws-2), *bin2-1*, *dwf12-1D*, *bri1*, *bri1-5, bin2-3, bil1, bil1 bin2-3, bin2-3 bil1 bil2*, *tdr1-1*, *arf7-1 bin2-1*, *arf7-1 arf19-1 bin2-1*, *tir1-1 bin2-1*, *bri1-5 35S–gBIN2–HA*, *35S–BSU1–HA, pBES1–bes1-D–HA, pBRI1–BRI1–GFP, bzr1-1D* and *msg2-1 bin2-1* progeny of heterozygous *dwf12-1D* or *bin2-1* mutants were vertically grown on 1/2 B5 medium containing 1% sucrose and 1.2% agar type-M (Sigma) for 12 days at 23 °C with a 16-h light/8-h dark photoperiod. The number of emerged LRs and primordial cells of the seedlings were counted using differential interference contrast microscopy (Axioplan 2, Carl Zeiss) after clearing with 90% lactic acid. For auxin response assays in primary root growth or LR formation, 7-day-old Col-0, Ws-2, *bin2-1*, *dwf12-1D, bin2-3,* and *bin2-3 bil1 bil2* seedlings were transferred to 1/2 B5 media containing 0, 30 and 60 nM, or 0, 100, 500 and 1,000 nM of IAA for 4–5 days. To test the effect of bikinin (4-(5-bromopyridin-2-yl)amino-4-oxobutanoic acid) (ChemBridge Corporation) on primary root growth and LR development, seedlings were grown on 1/2 B5 media containing 5 μM bikinin for 12 days. For gene expression analysis, 5-day-old seedlings grown in MS media were transferred to MS media containing 12.5 μM bikinin for 6 h. For the TDIF response assay in LR formation, 12-day-old Col-0, *tdr1-1*, *bin2-3* and *dwf12-1D* seedlings were grown on 1/2 B5 media containing 0, 0.1, 0.5 or 1 μM TDIF with or without 5 μM bikinin. The 12-day-old Col-0, *35S–ARF7–HA* and *35S–ARF7m–HA* seedlings were grown on 1/2 B5 media containing 0, 0.5 or 1 μM TDIF. To generate transgenic plants overexpressing *ARF7–HA* or *IAA19–HA*, the coding sequences were cloned into the *pCB302ES* vector containing the 35*S* promoter and HA epitope tag. The constructs for *ARF7–HA, ARF7m–HA* and *IAA19–HA* were transformed into the heterozygous *dwf12-1D* and *bin2-1* mutant using the *Agrobacterium*-mediated floral dipping method, respectively. The expression levels of ARF7 or IAA19 were verified by immunoblotting with a horseradish peroxidase (HRP)-conjugated high-affinity anti-HA antibody (1:2,000, Roche catalogue no. 12013819001).

Histochemical GUS assays. Transgenic *Arabidopsis* harbouring *β-glucuronidase* (*GUS*) fused to the 3.4-kb *Arabidopsis BIN2* promoter[17], 2.0-kb *TDR* promoter or synthetic *DR5* promoter were used for histochemical GUS assays. The *pDR5–GUS / bin2-1* plant was generated by genetic crossing. For determination of *BIN2* or *TDR* expression, 7-day-old seedlings were stained with GUS-staining buffer (100 mM Tris-HCl (pH 7.0), 2 mM ferricyanide and 1 mM X-Gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronidase)) for 6 h. For determination of *pDR5–GUS* expression, seedlings were grown for 72 h on 1/2 B5 medium containing 1% sucrose, 1.2% agar type-M (Sigma) and 10 μM NPA. The seedlings were transferred to 1/2 B5 medium containing 1 μM NAA for an additional 1, 3 and 5 h, and then stained with the GUS-staining solution. For quantification of GUS activity, seedlings were homogenized with extraction buffer (150 mM sodium phosphate butter (pH 7.0), 10 mM EDTA, 10 mM β-mercaptoethanol, 0.1% Triton X-100 and 140 μM phenylmethylsulphonyl fluoride) for 5 min at room temperature. Ten microlitres of extract was incubated with 130 μl of 2 mM MUG (4-methylumbelliferyl-β-D-glucuronide) in the extraction buffer for 20 min. The reaction was terminated with 200 μl of 200 mM sodium carbonate. The fluorescence was detected with a plate reader (Perkin-Elmer) with excitation/emission at 355 nm/460 nm. For microscopic analysis of the GUS-stained seedlings, the samples were treated with 0.24 N HCl in 20% methanol for 15 min at 57 °C and replaced with 7% NaOH and 7% hydroxylamine-HCl (Sigma) in 60% ethanol for 15 min. Seedlings were dehydrated for 10 min each in 40%, 20% and 10% ethanol, and analysed by differential interference contrast microscopy (Axioplan 2, Carl Zeiss). For anatomical analysis of GUS-stained roots, stained samples were fixed for 24 h with 3% glutaraldehyde and 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2). The fixed roots were counter-stained with 0.05% ruthenium red for 30 min and rinsed twice with 0.1 M phosphate buffer (pH 7.2). The samples were dehydrated for 1 h each in 40, 50, 60, 70, 80, 90 and 100% acetone. The specimens were infiltrated and embedded in Spurr's resin (Ted Pella) for 48 h at 65 °C using a two-step method[41]. Sections (2 μm) were cut with a MT-X ultramicrotome (RMC), mounted in 50% glycerol, and observed with a microscope (Axioplan2).

***In vivo* root confocal imaging.** DII–VENUS seedlings were grown vertically on sugar-free 1/2 MS media in 24 h light at 21 °C until 5–6 days after germination. Seedlings were transferred to 1/2 MS containing 15 μM bikinin or dimethylsulphoxide as a control and incubated overnight. Seedlings were mounted on glass slides and imaged using a Leica SP5 confocal microscope (Leica; a 514 nm detector using gain value 100%, offset value 28.98, averaged over 4 frames). Static images were taken of root tips and mature sections of primary root for each treatment. Fluorescence was quantified as the raw integrated density value, measured using FIJI software[42].

Protein–protein interaction, immunoblotting and *in vitro* kinase assays. For interaction between BIN2 and TDR kd (TDR kinase domain) in yeast cells, the yeast strain AH109 was transformed with *pGBKT7–BIN2* and *pGADT7–TDR*-kd by the LiAc method. Transformed yeasts were grown on synthetic medium lacking Leu, Trp and His containing 3 mM 3-aminotriazole or medium lacking Leu and Trp. For purification of GST-fused recombinant proteins, *GST–BIN2, GST–IAA1, GST–IAA3, GST–IAA14, GST–IAA18, GST–IAA19* or *d2-ARF7* (encoding the QSL-rich C-terminal domain of ARF7) were expressed in *Escherichia coli* BL21. Bacterial cells were grown in 200 ml of Luria broth medium at 37 °C until $D_{600\ nm} = 0.8$ and further incubated with 0.5 mM IPTG (isopropyl β-D-1-thiogalactopyranoide) for 3 h. The recombinant GST-tagged proteins were purified according to the manufacturer's protocol (GE Healthcare). For pulldown assays, HA-tagged *ARF7*, *TDR* or *ARF19* was transfected into protoplasts. Protoplasts were then incubated for 6 h to permit transgene expression. The transfected protoplasts were lysed using immunoprecipitation buffer (50 mM Tris-HCl (pH 7.5), 75 mM NaCl, 5 mM EDTA, 1 mM dithiothreitol, 1 × protease inhibitor cocktail (Roche) and 1% Triton X-100). Total protoplast lysates were incubated with 1 μg of recombinant GST or GST–BIN2 proteins for 1 h. To determine the binding affinities between phosphorylated ARF7 and AUX/IAAs, *ARF7–HA* was co-transfected with or without either *BIN2–HA* or *BIN2* ^{K69R}*–HA* into protoplasts. After 6 h of incubation, total lysates from the transfected protoplasts were incubated with 1 μg of GST or 100 ng GST–IAA1, GST–IAA3, GST–IAA14, GST–IAA18 or GST–IAA19 for 1 h in the presence of 10 μM MG132. For monitoring TIR1/ARF7/IAA19 triple complex formation, *ARF7–HA* and *TIR1–HA* were co-transfected into protoplasts with or without *BIN2–HA*. Protoplast lysates were incubated with 1 μg of GST or 100 ng of GST–IAA19 for 1 h in the presence of 10 μM MG132. The lysates were then precipitated with glutathione Sepharose 4B. The precipitated proteins were detected using a HRP-conjugated high-affinity anti-HA antibody. Calf intestine alkaline phosphatase treatment was performed in immunoprecipitation buffer for 20 min

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at 37 °C. For immunoblotting analyses, 3–20 μg of proteins from protoplasts or seedlings was analysed by 7.5% (or 10%) SDS–PAGE and visualized with HRP-conjugated high-affinity anti-HA antibody. For determination of IAA19 protein half-life, wild-type (Col-0) and *bin2-1* homozygote plants expressing *35S–IAA19–HA* were grown in 1/2 B5 media containing dimethylsulphoxide or 5 μM bikinin, and incubated for the indicated times with 100 μM cycloheximide. The proteins from seedlings were analysed by 10% SDS–PAGE and visualized with anti-HA or anti-actin (1:1,000, MP biomedical catalogue no. 69100) antibody. The half-life was calculated by a regression analysis[43]. For the *in vitro* kinase assay, typically, 10 μg of GST–ARF7 QSL-rich C-terminal domain was incubated with or without 5 μg of GST–BIN2 in a kinase buffer (20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 12 mM $MgCl_2$, 100 μM ATP and 10 μCi of [γ-^{32}P]ATP). After incubation for 2 h at 37 °C, the proteins were subjected to 10% SDS–PAGE and the phosphorylated proteins were visualized by autoradiography.

Chromatin immunoprecipitation. Two-week-old *35S–ARF7–GFP/arf7-1* seedlings (5 g) were treated with 1 μM NAA and 50 μM bikinin for 4 h. Two-week-old *35S–ARF7–HA* and *35S–ARF7–HA/dwf12-1D* (5 g) were also used for ChIP assay. ChIP assays for ARF19 were performed with Col-0 or *bin2-1-gof* using anti-ARF19 antibody after 2 h of 1 μM 2.4D treatment with or without 50 μM bikinin. The seedlings were crosslinked with 1% formaldehyde for 10 min and quenched with 125 mM glycine. The ground tissues were resuspended with nuclei isolation buffer (0.25 M sucrose, 15 mM PIPES (pH 6.8), 5 mM $MgCl_2$, 60 mM KCl, 15 mM NaCl, 1 mM $CaCl_2$, 1% Triton X-100 and 1 mM phenylmethylsulphonyl fluoride) for 30 min at 4 °C. The nuclear pellets were resuspended in nuclei lysis buffer (50 mM HEPES (pH 7.0), 150 mM NaCl, 1 mM EDTA, 1% SDS, 1% Triton X-100, 1 mM phenylmethylsulphonyl fluoride and 1 X Protease inhibitor cocktail for plant cell and tissue extracts (Sigma)) and sonicated to produce ~0.5–1 kb DNA fragments. After sonication, chromatin fractions were diluted tenfold with nuclei lysis buffer and pre-cleared with protein G agarose/salmon sperm DNA (Millipore) for 1 h at 4 °C. The protein–DNA complexes were immunoprecipitated with anti-GFP antibody (1:1,000, Santa-Cruz Biotech catalogue no. sc-8334), anti-HA antibody (1:1,000, Abcam catalogue no. ab9110) or 1–3 μg of anti-ARF19 antibody (1:1,000, home-generated) overnight at 4 °C and further incubated with protein G agarose/salmon sperm DNA for 1 h at 4 °C. After washing with low salt buffer (150 mM NaCl, 20 mM Tris-HCl (pH 8.0), 0.2% SDS, 0.5% Triton X-100 and 2 mM EDTA) for 5 min at 4 °C and high-salt buffer (500 mM NaCl, 20 mM Tris-HCl (pH 8.0), 0.2% SDS, 0.5% Triton X-100 and 2 mM EDTA) for 5 min at 4 °C, the immunocomplexes were eluted twice by elution buffer (0.5% SDS and 0.1 M $NaHCO_3$) and then reverse crosslinked with 200 mM NaCl for 6 h at 65 °C. After removing proteins with proteinase K, DNAs were purified by phenol–chloroform extraction and recovered by ethanol precipitation. Precipitated DNAs were resuspended with TE buffer (10 mM Tris-HCl (pH 8.0) and 1 mM EDTA) and used as PCR templates (primers listed in Supplementary Table 1).

Identification of phosphorylation sites. Recombinant GST–tARF7 proteins phosphorylated by GST–BIN2 for 12 h *in vitro* were subjected to in-solution digestion. The proteins were resolved in a digestion solution (6 M urea and 40 mM ammonium bicarbonate dissolved in HPLC-grade water). Protein reduction was performed with 5 mM dithiothreitol for 30 min, followed by an alkylation step with 25 mM iodoacetamide in the dark for 30 min at room temperature. The sample was digested with sequencing-grade modified trypsin (Promega) overnight at 37 °C. The digested proteins were collected and desalted with the C-18 spin column (Thermo). Phosphopeptides were enriched with the SwellGel gallium-chelated disc (Pierce) according to the manufacturer's instructions. Phosphopeptides were analysed on a linear ion trap LTQ XL mass spectrometer (Thermo) interfaced with a nano-electrospray ion source. Chromatographic separation of peptides was achieved using a NanoLC• 2D system (Eksigent), equipped with a capillary column (a 10 cm PicoTip emitter, 75 μm inner diameter) packed in-house with Magic C18AQ 5 μ 200 Å particles (Michrom BioResources). Peptides mixtures were loaded from an Eksigent auto sampler and separated using a linear gradient of ACN/water (2–40% ACN in 40 min), containing 0.1% formic acid, at a flow rate of 260 nl min^{-1}. A full MS scan was performed in the range of 300–2,000 m/z, and a data-dependent MS/MS (MS^2) scan on the four most intense precursor ions. The MS/MS spectra were searched against the TAIR 10 protein sequence database using SEQUEST algorithm in the Proteome Discoverer software (Thermo) with the following parameters: semi-trypsin, a mass tolerance of 2.0 and 1.0 Da for precursor and fragment ions, respectively, carbamidomethylation of cysteine (+57.021) as a fixed modification, and oxidation of methionine (+15.995) and phosphorylation of serine, threonine and tyrosine (+79.966) as variable modifications. The phosphorylated peptides were identified using XCorr cutoff scores of 1.5, 2.0 and 2.5 for singly, doubly and triply charged peptides, respectively. All phosphorylation sites were confirmed by manual interpretation.

Statistical analysis. Statistical analysis was performed using Student's t-test with a two-tailed distribution. No statistical method was used to predetermine sample sizes. No samples have been excluded. The experiments were not randomized. The investigators were not blinded to allocation during experiments and outcome assessment. To determine the appropriate statistical tests, the data were tested for normal distribution.

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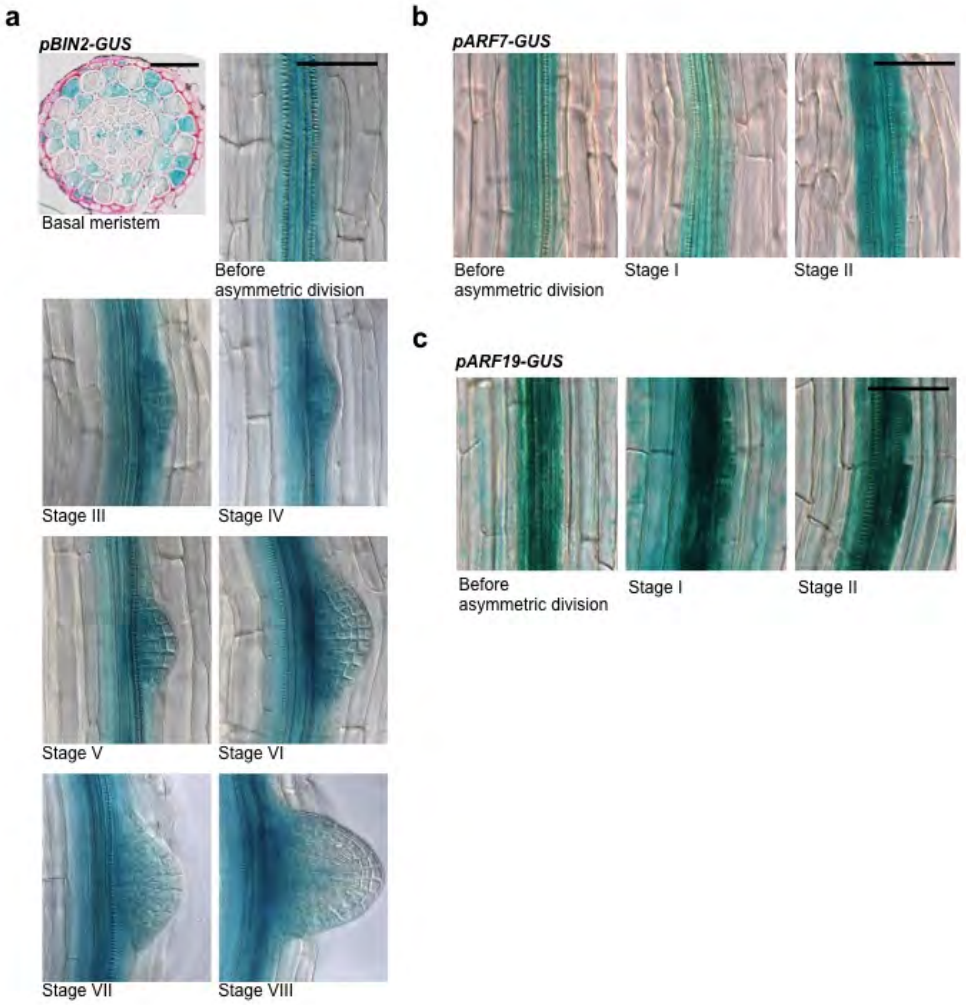

Supplementary Figure 1 The expression pattern of *BIN2, ARF7* and *ARF19* in root. (**a**) *BIN2* expression in basal meristem and LRP at different stages of lateral root development. Note that the expression is restricted to the basal part of LRP at stages IV-VII. Scale bar, 50 μm. (**b, c**) *ARF7* (**b**) and *ARF19* (**c**) expression in LRP at early stages of lateral root development. GUS activity driven by the *ARF7* or *ARF19* promoter was observed in early stages of LRP. Scale bar, 50 μm.

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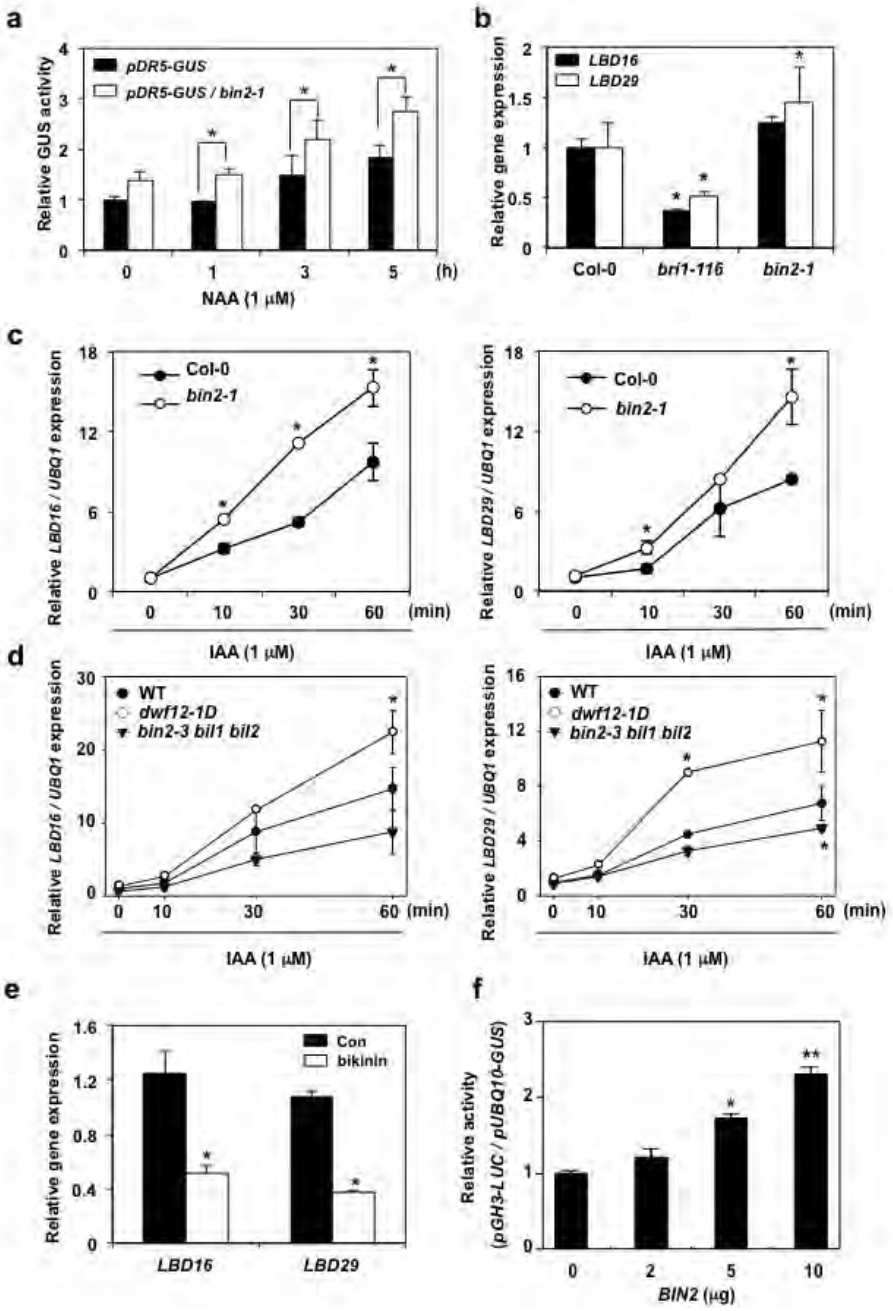

Supplementary Figure 2 BIN2 enhances auxin-response in plants. (**a**) Three-day-old seedlings of *pDR5-GUS* and *pDR5-GUS / bin2-1-gof* grown on 10 μM NPA-containing medium were transferred to 1 μM NAA-containing medium, and further treated for 1, 3, and 5 h. The GUS activity of the seedlings was measured. Error bars indicate SEM (n=3, 20 plants per n=1, * for p<0.05 by student's t-test). (**b**) The expression levels of *LBD16* and *LBD29* in 10-day-old *bri1-116* and *bin2-1-gof* (+/+) mutants were determined by quantitative RT-PCR (n=4, 10 plants per n=1, * for p<0.05 by student's t-tests). (**c, d**) The expression levels of *LBD16* (left panel) and *LBD29* (right panel) in 10-day-old WT and *bin2-1-gof* (**c**), *dwf12-1D-gof* (**d**), and *bin2-3 bil1 bil2* mutants were determined by quantitative RT-PCR after auxin treatment for the indicated times. Error bars indicate SEM (n=3, 10 plants per n=1, * for p<0.05 by student's t-tests). (**e**) Bikinin treatment represses *LBD16* and *LBD29* expression. Error bars indicate SEM (n=3, 10 plants per n=1, * for p<0.05 by student's t-tests). (**f**) BIN2 enhances the activity of *pGH3-LUC* auxin-responsive reporter in a dose-dependent manner. Protoplasts were co-transfected with *pGH3-LUC*, indicated amount of *BIN2-HA,* and *pUBQ10-GUS* (internal control). Error bars indicate the SEM (n = 4, 20,000 cells per n=1, * for p<0.05, ** for p<0.01 by student's t-test). Statistics source data found in Supplementary Table 2. Details on the statistical analysis are found in Methods.

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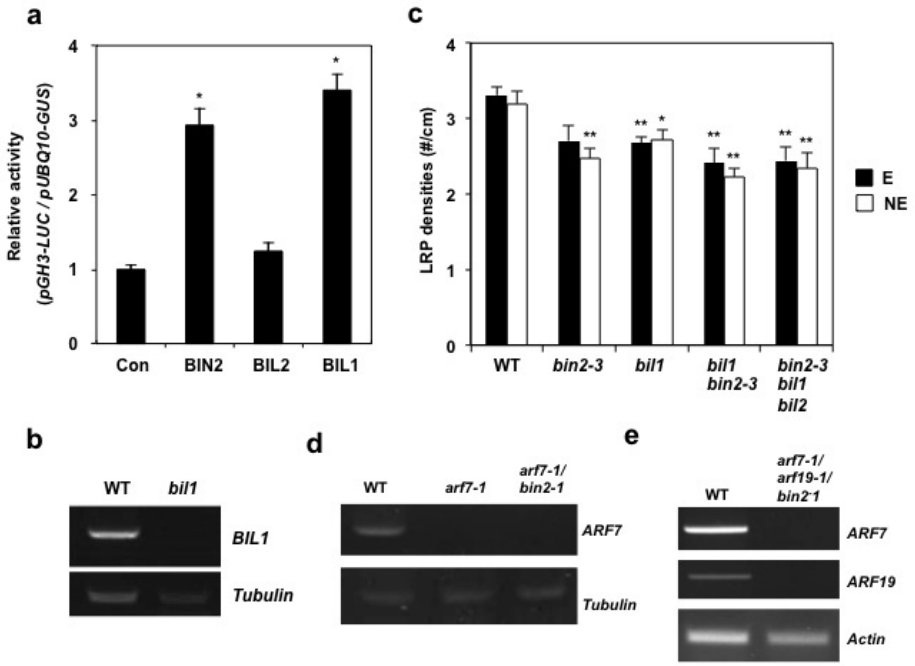

Supplementary Figure 3 BIN2 and BIL1 are involved in lateral root formation. (**a**) BIN2 and BIL1 enhance auxin-responsive *pGH3-LUC* activity. Protoplasts were cotransfected with *pUBQ10-GUS* (internal control), *pGH3-LUC* and an effector plasmid harboring *BIN2, BIL1* or *BIL2*. Error bars indicate SEM (n = 4, 20,000 cells per n=1, * for p<0.05 by student's t-test). (**b**) Isolation of *bil1* knockout mutant. The transcripts of *BIL1* in 7-day-old wild type (Ws-2) and *bil1* (FLAG_426B01) were determined by semi-quantitative PCR. (**c**) Lateral root densities of twelve-day-old seedlings of WT (Ws-2), *bin2-3-lof*, *bil1*, *bil1 bin2-3* and *bin2-3 bil1 bil2* mutant. E, emerged lateral roots; NE, non-emerged LRP. Error bars indicate SEM (n=15 plants, * for p<0.05, ** for p<0.01 by student's t-test). (**d**) The transcripts of *ARF7* in WT (Col-0), *arf7-1* and *arf7-1 bin2-1-gof* were determined by semi-quantitative PCR. (**e**) The transcripts of *ARF7* and *ARF19* in WT (Col-0) and *arf7-1 arf19-1 bin2-1-gof* were determined by semi-quantitative PCR. *Tubulin* and *Actin2* genes served as input controls. Details on the statistical analysis are found in Methods.

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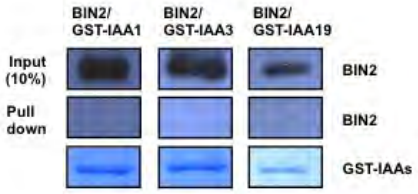

Supplementary Figure 4 BIN2 does not interact with AUX/IAA proteins. HA-tagged *BIN2* was expressed in protoplasts. Protoplast lysates were incubated with GST-IAA1, GST-IAA3, or GST-IAA19 proteins for 1 h, and then precipitated with glutathione sepharose 4B. BIN2 proteins were detected with anti-HA antibody (upper and middle panels), and GST-tagged IAA1, IAA3, or IAA19 proteins were visualized by Coomassie blue staining (lower panel).

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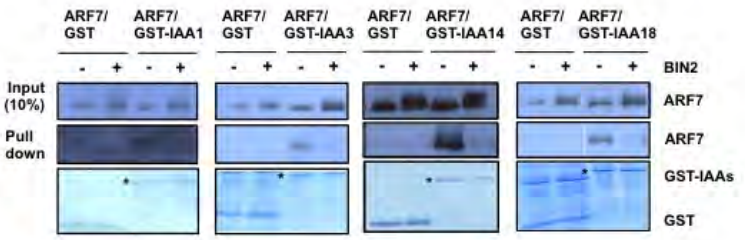

Supplementary Figure 5 BIN2-mediated phosphorylation of ARF7 attenuates its interaction with IAA1, IAA3, IAA14 and IAA18. Protoplast lysates overexpressing *ARF7-HA* or *ARF7-HA* and *BIN2-HA* were incubated with purified GST-tagged IAA1, IAA3, IAA14, or IAA18 proteins and pulled down with glutathione sepharose 4B. The pulled-down proteins were analyzed with anti-HA antibody. The GST protein was used as a negative control. An asterisk indicates GST-IAAs proteins.

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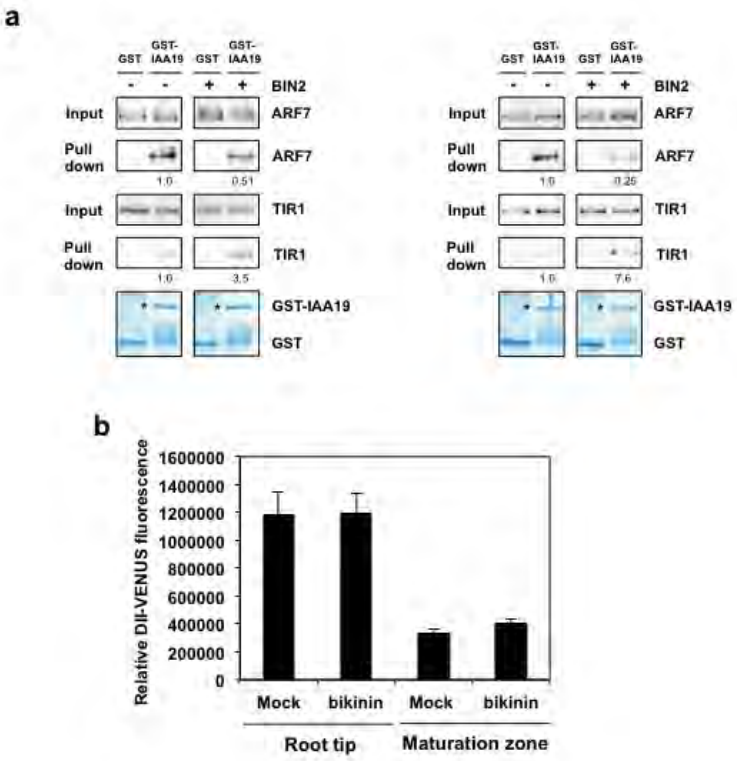

Supplementary Figure 6 (**a**) BIN2-mediated phosphorylation of ARF7 results in increased binding of IAA19 with TIR1. Two more independent pull down experiments conducted in Fig. 6a are presented. HA-tagged *ARF7* and *TIR1* were co-transfected into protoplasts with or without *BIN2-HA*. After 6 h of incubation, protoplast lysates were incubated with purified GST-IAA19 proteins and 10 μM of MG132 for 1 h, and then pulled down with glutathione sepharose 4B. GST-IAA19-bound proteins were visualized with anti-HA antibody. GST protein was included as a negative control. Asterisks indicate GST-IAA19 proteins. The levels of GST-IAA19 pulled-down proteins were normalized to those of the input proteins. The normalized intensities of the pulled-down proteins in the absence of BIN2 were set to 1, and the relative protein intensities were presented under the corresponding protein bands. (**b**) Relative DII-VENUS intensities in seedlings of mock or 15 μM bikinin treated WT (Col-0). (n=20 plants).

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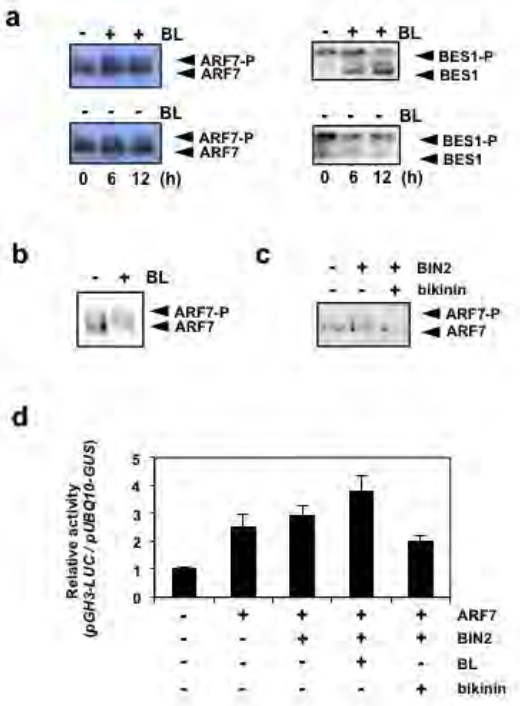

Supplementary Figure 7 Brassinosteroid is not involved in BIN2-mediated ARF7 activation. (**a, b**) Brassinosteroid does not affect the phosphorylation status of ARF7. Seven-day-old transgenic seedlings harboring *35S-ARF7-HA* (left panel), *35S-ARF7-HA / dwf12-1D-gof* (**b**), or *35S-BES1-HA* (a positive BR-responsive control, right panel) were incubated with or without 20 nM of epi-BL for 6 and 12 h. The phosphorylation status of HA-tagged ARF7 and BES1 was determined as the protein mobility shift with anti-HA antibody. (**c**) Bikinin inhibits BIN2-induced phosphorylation of ARF7. *ARF7-HA* was co-transfected with *BIN2-HA* into protoplasts and incubated with or without 30 μM of bikinin for 6 h. ARF7 proteins were visualized with anti-HA antibody. (**d**) Brassinosteroid could not suppress the BIN2 actions for enhancing the transcriptional activity of ARF7. *ARF7-HA, pGH3-LUC* and *pUBQ10-GUS* were cotransfected into protoplasts with or without *BIN2-HA.* The protoplasts were incubated with or without 1 μM epi-BL and 30 μM bikinin for 6 h. Error bars indicate SEM (n=4, 20,000 cells per n=1).

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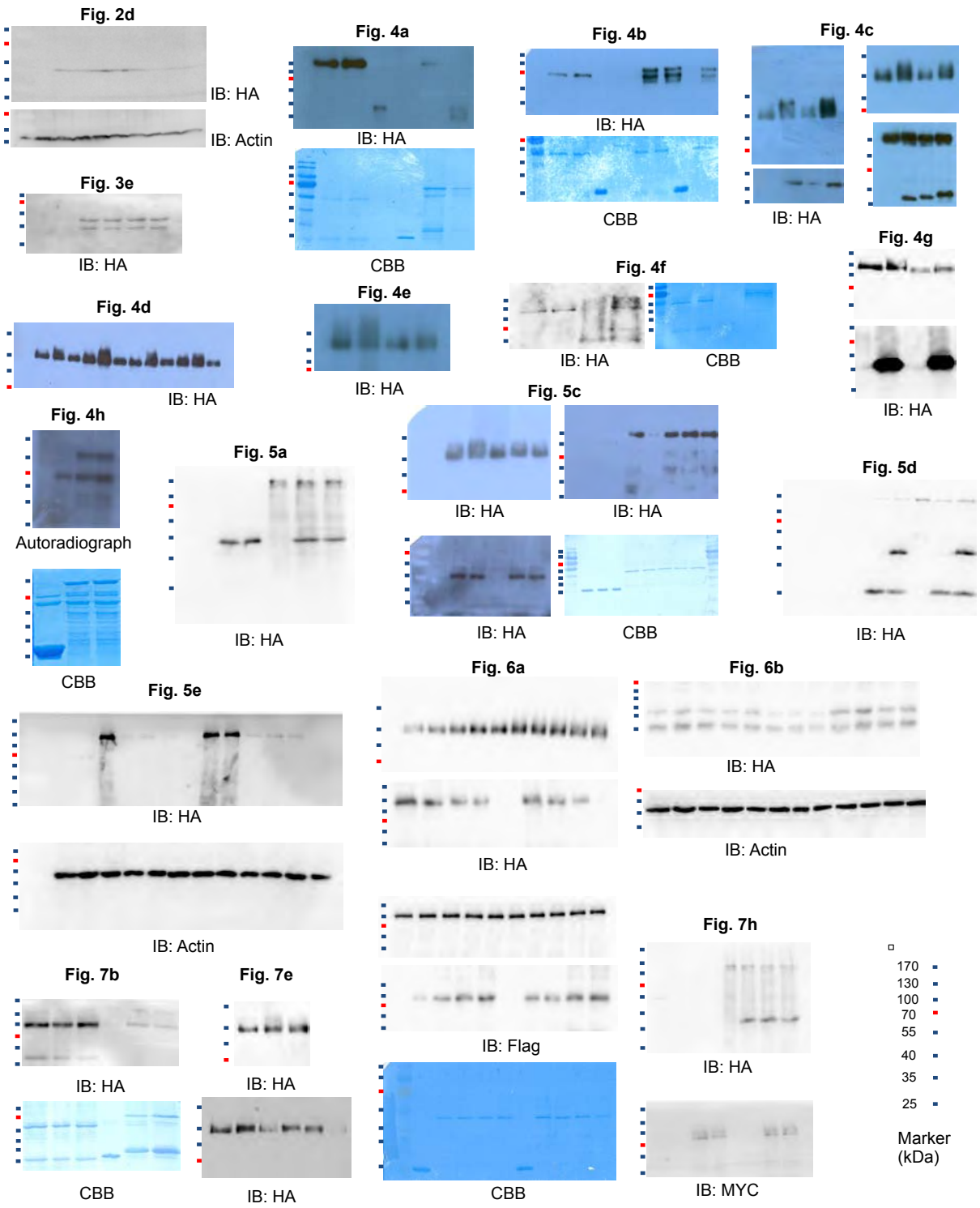

Supplementary Figure 8 Full scan data of immunoblot and in vitro kinase assay

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Supplementary Table Legends

Supplementary Table 1 Primer combinations for quantitative PCR

Supplementary Table 2 Statistics source data

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APPENDIX X

The small signalling peptide CEP5 attenuates the dynamic AUX/IAA equilibrium

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Unpublished manuscript.

*Asterisk denotes joint primary authorship.

The small signalling peptide CEP5 attenuates the dynamic AUX/IAA equilibrium

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FOOTNOTES

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RUNNING TITLE

CEP5 affects AUX/IAA stability

INTRODUCTORY PARAGRAPH

Root architecture is vital for plant growth and largely depends on primary root growth and lateral root development. Several plant hormones have been shown to affect root architecture among which auxin has been granted a central role. Lately, small signalling peptides also emerged as potential molecular components regulating root growth and development. Here, we identified C-TERMINALLY ENCODED PEPTIDE 5 (CEP5) as a novel, phloem pole-expressed paracrine signal for lateral root initiation. Our genetic, biochemical and pharmacological results show that CEP5 counteracts auxin signalling by stabilizing AUXIN/INDOLE ACETIC ACID (AUX/IAA) transcriptional repressors, suggesting the existence of an additional control mechanism through which plants can attenuate auxin signalling in a developmental context. Reducing *CEP5* expression levels resulted in an increased auxin response and subsequently interfered with the normal progression through lateral root developmental stages.

Coordinated positioning and development of lateral roots is central to shape root system architecture allowing plants to adapt their below-ground organs for optimal soil exploration[1-4]. Lateral root primordia are formed from approximately three pairs of xylem pole pericycle cells arranged in neighbouring cell files that undergo asymmetric cell division and subsequently form a new organ[5-9]. Close to the primary root tip – in the basal meristem – and before any asymmetric cell division, a periodic transcriptional mechanism specifies on a regular basis pre-branch sites that are competent to form lateral roots[7,10-12].

The phytohormone auxin regulates many plant growth and developmental processes and is prominently involved in lateral root development[13,14]. To generate different auxin-mediated developmental outputs, a complex signalling mechanism involving spatio-temporal expression of *ARFs* and *AUX/IAAs*, variation in auxin sensitivity of AUX/IAA−TIR1/AFB co-receptor complexes, and phospho-regulation of ARF-AUX/IAA interactions is required[15-19]. However, fine-tuning temporal and spatial developmental responses at the protein level most likely requires additional mechanisms. For example, small signalling peptides are important in cell-cell communication to coordinate and integrate cellular functions[20,21] and we recently demonstrated that the TRACHEARY ELEMENT DIFFERENTIATION INHIBITORY FACTOR (TDIF) − TDIF RECEPTOR (TDR) − BRASSINOSTEROID-INSENSITIVE2 (BIN2) signalling cascade can interfere with ARF − AUX/IAA interactions[19].

In this work, we identified *C-TERMINALLY ENCODED PEPTIDE 5* (*CEP5*), which encodes a member of the CEP family with unknown mode-of-action[22]. We demonstrated that CEP5-dependent signalling leads to the stabilization of AUX/IAA transcriptional repressors, arguing for the existence of a novel peptide-dependent control mechanism that contributes to the fine-tuning of auxin signalling during root development.

RESULTS

CEP5 expression is associated with early stages of lateral root development

Since the plant hormone auxin is a major regulator of primary root growth and lateral root development[5,23], several transcript profiling studies based on auxin treatments have been performed in order to identify the molecular players involved[24-26]. However, because of the pleiotropic effects caused by exogenous auxin application, such datasets risk to compromise the required spatiotemporal resolution when looking for components specific for a single developmental process. To circumvent this, we searched for putative novel early lateral root formation components by screening a dataset obtained through a highly focused transcript profiling analysis on seedling roots treated with the synthetic molecule naxillin, which does not produce the typical pleiotropic effects of exogenous auxin application[27]. We identified *CEP5* as differentially regulated between non-treated and naxillin-treated seedling roots[27]. The *CEP5* gene encodes a small protein of 105 amino acids and contains a conserved 15 amino acid C-terminal CEP domain that gives rise to a small signalling peptide[22,28].

Using a *pCEP5::nls:GUS:GFP* reporter line[22], we observed regularly spaced patches of *CEP5* expression associated with lateral root primordia, confirming its potential involvement in this process (**Fig. 1a-c**). We did not detect *CEP5* expression in the primary root stem cell niche, however *CEP5* was expressed in the basal meristem (**Fig. 1d**). The latter is important in the context of lateral root initiation as this region is defined as part of the oscillation zone where prebranch sites are established[7,11,12]. Tissue-specific analyses showed that both in the basal meristem and during early stages of lateral root development, *CEP5* was predominantly expressed in the phloem pole pericycle, but also – although weaker – in the adjacent phloem (**Fig. 1e-g, Supplementary Fig. 1 and Supplementary Movie 1**). This

CEP5 expression pattern does not overlap with the well-documented sites of high auxin response in the root or during lateral root initiation, which in *Arabidopsis* occurs in pericycle cells at the xylem pole[7,29,30]. In addition, treatment of wild-type roots with different concentrations of the synthetic auxin 1-naphthalene-acetic acid (NAA) revealed that *CEP5* expression was down regulated by auxin (**Fig. 1h**). Taken together, the tissue where *CEP5* is expressed and its regulation by auxin suggested a negative correlation with auxin activity.

Altering CEP5 levels affects root architecture

Given the correlation of *CEP5* expression with lateral root initiation and development, we assessed if CEP5 loss-of-function affected this process. Detailed analyses of lateral root initiation revealed increased stage I lateral root primordia in a CaMV *35S* promoter-driven *CEP5* RNAi knockdown line ($CEP5^{RNAi}$)[22] compared to the control (**Fig. 2a and Supplementary Fig. 2**). Additionally, in a root bending assay[31], the $CEP5^{RNAi}$ line progressed faster through lateral root developmental stages than wild type (**Fig. 2b**). The $CEP5^{RNAi}$ line did, however, not display a striking difference in primary root length compared to the control (**Supplementary Fig. 3**).

Next, we analysed a line with CaMV *35S* promoter-driven constitutive overexpression of *CEP5* ($CEP5^{OE}$)[22], which displayed shorter primary roots (similar to other independent $CEP5^{OE}$ lines) and a short primary root meristem as compared to wild type (**Fig. 2c-d and Supplementary Fig. 2 and 3**). Furthermore, the $CEP5^{OE}$ line displayed a decrease in total lateral root density (22% decrease), with fewer non-emerged lateral roots, compared to wild type (**Fig. 2e**). Detailed analyses of lateral root developmental stages showed that this was mainly due to fewer initiation events (**Fig. 2e**). At later stages of lateral root development, we also observed closely spaced lateral root primordia in $CEP5^{OE}$ lines (**Supplementary Fig. 3**).

Our analyses further revealed no obvious differences in *CEP5*^{OE} and *CEP5*^{RNAi} lines compared to the control with respect to the radial vascular pattern and xylem differentiation (**Supplementary Fig. 4**), ruling out that vascular defects would be responsible for the lateral root initiation phenotype.

Synthetic CEP5 peptide affects root architecture

CEP5 contains a conserved C-terminal CEP domain containing three proline (Pro) residues that gives rise to a small peptide (CEP5p)[22,28] (**Fig. 2f**). However, small signalling peptides are often post-translationally modified, thereby modulating the binding ability and specificity of peptides to their targets[32]. In this context, it was previously shown that members of the CEP family give rise to a peptide containing hydroxyproline (Hyp) residues[28,33,34]. To confirm that a 15 amino acid CEP5 peptide with three Hyp residues (CEP5p^{Hyp}) (**Fig. 2f**) is indeed present in seedlings overexpressing *CEP5*, we performed selected reaction monitoring (SRM) on a *CEP5*^{OE} line. Indeed, in the *CEP5*^{OE} proteome spiked with a chemically synthesized version of CEP5p^{Hyp} containing an isoleucine residue with heavy, stable isotopes, transitions for both the heavy, spiked-in CEP5p^{Hyp} and the light, naturally occurring CEP5^{Hyp} peptide could be detected (**Supplementary Fig. 5**).

To subsequently assess the activity of chemically synthesised CEP5p^{Hyp} (**Fig. 2f**), we analysed its effect on primary root growth, which has previously been shown to be a straightforward, although possibly nonspecific, assay to test the activity of small post-translationally modified peptides[28,33,35-37]. Indeed, seedlings grown in the presence of CEP5p^{Hyp} displayed shorter roots compared to the mock-treated control and compared to a synthetic variant with 4 randomly positioned, but not very unlikely amino acid substitutions based on a BLOSUM62 substitution matrix within the 15 amino acid CEP5 peptide sequence,

while retaining the Hyp residues at the same positions (mCEP5p^{Hyp}) (**Fig. 2f-h**). To relate the shorter primary root to meristematic activity, we analysed the size of the primary root apical meristem. This revealed a CEP5p^{Hyp} concentration-dependent primary root meristem inhibition (**Supplementary Fig. 3**). Next, to gain further insight in the structure-activity relationship within the CEP5p sequence, we used synthetic peptides where Hyp residues were replaced by Pro or where each residue was replaced by alanine (Ala) (**Supplementary Fig. 6**). This revealed that interfering with the Hyp residues or overall residue composition of CEP5p^{Hyp} reduces or abolishes activity in most cases (**Supplementary Fig. 6**). Overall, this is in agreement with observations on other small signalling peptides where bioactivity is increased upon hydroxyprolination[34,38-41]. In two cases (CEP5p$^{Hyp(Thr6Ala)}$ and CEP5p$^{Hyp(His9Ala)}$) the synthetic CEP5p variants retained activity and in one case (CEP5p$^{Hyp(Hyp11Ala)}$) we observed increased activity in our primary root growth assay (**Supplementary Fig. 6**). This analysis pinpointed that Hyp and several other residues within the CEP5 peptide sequence are important for CEP5p activity.

Next, we addressed the effect of synthetic CEP5p^{Hyp} on lateral root formation. Seedlings grown in the presence of different low concentrations of CEP5p^{Hyp} displayed changes in total lateral root density (19% and 29% decrease for 10 nM and 100 nM, respectively), which is mainly due to a significant reduction in lateral root initiation events (**Fig. 2i**). Conversely, this did not occur in mCEP5p^{Hyp}-treated seedlings (**Supplementary Fig. 3**). When lateral root initiation occurred, we occasionally observed regions of ectopic and/or aberrant pericycle cell divisions resulting in malformed lateral root primordia or closely spaced primordia in CEP5p$^{Pro/Hyp}$-treated seedlings, which differed from regularly spaced lateral roots in wild type (**Supplementary Fig. 3**).

Taken together, the similarities in primary and lateral root phenotypes between CEP5p treatment and *CEP5*^{OE} indicate that the chemically synthesised CEP5 peptide has the same bioactivity as the overexpressed *CEP5*.

Differential proteomics identifies changes downstream of CEP5

To gain insight in the molecular changes downstream of CEP5 activity, we quantified differences in proteomes of wild type, *CEP5*^{OE}, and CEP5p^{Hyp} or mCEP5p^{Hyp}-treated seedlings using label-free mass spectrometry-based proteomics. This analysis allowed the identification and quantification of a total of 4564 proteins over three biological and two technical replicates. After filtering for proteins that were detected in all biological replicates of at least one group, we retained 3719 proteins for further data analysis. An ANOVA test ($p<0.01$ and permutation-based FDR<0.05) marked 193 proteins with significantly different abundance, with the most dramatic effects observed upon overexpression of *CEP5*, compared to treatment with CEP5p^{Hyp} or mCEP5p^{Hyp}, which only moderately influenced the protein levels (**Fig. 3 and Supplementary Table 1**). This dataset showed enrichment on the level of nitrate/nitrogen-related proteins (19%, 36/193) (**Supplementary Table 1**), a connection that is in agreement with earlier observations on CEP family peptides[28]. However, in addition to this, we also observed a set of differentially abundant proteins that have an association with hormone biology, more specifically with auxin, abscisic acid, brassinosteroids, ethylene, gibberellins, and jasmonic acid (8%, 15/193) (**Supplementary Table 1**).

CEP5 affects auxin response

Given the connection with auxin biology through the effect of CEP5 on primary root growth and lateral root development (both representing auxin-mediated processes[5,23]), expression analyses and quantitative proteomics, we further assessed if CEP5p affected auxin signalling and/or response. First, we analysed auxin-mediated root phenotypes in seedlings with increased *CEP5* levels. We observed that a *CEP5*^{OE} line was less sensitive to auxin with respect to primary root growth inhibition (**Fig. 4a**) and induction of lateral root initiation (**Fig. 4b**). Next, we tested the effect of CEP5 on auxin response by analysing the auxin response marker *pDR5::GUS* in the root.We observed reduced *pDR5::GUS* activity in the root tip and in the basal meristem following CEP5p^{Hyp} treatment and in the *CEP5*^{OE} line (**Fig. 4c-d**). In this context, the earliest known event associated with lateral root initiation occurs in the basal meristem, where also *CEP5* is expressed (**Fig. 1d**), and can be monitored *in vivo* through the oscillating expression of *pDR5::LUC*[7,11]. Under our control conditions, transcriptional auxin-response oscillations visualised through *pDR5::LUC* occur with an average period of 3.67 ± 0.78 hours[12], but CEP5p^{Hyp}-treated seedlings displayed an increased average period (4.51 ± 1.74 hours) and a disturbed peak distribution (**Fig. 4e-f and Supplementary Movies 2 and 3**). In addition, the overall average intensity of the LUCIFERASE signal was severely reduced in the root (**Fig. 4g and Supplementary Movies 2 and 3**), supporting our observations with *pDR5::GUS*. In addition to basal auxin response, also auxin-inducible expression of *IAA18*, *LBD16*, *LBD29*, and *PIN1* was reduced in auxin-treated *CEP5*^{OE} roots compared to the control (**Fig. 4h**). Finally, we analysed if CEP5p interfered with the establishment of the differential auxin distribution that is established during gravitropic response[42]. We observed a quick change in activity of the AUX/IAA protein-based auxin sensor *p35S::DII:VENUS*[30,43] in gravistimulated control primary root tips, which was delayed in the presence of CEP5p^{Hyp} (**Fig. 4i**). Ultimately, following gravistimulation, a bend is induced in seedling primary root tips, progressing from a theoretical maximum bend angle of 180° (vertical growth) to a

theoretical minimum bend angle of 90°. CEP5p^{Hyp}-treated wild type seedling displayed a slower gravitropism-induced bending response of primary root tips relative to the untreated control (**Fig. 4j**). Taken together, our results suggested that CEP5 affects auxin response, either directly through interfering with signalling components or indirectly through affecting (free) auxin levels and/or auxin distribution patterns.

CEP5 leads to stabilization of AUX/IAAs

Since auxin response is intimately correlated with auxin levels, it is possible that increased or decreased CEP5 levels lead to lower or higher auxin levels, respectively, which in turn would result in decreased or increased auxin response. To investigate this, we compared auxin levels in wild-type, *CEP5*^{OE} and *CEP5*^{RNAi}, but this revealed no striking differences in free auxin (IAA, indole-3-acetic acid) content (**Supplementary Fig. 7**).

Transcriptional responses to auxin depend principally on the auxin-activated SKP1−CUL1/CDC53−F-BOX (SCF)$^{TIR1/AFB}$-dependent degradation of AUX/IAAs[13]. Auxin concentration and/or activity level of the SCF$^{TIR1/AFB}$ complex can be inferred from the decrease in DII:VENUS fluorescence levels[30,42]. In the presence of CEP5p^{Hyp} and in a *CEP5*^{OE} line, DII:VENUS fluorescence was significantly increased compared to the control, and this did not appear to be caused by an equally strong up-regulation of *DII:VENUS* expression (**Fig. 5a-b and Supplementary Fig. 8**). In comparison, the related CEP1p^{Hyp} only had a minor, not significant impact on DII:VENUS fluorescence (**Supplementary Fig. 9**). Moreover, for CEP5p^{Pro} and CEP5p^{Hyp}, a stabilization of DII:VENUS was already observed within 60 minutes, while for mock or mCEP5p^{Hyp}-treated seedlings a gradual decrease in DII:VENUS signal was observed (**Supplementary Fig. 10**). In contrast, the *CEP5*^{RNAi} line displayed significantly lower DII:VENUS levels than the control (**Fig. 5c**), which did not

appear to be caused by an equally strong down-regulation of *DII:VENUS* expression (**Supplementary Fig. 8**). Furthermore, co-incubation of auxin (IAA or NAA) with CEP5p^{Hyp} resulted in a significant delay of DII:VENUS degradation compared to auxin alone, while mCEP5p^{Hyp} does not affect DII:VENUS degradation (**Fig. 5d-e and Supplementary Fig. 11**).

Given the similar effect of CEP5 on IAA and NAA-induced DII-VENUS degradation (two auxins with different transport properties), CEP5 probably has no direct effect on auxin uptake or transport. Furthermore, since CEP1 was shown to affect *NITRATE TRANSPORTER* (*NRT*) expression levels[28] and since NRT1.1/CHLORINA1 (CHL1) not only transports nitrate but also facilitates uptake of auxin[44], we evaluated this in the context of CEP5. While *NRT* expression levels are indeed up-regulated in *CEP5^{OE}*, we did not observe any obvious insensitivity of *chl1-5* (a knockout mutant for *NRT1.1*)[45] to CEP5p^{Hyp} in our primary root growth assay (**Supplementary Figure 12**). This suggests that NRT1.1 is not directly involved in the CEP5-dependent regulation of auxin response.

To validate that CEP5 also affects full length AUX/IAAs, we analysed plants expressing *IAA19:HA*[19] and *pBODENLOS (BDL)::BDL:GUS*[46]. Indeed, CEP5p^{Hyp}-treatment of these seedlings resulted in a quick stabilization or accumulation of IAA19:HA or BDL:GUS compared to mock or mCEP5p^{Hyp} treatment as revealed by western blot analysis or GUS staining (**Fig. 5f and Supplementary Fig. 13**). Interestingly, accumulation of BDL/IAA12 in gain-of-function lines expressing *bdl* results in similar lateral root phenotypes as observed for increased CEP5p levels[47] (**Supplementary Fig. 3**), further supporting that CEP5p affects AUX/IAA levels and disturbs auxin-dependent lateral root patterning. To rule out that CEP5 leads to increased AUX/IAA levels by transcriptional down-regulation and/or up-regulation of *TIR1/AFBs* and/or *AUX/IAAs,* respectively, we checked their expression levels in a *CEP5^{OE}* line or in CEP5p-treated seedlings. This actually revealed a small increase

in *TIR1* and *AFB2* to *AFB5* expression levels in *CEP5*^{OE} roots and no obvious effect on *IAA12* and *IAA18* expression in CEP5p^{Pro} or CEP5p^{Hyp}-treated seedlings compared to the control (**Supplementary Fig. 14**). Taken together, these results suggested that CEP5 modulates auxin-regulated AUX/IAA stability, which impacts on overall root architecture.

XIP1 regulates lateral root initiation and auxin response

Recently, XYLEM INTERMIXED WITH PHLOEM1 (XIP1)/CEP RECEPTOR1 (CEPR1) and CEPR2 were proposed to act as receptors for CEP1 and other members of the CEP family[28]. To gain further insight into the CEP5 signalling cascade, we explored this connection in the context of lateral root initiation and auxin response. Detailed analyses of a previously described *pXIP1::GUS* line[48] showed that *XIP1* is expressed in the root from the basal meristem onward (**Fig. 6a**), which is similar to *CEP5* (**Fig. 1**). Furthermore, tissue-specific analyses showed that *XIP1* is expressed in the phloem pole pericycle and in the adjacent phloem (**Fig. 6b**), overlapping with *CEP5* expression (**Fig. 1e**), and is excluded from early stages of lateral root development (**Supplementary Fig. S15**), similar to *CEP5* (**Fig. 1c**). This expression pattern suggested that XIP1 could be a receptor for CEP5 in the root and regulate lateral root initiation and auxin response. To further explore this, we assessed lateral root stages and density of the previously described *xip1-1* mutant[48]. This revealed a reduced total lateral root density in *xip1-1* in comparison with the control, which seemed mainly due to a reduction in stage I and II lateral root primordia (**Fig. 6c**). This root architecture phenotype is similar to *CEP5*^{OE} or CEP5p^{Hyp}-treated seedlings (**Fig. 1c-i**), further supporting a connection between XIP1 and CEP5. However, based on these results, it appears that CEP5 negatively regulates XIP1 in the context of lateral root development.

To subsequently connect XIP1 to auxin response, we evaluated expression levels of a selection of auxin responsive, lateral root-associated genes in *xip1-1*. This revealed reduced auxin-inducibility of lateral root initiation-associated genes, such as *PIN1*, *LBD16*, *LBD29*, and *ARF19*, in *xip1-1* compared to the control (**Fig. 6d and Supplementary Fig. S16**). The above results suggested that CEP5 likely acts through XIP1/CEPR1 in regulating its downstream effect on auxin response and lateral root initiation, and that this could already be at the level of the basal meristem.

DISCUSSION

Previously, a role for CEPs in regulating aspects of root architecture was proposed, and specifically, CEPs appear to act as a root-derived ascending N-demand signal to the shoot, where its perception by CEPRs leads to the production of a putative shoot-derived descending signal that up-regulates nitrate transporter genes in the roots[28,33-35]. However, the downstream mechanism remained largely elusive and other potential mechanisms have not been explored. Here, our genetic, biochemical and pharmacological studies have revealed that CEP5 is involved in lateral root initiation and development and in attenuating auxin response through stabilising AUX/IAAs. During lateral root formation, CEP5 might assist in dampening auxin responses in neighbouring tissues of xylem pole pericycle cells undergoing lateral root initiation and thereby repressing cell division in the flanking cells. The antagonistic relationship between auxin and CEP5 could be important in regulating auxin response thresholds and fine-tuning (very sensitive) auxin responses during growth and development through stabilising AUX/IAAs. It remains, however, to be investigated if CEP5 acts directly on auxin signalling components or if a classical signalling cascade through a membrane-associated receptor kinase impacts on AUX/IAA levels. In this context, the recent

identification of likely CEP receptors, XIP1/CEPR1 and CEPR2[28], and the involvement of XIP1 in lateral root initiation and auxin response support the latter (our data). However, at the moment it is not known how CEP5 and XIP1 act on AUX/IAA levels and/or auxin response. In contrast to the effect of TDIF[19], we have – up to now – not been able to detect phosphorylation of ARF7, suggesting another mechanism. It is at least intriguing that a phloem-derived signal downstream of CEP5 and XIP1 has such an impact on lateral root initiation and development at the xylem pole. Given the expression patterns of the *CEP* family[22], and especially *CEP5* which appears to mirror areas of increased auxin response, the regulation of auxin response may prove to be a general mechanism for these small signalling peptides throughout growth and development. However, our data suggest that – at least with respect to stabilizing DII:VENUS – CEP1 is less potent, so there are likely differences between family members. This is likely due to subtle differences in their mature peptide sequence, as single amino acid changes can impact on bioactivity and/or specificity. In conclusion, our results support a new mechanism of regulating AUX/IAA stability during growth and development, and future studies are required to reveal all the actors involved.

METHODS

Detailed information on experimental procedures used in this study is provided in Supplementary information.

Plant materials. Details on transgenic lines and mutants in Supplementary information.

Plant growth and treatment conditions. Plant growth details in Supplementary information. For peptide treatments, media was supplemented with CEP5p^{Pro}, CEP5p^{Hyp}, or mCEP5p^{Hyp} peptide to a concentration as indicated in the text and/or figure legends.

Primary and lateral root phenotyping. At the indicated time, images of plates with seedlings were taken and roots measured using ImageJ (http://rsbweb.nih.gov/ij/index.html) or FIJI software[49]. For detailed staging of lateral roots and for meristem size measurements, samples were cleared by mounting in 90% lactic acid or by clearing as described previously[50] and analysed by differential interference contrast microscopy (Olympus BX51). Additional details in Supplementary information.

Histochemical GUS assays. Details for GUS assays in Supplementary information. For microscopic analysis, samples were cleared with 90% lactic acid or as previously described[50]. Samples were analysed by differential interference contrast microscopy (Olympus BX53) and a stereomicroscope (Leica MZ16). For anatomical analysis (microtome transversal sectioning) of GUS-stained roots, stained samples were processed as previously described[51].

LUCIFERASE imaging and expression analysis. The LUCIFERASE images were taken by a Lumazone machine carrying a CCD camera (Princeton instrument). The CCD camera with macro lens is controlled by WinView/32 software, and *LUCIFERASE* expression movies were taken automatically every 10 min with 10 min exposure time for ~ 24 hours. Before imaging, plates containing ½ MS were sprayed with 1 mM D-Luciferin (Duchefa Biochemie). The expression level of *pDR5::LUC* was measured by selecting the region of interest and quantifying the analog-digital units (ADU) per pixel using ImageJ. To quantify the prebranch sites number (static *DR5* expression sites above the oscillation zone), seedlings harboring *pDR5::LUC* were sprayed with D-Luciferin, and imaged by Lumazone with 15 minute exposure.

qPCR analyses. Details and primers in Supplementary information.

DII :VENUS fluorescence quantification. Seedlings were imaged on a Leica SP5 confocal microscope (Leica, Wetzlar, Germany). Static images of each seedling were taken and fluorescence was quantified by calculating raw integrated density values for each image.

Measurements were done using FIJI software[49]. Background fluorescence was removed using a threshold and only fluorescence coming from the nuclei was quantified. A zone just above the root hair initiation zone was used for analyses. Alternatively, seedlings were imaged on an inverted Nikon eclipse Ti-U confocal microscope (Nikon, Japan) with a fixed delay of 2 minutes over a minimum of 12 hours. Background fluorescence was removed using a threshold and only fluorescence coming from the nuclei was quantified. Plots presented show changes in raw integrated density values over time, measured using FIJI software[49]. A minimum of 3 seedlings (~80 nuclei) were independently quantified for each condition.

Auxin measurements. 500 pg 13C6-IAA internal standard was added to each sample, and extraction and purification was done as previously described[52], with minor modifications. Quantification of free IAA was then performed by gas chromatography - tandem mass spectrometry (GC-MS/MS).

Quantitative, label-free proteomics. Details in Supplementary information.

Immunoblotting. Details in Supplementary information.

AUTHOR CONTRIBUTIONS

B.D.R. initiated experimental work; I.R., S.S., E.S, W.X., A.S, H.C., A.L., L.D.V., B.G., J.M.G., A.R., S.R.H., B.V.D.C., H.D., S.V., G.K.K., J.L., E.V. performed experiments; I.R., S.S., S.V., L.M., Y.S., D.A., J.F., G.F., R.S., M.J.B., A.B., K.L., S.K., S.R., J.N., I.H., K.G., T.B., and I.D.S. designed experiments, analysed data and/or commented on the manuscript, and T.B. and I.D.S. wrote the manuscript.

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FIGURE LEGENDS

Fig. 1. *CEP5* expression in the *Arabidopsis* root. (a-g) *CEP5* expression (monitored through *GUS* expression in a *pCEP5::nls:GUS:GFP* transgenic line) in a complete seedling (overstained for illustrative reasons) (a), in a part of the root from the seedling in panel a (b), in one lateral root primordium (c), in the root apex (d), in the basal meristem on transverse section (e), at a site of lateral root formation (f-g) with the lateral root primordium pointing to the right (outlined with dotted black line) (f), and on transverse section through a lateral root primordium (outlined with dotted black line) (g). Seedlings are 5-6 days after germination. *, lateral root primordium; arrowheads separate individual cells (f); P, phloem; X, xylem; Pe, pericycle; En, endodermis; C, cortex; b.m., basal meristem. **(h)** *CEP5* expression in 5 day old root tips of ~5 mm (including the basal meristem) following 2 hours auxin treatment analysed through qRT-PCR analysis (n = 3 biological replicates). *, $p < 0.05$ according to Student's *t*-test compared to 0 μM NAA.

Fig. 2. Effect of altered CEP5 peptide levels on root architecture. (a-b) Effect of reduced *CEP5* levels on lateral root development. Stage I lateral root primordia at indicated seedlings age (n = 10) (a) and progression through lateral root stages at indicated hours post gravisimulus (n ≥ 14) (b) in a $CEP5^{RNAi}$ line versus Col-0. *, $p < 0.05$ and **, $p < 0.075$ according to Student's *t*-test compared to Col-0. **(c-e)** Effect of increased *CEP5* levels on primary root growth and lateral root development. Representative picture of a $CEP5^{OE}$ line at 12 days after germination (c) and quantification of primary root length at 12 days after germination (d). (e) Lateral root stages I to VII according to ref. [50] in $CEP5^{OE}$ line (n ≥ 15) at 7 days after germination. The % reduction in total lateral root density is indicated. E, emerged lateral roots; NE, non-emerged lateral roots; Total, sum of E and NE. *, $p < 0.05$ according to

Student's *t*-test compared to Col-0. **(f)** 15 amino acid CEP5 sequence for synthetic variants. Unmodified ($CEP5p^{Pro}$), with proline hydroxylation modifications on P4, P7 and P11 ($CEP5p^{Hyp}$), and hydroxyprolinated mutated CEP5 sequence with four residue substitutions (R3>L, T5>H, S10>V and G14>S, indicated in red) ($mCEP5p^{Hyp}$). **(g-i)** Effect of synthetic CEP5p on primary root growth and lateral root development. Representative picture of Col-0 *Arabidopsis* seedlings grown on 1 μM $CEP5p^{Hyp}$ for 9 days after germination (g) and quantification of primary root length of Col-0 seedlings treated with 5 μM $mCEP5p^{Hyp}$ compared with mock treatment and 5 μM $CEP5p^{Hyp}$ at 12 days after germination ($n \geq 15$ per condition). The % reduction in primary root length is indicated. (i) Lateral root stages I to VII according to ref. [50] upon mock or $CEP5p^{Hyp}$ treatment at different concentrations at 9 days after germination (data from newly grown root part of 5 day old seedlings transferred to $CEP5p^{Hyp}$ for 4 days, $n \geq 32$). E, emerged lateral roots; NE, non-emerged lateral roots; Total, total lateral roots. *, $p < 0.05$ according to Student's *t*-test compared to mock. The % reduction in total lateral root density is indicated. In all cases, mock refers to medium with water as used to dissolve CEP5p. All graphs show average ± standard error.

Fig. 3. Differential protein levels in seedlings with increased CEP5 levels. Heat map showing differences in a subset of 193 proteins identified as statistically significantly different between wild type seedlings treated with mock (water as used to dissolve CEP5p), 1 μM $CEP5p^{Hyp}$, or 1 μM $CEP5p^{Hyp}$, or $CEP5^{OE}$ seedlings. Topology of the heat map is the results of K-means clustering. Values are an average of 3 biological and 2 technical repeats per treatment or genotype and are scaled to interval for graphical representation.

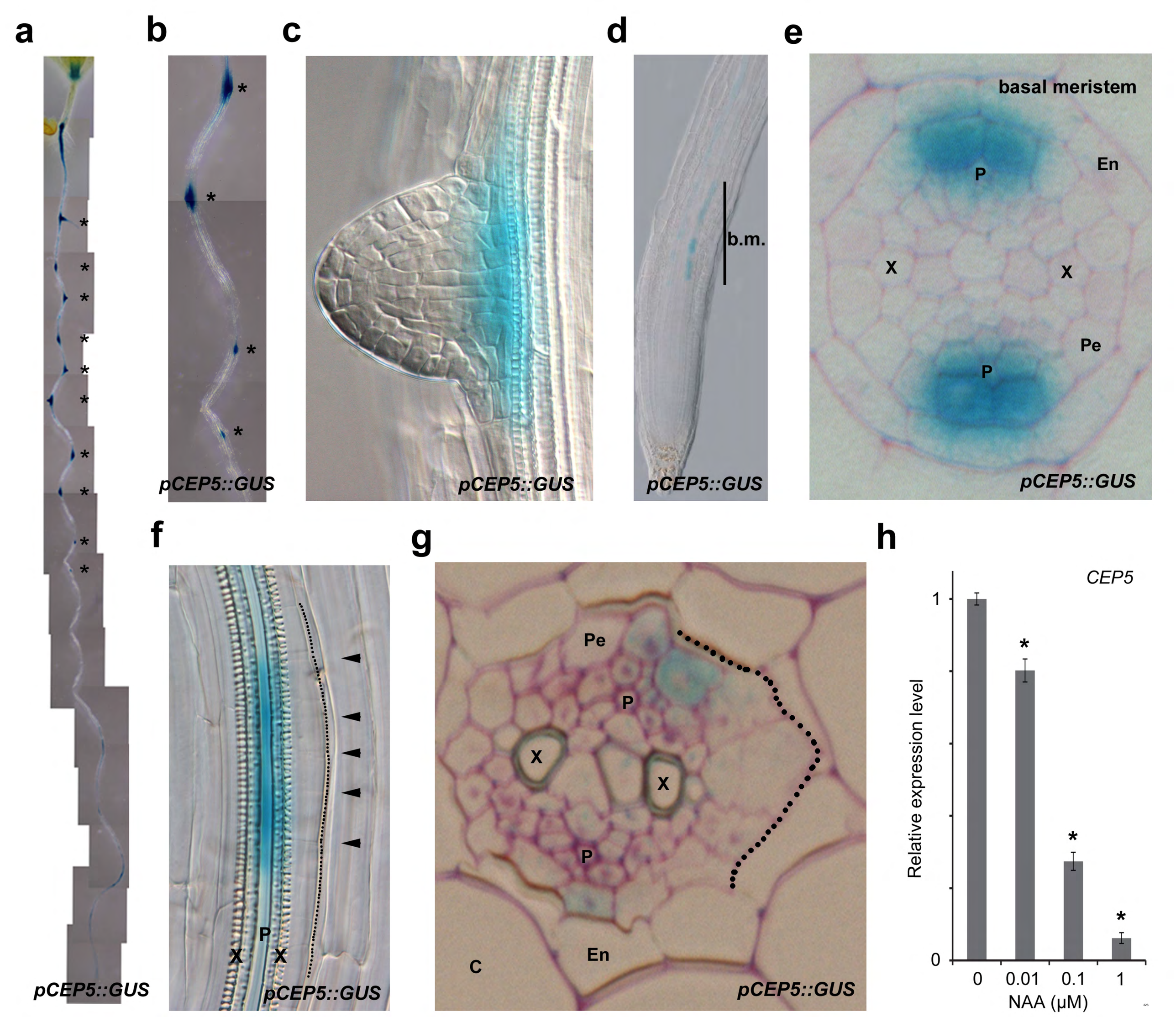
Fig. 4. Effect of CEP5 on auxin response. (a-b) Effect of NAA (at indicated concentrations) on primary root growth (a) and lateral root initiation (b) in a $CEP5^{OE}$ line at

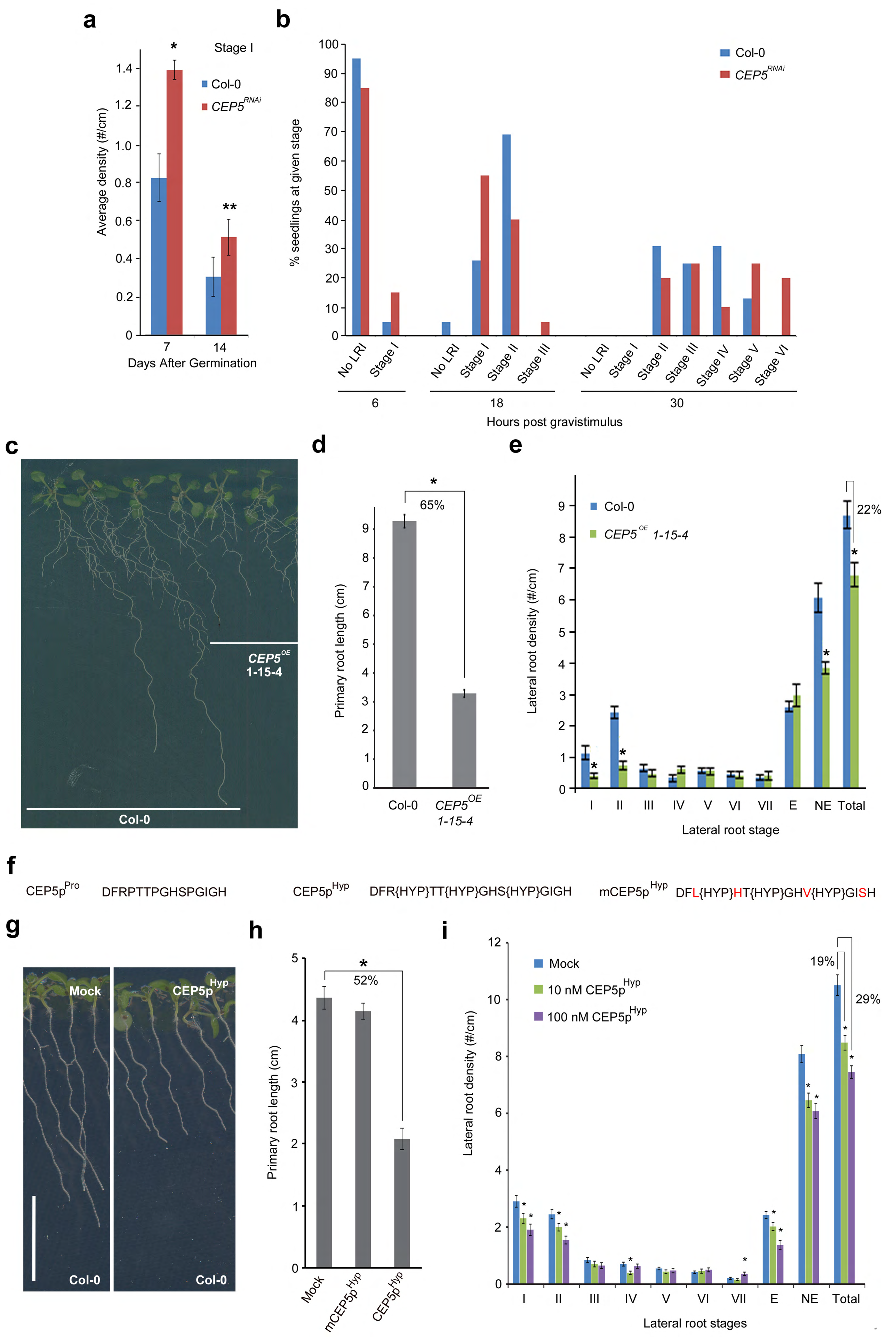
13 days after germination (n ≥ 22). Blue, Col-0 wild type; Red, *CEP5*^{OE} ; *, $p < 0.05$ according to Student's *t*-test compared to Col-0. **(c-d)** *pDR5::GUS* activity in the primary root tip of 5-day old seedlings transferred to mock (left) or 1 μM CEP5p^{Hyp} (right) for 4 days (c) or in the root tip of *CEP5*^{OE} at 7 days after germination (d)**. (e-g)** *pDR5::LUC* in the root of 3-day old seedlings treated with mock (left) or CEP5p^{Hyp} (right). Arrowhead indicates root tip (e). *pDR5::LUC* peak distribution in the oscillation zone following mock (blue) or 1 μM CEP5p^{Hyp} treatment (green) (n ≥ 14) (f). Total relative LUC activity/cm in *pDR5::LUC* following 1 μM CEP5p^{Hyp} treatment (g). **(h)** Auxin-inducible expression of *IAA18, LBD16, LDB29,* and *PIN1* (as determined through qRT-PCR analysis) in Col-0 and *CEP5*^{OE} 5 day old seedling roots treated with 1 μM NAA or mock (DMSO) for 6 hours. Ratio calculated from average of 2 (Col-0 + 1 μM NAA) or 3 biological repeats (Col-0, *CEP5*^{OE} and *CEP5*^{OE} + 1 μM NAA). Percentage difference in reduced auxin-inducibility is indicated. **(i)** The ratio of VENUS fluorescence in the lower (orange lines) versus upper (green lines) horizontal sections of roots from DII:VENUS reporter line subjected to a gravitropic stimulus and mock or CEP5p^{Hyp}-treated. The percentage share of the total root fluorescence at the indicated hours post gravistimulus is shown. RID, raw integrated density. Arrows indicate ~1 hr delay in response. **(j)** Angle of gravistimulated mock or 5 μM CEP5p^{Hyp}-treated Col-0 root at indicated minutes post gravistimulus. *, $p < 0.05$ according to Student's *t*-test compared to mock. In all cases, mock refers to medium with water as used to dissolve CEP5p. All graphs show average ± standard error.

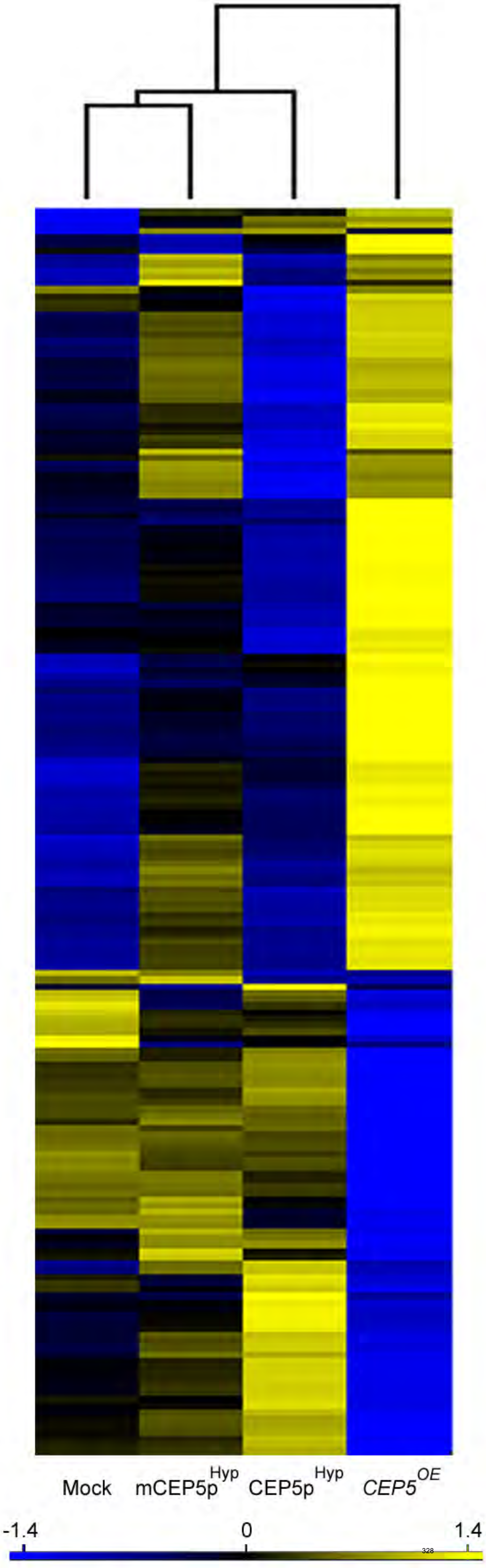
Fig. 5. Effect of CEP5 on AUX/IAA stability. (a-e) Relative VENUS fluorescence in DII:VENUS reporter line following 18 hrs incubation with 5 μM CEP5p^{Hyp} compared with mock treatment at 5-6 days after germination (n ≥ 83) (a), in Col-0, a *CEP5*^{OE} line and a *CEP5*^{RNAi} line at 5-6 days after germination (n ≥ 15) (b-c), and in seedlings treated with 1 μM

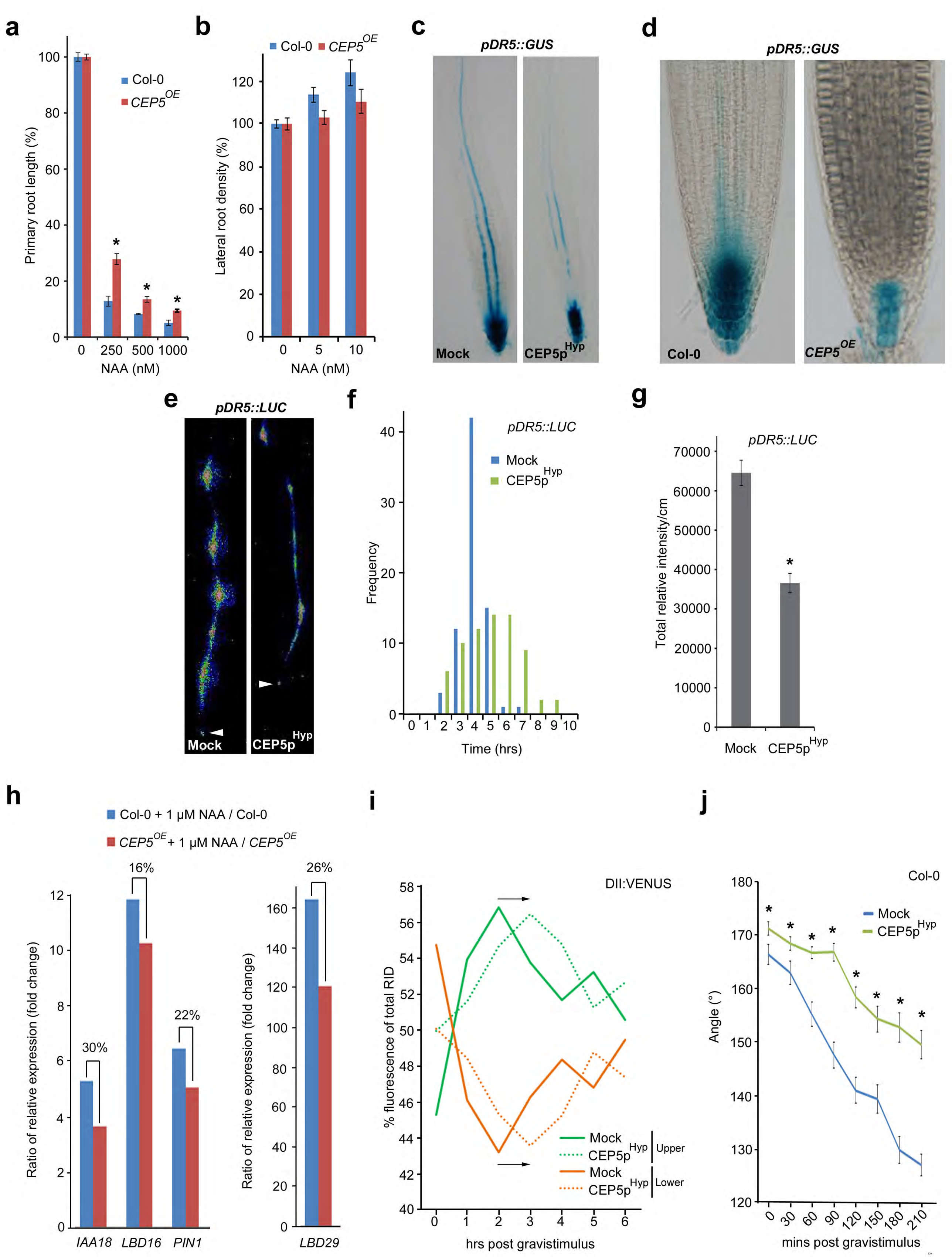
NAA and co-treated with 5 μM CEP5p^{Hyp} without (d) or with pre-treatment for 18 hrs (n ≥ 4) (e). Normalized ratio of average top 500 pixel intensity, compared to 0 min, is indicated (d). Graphs show average ± standard error. *, $p < 0.05$ according to Student's *t*-test compared to mock (medium with water as used to dissolve CEP5p) or Col-0. **(f)** Representative Western blot of IAA19:HA levels (anti-HA) in 10 day old seedlings grown in the presence of 5 μM CEP5p^{Hyp} or 5 μM mCEP5p^{Hyp}.

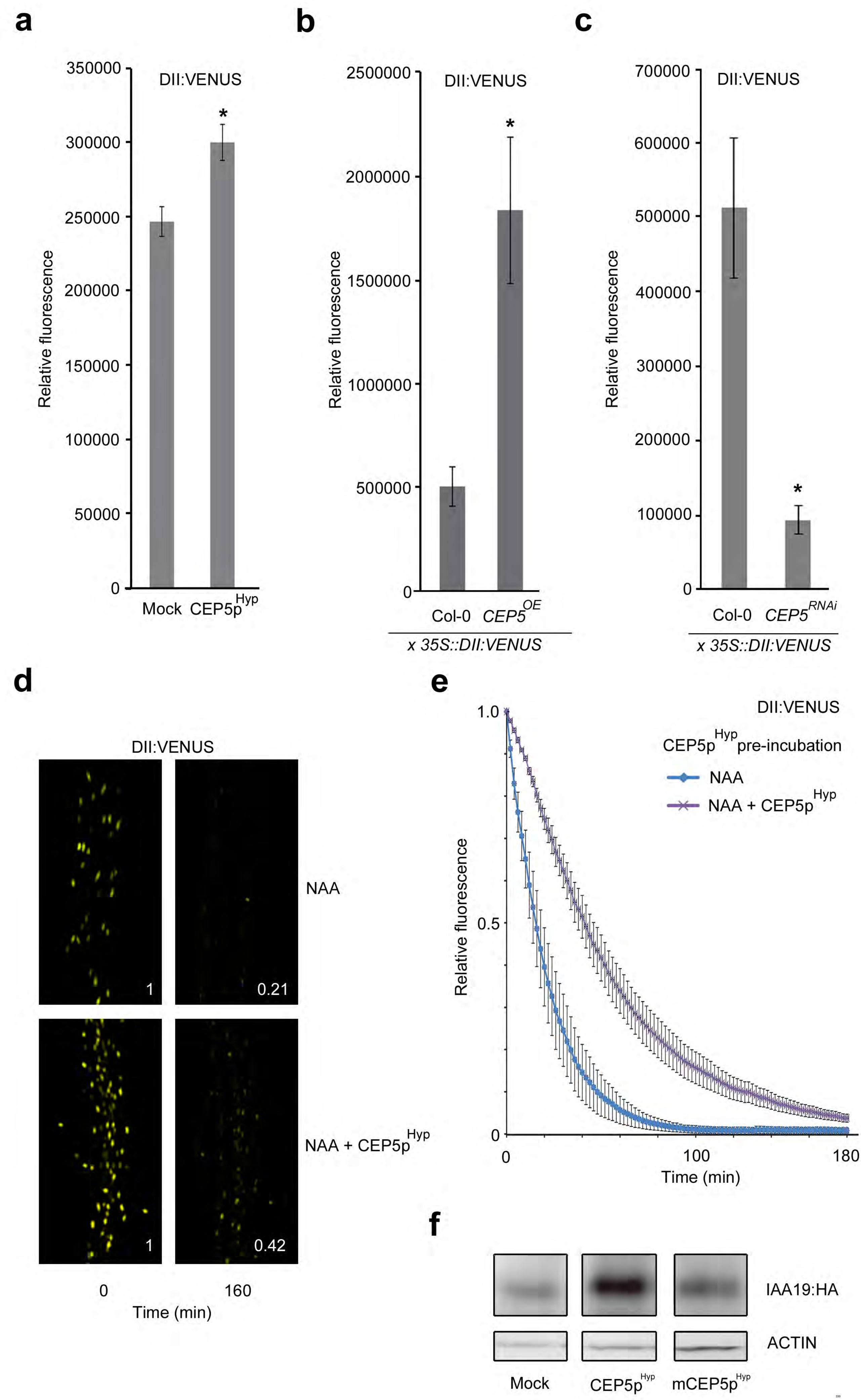
Fig. 6. Role of XIP1 in lateral root initiation and auxin response. (a-b) *XIP1* expression (monitored through *GUS* expression in a *pXIP1::GUS* transgenic line) in the root tip (a) and in the basal meristem on transverse section (b). P, phloem; X, xylem; Pe, pericycle; En, endodermis; b.m., basal meristem. **(c)** Lateral root stages I to VII according to ref. [50] in Col-0 and *xip1-1* at 5 days after germination (n ≥ 14). E, emerged lateral roots; NE, non-emerged lateral roots; Total, total lateral roots. Graph shows average ± standard error. *, $p < 0.05$ according to Student's *t*-test compared to Col-0. The % reduction in total lateral root density is indicated. **(d)** Auxin-inducible expression of *PIN1* and *ARF19* (as determined through qRT-PCR analysis) in Col-0 and *xip1-1* 7 day old seedling roots treated with mock (DMSO) or 1 μM NAA for indicated time. Graph shows average ± standard error of 3 biological repeats. Fold change difference in reduced auxin-inducibility is indicated. *, $p < 0.05$ according to Student's *t*-test compared to mock.

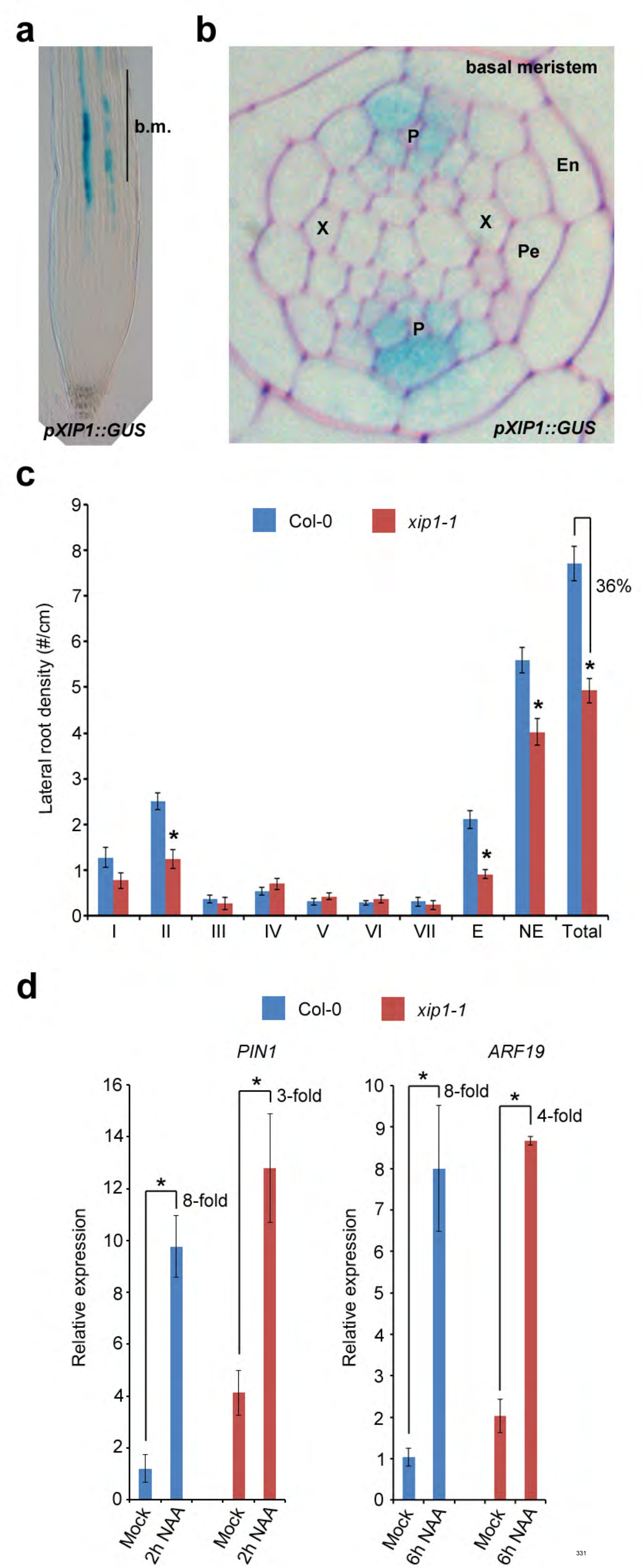

a
pCEP5::GUS
b
pCEP5::GUS
c
pCEP5::GUS
d
b.m.
pCEP5::GUS
e
basal meristem
En
P
X
X
Pe
P
pCEP5::GUS
f
P
X
X
pCEP5::GUS
g
Pe
P
X
X
P
En
C
pCEP5::GUS
h
CEP5
Relative expression level
1
0
0
0.01
0.1
1
NAA (μM)


a
Stage I
Col-0
CEP5RNAi
Average density (#/cm)
Days After Germination
b
Col-0
CEP5RNAi
% seedlings at given stage
No LRI
Stage I
Stage II
Stage III
Stage IV
Stage V
Stage VI
Hours post gravistimulus
c
CEP5OE 1-15-4
Col-0
d
65%
Primary root length (cm)
Col-0
CEP5OE 1-15-4
e
Col-0
CEP5OE 1-15-4
22%
Lateral root density (#/cm)
Lateral root stage
f
CEP5pPro DFRPTTPGHSPGIGH
CEP5pHyp DFR{HYP}TT{HYP}GHS{HYP}GIGH
mCEP5pHyp DFL{HYP}HT{HYP}GHV{HYP}GISH
g
Mock
CEP5pHyp
Col-0
Col-0
h
52%
Primary root length (cm)
Mock
mCEP5pHyp
CEP5pHyp
i
Mock
10 nM CEP5pHyp
100 nM CEP5pHyp
19%
29%
Lateral root density (#/cm)
Lateral root stages


Mock
mCEP5p Hyp
CEP5p Hyp
CEP5 OE
-1.4
0
1.4


a
Primary root length (%)
Col-0
CEP5OE
NAA (nM)
b
Lateral root density (%)
Col-0 CEP5OE
NAA (nM)
c
pDR5::GUS
Mock
CEP5pHyp
d
pDR5::GUS
Col-0
CEP5OE
e
pDR5::LUC
Mock
CEP5pHyp
f
pDR5::LUC
Mock
CEP5pHyp
Frequency
Time (hrs)
g
pDR5::LUC
Total relative intensity/cm
Mock
CEP5pHyp
h
Col-0 + 1 µM NAA / Col-0
CEP5OE + 1 µM NAA / CEP5OE
Ratio of relative expression (fold change)
30%
16%
22%
26%
IAA18
LBD16
PIN1
LBD29
i
DII:VENUS
% fluorescence of total RID
Mock
CEP5pHyp
Upper
Mock
CEP5pHyp
Lower
hrs post gravistimulus
j
Col-0
Angle (°)
Mock
CEP5pHyp
mins post gravistimulus


a
DII:VENUS
Relative fluorescence
350000
300000
250000
200000
150000
100000
50000
0
*
Mock
CEP5p Hyp
b
DII:VENUS
Relative fluorescence
2500000
2000000
1500000
1000000
500000
0
*
Col-0
CEP5 OE
x 35S::DII:VENUS
c
DII:VENUS
Relative fluorescence
700000
600000
500000
400000
300000
200000
100000
0
*
Col-0
CEP5 RNAi
x 35S::DII:VENUS
d
DII:VENUS
NAA
1
0.21
NAA + CEP5p Hyp
1
0.42
0
160
Time (min)
e
DII:VENUS
CEP5p Hyp pre-incubation
NAA
NAA + CEP5p Hyp
Relative fluorescence
1.0
0.5
0
0
100
180
Time (min)
f
IAA19:HA
ACTIN
Mock
CEP5p Hyp
mCEP5p Hyp


a
b.m.
pXIP1::GUS
b
basal meristem
P
En
X
X
Pe
P
pXIP1::GUS
c
Col-0
xip1-1
Lateral root density (#/cm)
36%
I
II
III
IV
V
VI
VII
E
NE
Total
d
Col-0
xip1-1
PIN1
ARF19
Relative expression
8-fold
3-fold
8-fold
4-fold
Mock
2h NAA
Mock
2h NAA
Mock
6h NAA
Mock
6h NAA

APPENDIX XI

CEP5 and XIP1/CEPR1 regulate lateral root initiation in *Arabidopsis.*

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CEP5 and XIP1/CEPR1 regulate lateral root initiation in *Arabidopsis*

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Running title: CEP5 and lateral root initiation

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Short statement: We identified C-TERMINALLY ENCODED PEPTIDE 5 (CEP5) as a novel, auxin-repressed and phloem pole-expressed signal assisting in the formation of lateral roots.

ABSTRACT

Roots explore the soil for water and nutrients through the continuous production of lateral roots. Lateral roots are formed at regular distances in a steadily elongating organ but how future sites for lateral root formation become established is not yet understood. Here, we identified C-TERMINALLY ENCODED PEPTIDE 5 (CEP5) as a novel, auxin-repressed and phloem pole-expressed signal assisting in the formation of lateral roots. In addition, based on genetic and expression data, we found evidence for the involvement of its proposed receptor, XYLEM INTERMIXED WITH PHLOEM 1 (XIP1)/CEP RECEPTOR 1 (CEPR1), during the process of lateral root initiation. In conclusion, we report here on the existence of a peptide ligand−receptor kinase interaction that impacts lateral root initiation. Our results represent an important step towards the understanding of the cellular communication implicated in the early phases of lateral root formation.

Key words: CEP5, XIP1, lateral root initiation, Arabidopsis, post-translationally modified peptide, receptor kinase

INTRODUCTION

Coordinated positioning and development of lateral roots is central to shape root system architecture allowing plants to adapt their below-ground organs for optimal soil exploration (De Smet, 2012; Kong *et al.*, 2014; Smith and De Smet, 2012; Tian *et al.*, 2014). Lateral root primordia are formed from approximately three pairs of xylem pole pericycle (XPP) cells arranged in neighbouring cell files that undergo asymmetric cell division and subsequently form a new organ (De Smet *et al.*, 2007; De Smet *et al.*, 2006; Dubrovsky *et al.*, 2001; Kurup *et al.*, 2005; Lavenus *et al.*, 2013; Péret *et al.*, 2009). In the basal meristem, close to the primary root tip and before any asymmetric cell division, a periodic transcriptional mechanism specifies pre-branch sites that are competent to form lateral roots in a regular pattern (De Smet *et al.*, 2007; Moreno-Risueno *et al.*, 2010; Van Norman *et al.*, 2013; Xuan *et al.*, 2015; Xuan *et al.*, 2016).

Several plant hormones have been shown to affect root architecture among which auxin has been granted a central role (Lau *et al.*, 2008; Vanneste and Friml, 2009). In addition, a number of transcription factors and miRNAs have been shown to affect lateral root development (Satbhai *et al.*, 2015). However, several recent studies are beginning to reveal the importance of different classes of small signalling peptides during the process of lateral root development (Araya *et al.*, 2014; Bergonci *et al.*, 2014; Czyzewicz *et al.*, 2015; Delay *et al.*, 2013; Fernandez *et al.*, 2013; Fernandez *et al.*, 2015; Kumpf *et al.*, 2013; Ohyama *et al.*, 2008). However, in *Arabidopsis*, very few small signalling peptides have been linked to a receptor (Czyzewicz *et al.*, 2013; Murphy *et al.*, 2012), and very few receptors involved in lateral root development have been identified (Araya *et al.*, 2014; Cho *et al.*, 2014; De Smet *et al.*, 2008; De Smet *et al.*, 2009; Kumpf *et al.*, 2013; Tabata *et al.*, 2014; Wierzba and Tax, 2013). Recently, the leucine-rich repeat (LRR) receptor kinases XYLEM INTERMIXED

WITH PHLOEM 1 (XIP1) / C-TERMINALLY ENCODED PEPTIDE (CEP) RECEPTOR 1 (CEPR1, At5g49660) and CEPR2 (At1g72180) were proposed to act as receptors for CEP1 and other members of the CEP family (Tabata *et al.*, 2014). Both XIP1/CEPR1 and CEPR2 contain a short secretory signal peptide sequence, an N-terminal extracellular LRR receptor domain with 21 LRR-repeats, a single helical transmembrane region and a C-terminal cytoplasmic serine/threonine kinase domain. It was previously shown that a loss-of-function *xip1* mutant displays anthocyanin accumulation in the leaves, xylem-like lignification of phloem in inflorescence stems, disrupted xylem vessel formation, phloem cells sometimes located adjacent to xylem cells, and shorter inflorescence stems (Bryan *et al.*, 2012) and that the *cepr1 cepr2* double mutant displays a pleiotropic phenotype, including pale green leaves, smaller rosette leaves, shorter floral stems, anthocyanin accumulation, enhanced lateral root elongation, decreased expression of nitrate transporters and reduced nitrate uptake activity (Tabata *et al.*, 2014). Interestingly, the *Medicago truncatula compact root architecture* (*cra2*) mutant is also affected in its root system architecture, and CRA2 was shown to be closely related to XIP1 (Huault *et al.*, 2014).

The post-translationally modified CEP family members contain an N-terminal signal peptide sequence and a C-terminal conserved CEP domain from which the mature 15 amino acid peptide is processed (Delay *et al.*, 2013; Ohyama *et al.*, 2008; Roberts *et al.*, 2013; Tabata *et al.*, 2014). Some members of the (CEP) family have already been shown to regulate lateral root development (Delay *et al.*, 2013; Mohd-Radzman *et al.*, 2015a; Ohyama *et al.*, 2008), but in this work we functionally characterized *C-TERMINALLY ENCODED PEPTIDE5* (*CEP5*, At5g66815) in the context of lateral root initiation. Furthermore, we explored the involvement of XIP1/CEPR1 in lateral root initiation, and could show that CEP5 and XIP1 are co-expressed during early stages of lateral root initiation, and that both affect this process.

MATERIALS AND METHODS

Plant materials

The following transgenic lines and mutants were described previously: *pCEP5::NLS:GFP:GUS*, *CEP5*^{OE} and *CEP5*^{RNAi} (Roberts et al., 2013), *xip1-1* and *pXIP1::GUS* (Bryan et al., 2012).

Plant growth and treatment conditions

Unless mentioned otherwise, seedlings were grown at 21 °C under continuous light (110 µE m^{-2} s^{-1} photosynthetically active radiation, supplied by cool-white fluorescent tungsten tubes, Osram) on square Petri plates (12 x 12 cm) containing 50 ml solid half-strength MS growth medium supplemented with sucrose (per liter: 2.15 g MS salts, 0.1 g *myo*-inositol, 0.5 g MES, 10 g sucrose, 8 g plant tissue culture agar; pH adjusted to 5.7 with KOH). For peptide treatments, media was supplemented with CEP5p^{Pro} (DFRPTTPGHSPGIGH), CEP5p^{Hyp} (DFR{HYP}TT{HYP}GHS{HYP}GIGH), or mCEP5p^{Hyp} (DFL{HYP}HT{HYP}GHV{HYP}GISH) peptide to a concentration as indicated in the text and/or figure legends. Synthetic peptides (CEP5p^{Pro}, CEP5p^{Hyp} and mCEP5p^{Hyp}) were obtained from GenScript (www.genscript.com/peptide-services.html?src=home), and were supplemented to growth medium with concentrations as indicated in the text and/or figure legends. For auxin treatments, media was supplemented with indole-3-acetic acid (IAA) or 1-naphthaleneacetic acid (NAA) to a concentration as indicated in the text and/or figure legends.

Transcriptome profiling data

The naxillin-treatment transcriptome data from (De Rybel *et al.*, 2012) can be searched in the Lateral Root Initiation eFP Browser (bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi?dataSource=Lateral_Root_Initiation) (Winter *et al.*, 2007).

Primary and lateral root phenotyping

At the indicated time, images of plates with seedlings were taken and roots were measured using ImageJ (http://rsbweb.nih.gov/ij/index.html) or FIJI software (Schindelin *et al.*, 2012). For detailed staging of lateral roots, samples were cleared as described previously (Malamy and Benfey, 1997) and analysed by differential interference contrast microscopy (Olympus BX53).

Histochemical GUS assays

For GUS assays, plants were put overnight in 90% acetone, then transferred to a GUS-solution [1 mM X-Glc, 0.5% (v/v) dimethylformamide (DMF), 0.5% (v/v) Triton X-100, 1 mM EDTA (pH 8), 0.5 mM potassium ferricyanide ($K_3Fe(CN)_6$), 0.5% potassium ferrocyanide ($K_4Fe(CN)_6$), 500 mM phosphate buffer (pH 7)] and incubated at 37 °C for GUS staining, and finally washed in 500 mM phosphate buffer (pH 7). For microscopic analysis, samples were cleared with 90% lactic acid or as described previously (Malamy and Benfey, 1997). Samples were analyzed by differential interference contrast microscopy (Olympus BX53) and a stereomicroscope (Leica MZ16). For anatomical analysis (microtome transversal

sectioning) of GUS-stained roots, stained samples were processed as described previously (De Smet *et al.*, 2004).

Real-time qRT-PCR analyses

For the analysis of *CEP5* expression, RNA was extracted by first performing an RNA extraction with TRI Reagent® from Sigma-Aldrich according to the manufacturer's protocol, followed by an extra RNA extraction procedure with the Plant RNeasy Mini kit from Qiagen according to the manufacturer's protocol to further clean up the RNA. Next, 1 μg of total RNA was used for cDNA synthesis using the iScript cDNA synthesis kit from BIORAD according to the manufacturer's protocol. The real-time qRT-PCR reaction was carried out on the LightCycler 480 from Roche Applied Science with the LightCycler 480 SYBR Green I Master Mix from Roche Applied Science. The expression of *CEP5* (CCATGGACGAACCCTAAAAG and TGCCATCATCGTCTTGCTAT) was determined using at least three biological repeats and the reference genes *EEF-1α4* (CTGGAGGTTTTGAGGCTGGTAT and CCAAGGGTGAAAGCAAGAAGA) and *At2g32170* (GGACCTCTGTTGTATCATTTTGCG and CAACCCTCTTTACATCCTCCAAAC).

SRM analysis of the CEP5 peptide

For SRM experiments, the CEP5 peptide containing an isoleucine residue with heavy, stable isotopes, NH_2-DFRP<hydroxy>TTP<hydroxy>GHSP<hydroxy>GI($^{13}C_6{}^{15}N$)GH-COOH, was in-house synthesized by Fmoc (N-(9-fluorenyl)methoxycarbonyl) chemistry on a 433A peptide synthesizer (Applied Biosystems, Framingham, MA, USA). Frozen five-day-old

35S::CEP5 seedlings were ground to a fine powder in liquid N_2 and proteins were extracted in 50 mM triethylammonium bicarbonate (TEAB) buffer containing 8 M urea and the suggested amounts of protease and phosphatase inhibitors according to the manufacturer's instructions (cOmplete protease inhibitor cocktail tablet and PhosStop phosphatase inhibitor cocktail tablet, Roche). After determining the protein concentration using the Bradford assay and diluting the protein extract twice with 50 mM TEAB buffer, a total of 500 µg of protein material was filtered over a 3 kDa cut-off filter (Pall Nanosep® centrifugal devices, Sigma-Aldrich) to retain only peptides with masses less than 3 kDa in the filtrate. This peptide mixture was spiked with 10 pmol of the synthetic heavy CEP5 peptide and vacuum dried. Next, the sample was re-dissolved in 2% acetonitrile (ACN) with 0.1% trifluoroacetic acid (TFA) and used for SRM analysis. SRM analysis was performed on an Ultimate 3000 RSLC nano HPLC system (Thermo Fisher scientific, Bremen, Germany) coupled to a TSQ Vantage (Thermo Fisher Scientific, Bremen, Germany). The nano-LC system was configured with a trapping column (made in-house, 100 µm internal diameter (I.D.) x 20 mm, 5 µm beads, C18 Reprosil-HD (Dr. Maisch, Ammerbuch-Entringen, Germany)) and an analytical column (made in-house, 75 µm I.D. x 150 mm, 3 µm beads, C18 Reprosil-HD (Dr. Maisch, Ammerbuch-Entringen, Germany)). The loading solvent consisted of 0.1% TFA in 2:98 ACN:H_2O, and the nano-LC was run with 0.1% formic acid as nano-LC solvent A and 0.1% formic acid in 80:20 ACN:H_2O as nano-LC solvent B. The needle voltage in the nano-ESI source was set at 1300 V and the capillary temperature at 275°C. Of each sample, 5 µl was injected using a full loop injection. Injection was at 10 µl/min in loading solvent. After loading, the trapping column was flushed for 4 min in order to pre-concentrate the components while removing buffer components, before it was put in-line with the analytical column. Compounds were eluted at 300 nl/min with an ACN gradient of 30 min from 2% to 35% of nano-LC solvent B. The column was washed with 90% of nano-LC solvent B for 1

min and equilibrated with nano-LC solvent A for 9.5 min before analysis of the next sample. A dwell time of 120 ms for each transition was applied. Seven transitions were monitored for both the heavy and the light form of the CEP5 peptide with the doubly charged precursor as the first mass filter. Data analysis was performed through the Skyline software (MacLean *et al.*, 2010).

RESULTS AND DISCUSSION

Focused transcript profiling data identifies *CEP5* as a putative regulator of lateral root development

Since the plant hormone auxin is a major regulator of primary root growth and lateral root development (Lavenus *et al.*, 2013; Overvoorde *et al.*, 2010), several transcript profiling studies based on auxin treatments have been performed in order to identify the molecular players involved (De Smet *et al.*, 2008; Himanen *et al.*, 2004; Vanneste *et al.*, 2005). However, because of the pleiotropic effects caused by exogenous auxin application, such datasets risk compromising the spatiotemporal resolution required when looking for components specific for a single developmental process. To circumvent this, we searched for putative novel early lateral root formation regulators by screening a dataset obtained through a highly focused transcript profiling analysis on seedling roots treated with the synthetic molecule naxillin. Naxillin specifically induces an auxin response in the basal meristem associated with lateral root initiation through enhancing indole-3-butyric acid (IBA) to indole-3-acetic acid (IAA) conversion in the root cap (De Rybel *et al.*, 2012). Driven by the recurrent programmed cell death of the outermost lateral root cap cells, a periodic input of the converted auxin into the main root contributes to a fine-tuned mechanism that results in an

evenly spaced lateral root distribution pattern (Xuan *et al.*, 2016). Importantly, through its local activity, naxillin does not display the typical pleiotropic effects of exogenous application of auxin or auxin-like molecules (De Rybel *et al.*, 2012). In order to identify novel putative early lateral root formation regulators, seedlings were grown for 72 hours on growth medium supplemented with the polar auxin transport inhibitor N-1-naphthylphthalamic acid (NPA), which prevents lateral root initiation, followed by a transfer to growth medium supplemented with naxillin to synchronously trigger the priming event in the basal meristem. In a genome-wide transcript profiling analysis, we identified *CEP5* (At5g66815) as differentially early up-regulated between non-treated and naxillin-treated seedling roots (De Rybel *et al.*, 2012) (**data not shown**; see Lateral Root Initiation eFP Browser; (Winter *et al.*, 2007)). The *CEP5* gene encodes a small protein of 105 amino acids and contains a conserved 15 amino acid C-terminal CEP domain that gives rise to a small signalling peptide (Ohyama *et al.*, 2008; Roberts *et al.*, 2013; Tabata *et al.*, 2014).

***CEP5* expression is regulated by auxin**

Since *CEP5* is transcriptionally regulated following naxillin treatment, we subsequently checked if *CEP5* expression is also auxin-regulated. Treatment of wild-type roots with different concentrations of the synthetic auxin 1-naphthalene-acetic acid (NAA) or with indole-3-acetic acid (IAA) revealed that *CEP5* expression was down regulated by auxin (**Figure 1A-B).** These results suggested that *CEP5* expression is (directly or indirectly) regulated by auxin.

***CEP5* expression is associated with early stages of lateral root development**

Based on its naxillin-regulated expression profile, CEP5 represents a candidate peptide to be involved in the early developmental steps toward lateral root development. Using a *pCEP5::NLS:GFP:GUS* reporter line (Roberts *et al.*, 2013), we observed regularly spaced patches of *CEP5* expression associated with lateral root primordia, confirming its potential involvement in this process (**Figure 2A-C**). We did not detect *CEP5* expression in the primary root stem cell niche, however *CEP5* was expressed in the basal meristem (**Figure 2D**). The latter is important in the context of lateral root initiation as this region is defined as part of the oscillation zone where prebranch sites are established by the input of auxin derived from the lateral root cap (De Smet *et al.*, 2007; Moreno-Risueno *et al.*, 2010; Xuan *et al.*, 2016). Tissue-specific analyses showed that both in the basal meristem and during early stages of lateral root development, *CEP5* was predominantly expressed in the phloem pole-associated pericycle (PPP) cells, but also – although weaker – in the adjacent phloem (**Figure 2E-G, Supplementary Figure S1 and Supplementary Movie 1**). This *CEP5* expression pattern does not overlap with the well-documented sites of high auxin response in the primary root or during lateral root initiation, which in *Arabidopsis* occurs in xylem pole-associated pericycle (XPP) cells (De Smet *et al.*, 2007). To check if the expression pattern of *CEP5* is perturbed under conditions of altered auxin response in the XPP cells, the *pCEP5::NLS:GFP:GUS* reporter line was grown on NPA. Under these conditions, we did not observe any change in the *CEP5* expression pattern (such as radial expansion) compared to control conditions (**Supplementary Figure S1**). Taken together, *CEP5* is negatively regulated by auxin and specifically expressed in the PPP cells that are closely associated with the lateral root development process, suggesting a negative correlation with auxin activity. However, what the specific cellular threshold is, is at the moment not known.

Altering *CEP5* expression levels affects root architecture

Given the spatial (appearing in common regions of the root, although not in the same cells) and temporal (being induced at the same time points) correlation of *CEP5* expression with lateral root initiation and development, we assessed if CEP5 loss-of-function affected this process. A CaMV *35S* promoter-driven *CEP5* RNAi knockdown line ($CEP5^{RNAi}$) (Roberts *et al.*, 2013) displayed a significant difference in primary root length compared to the control (**Figure 3A-B**). In addition, detailed analyses of lateral root initiation in this $CEP5^{RNAi}$ line revealed an increased number of stage I and II lateral root primordia compared to the control (**Figure 3C and Supplementary Figure S2**). Additionally, in a root bending assay (Péret *et al.*, 2012), the $CEP5^{RNAi}$ line progressed faster through lateral root developmental stages than wild type (**Figure 3D**). These loss-of-function data, together with the *CEP5* expression pattern, indicate that CEP5 plays a role in early lateral root initiation events.

Next, we analysed a line with CaMV *35S* promoter-driven constitutive overexpression of *CEP5* ($CEP5^{OE}$) (Roberts *et al.*, 2013), which displayed shorter primary roots (similar to other independent $CEP5^{OE}$ lines) as compared to wild type (**Figure 4A-B and Supplementary Figure S2**). Furthermore, the $CEP5^{OE}$ line displayed a decrease in total lateral root density, with fewer non-emerged lateral roots, compared to wild type (**Figure 4C**). Detailed analyses of lateral root developmental stages showed that this was mainly due to fewer initiation events (**Figure 4C and Supplementary Figure S3**). At later stages of lateral root development, we also observed closely spaced lateral root primordia in $CEP5^{OE}$ lines, which we never observed as such in wild type roots (**Figure 4D**). This gain-of-function approach further suggested that CEP5 impacts root architecture, but does not exclude that this is an indirect and/or non-specific effect due to ectopic expression.

CEP5 gives rise to CEP5p^{Hyp}

CEP5 has a conserved C-terminal CEP domain, containing three proline (Pro) residues and a predicted N-terminal signal peptide cleavage site that undergoes proteolytic processing to form a mature CEP5 peptide of 15 amino acids (CEP5p) (Roberts *et al.*, 2013; Tabata *et al.*, 2014) (**Figure 5A**). However, small signalling peptides are often post-translationally modified, thereby modulating – amongst others – the binding ability and specificity of peptides to their targets (Murphy *et al.*, 2012). In this context, it was previously shown that members of the CEP family give rise to a peptide containing hydroxyproline (Hyp) residues (Tabata *et al.*, 2014). To confirm that a 15 amino acid CEP5 peptide with three Hyp residues (CEP5p^{Hyp}) (**Figure 5A**) is indeed present in seedlings overexpressing *CEP5*, we performed selected reaction monitoring (SRM) on a *CEP5*^{OE} line. SRM is a mass spectrometry technique that allows detection and quantification of specific (low abundant) peptides in total protein preparations (Picotti and Aebersold, 2012). Indeed, in the *CEP5*^{OE} proteome spiked with a chemically synthesized version of CEP5p^{Hyp} containing an isoleucine residue with heavy, stable isotopes, transitions for both the heavy, spiked-in CEP5p^{Hyp} and the light, naturally occurring CEP5^{Hyp} peptide could be detected (**Figure 5B-E**). These results supported that a CEP5 peptide with three Hyp residues can be present *in planta*.

Synthetic CEP5 peptide affects root architecture

Based on previous studies (Tabata *et al.*, 2014) and the above-described results, a synthetic CEP5p^{Hyp} peptide was generated for further analysis of CEP5 function (**Figure 5A**). To assess the activity of synthesized CEP5p^{Hyp}, we first analysed its effect on primary root growth, which has previously been shown to be a straightforward, although possibly nonspecific,

assay to test the activity of small post-translationally modified (CEP) peptides (Delay *et al.*, 2013). Indeed, seedlings grown in the presence of CEP5p^{Hyp} (also at low concentrations) displayed shorter roots compared to the mock-treated control and compared to a synthetic variant with 4 randomly positioned, but not very unlikely amino acid substitutions based on a BLOSUM62 substitution matrix within the 15 amino acid CEP5 peptide sequence, while retaining the Hyp residues at the same positions (mCEP5p^{Hyp}) (**Figure 5A and 6A-B and Supplementary Figure S4**). Next, we addressed the effect of synthetic CEP5p^{Hyp} on lateral root formation. Seedlings grown in the presence of different low concentrations of CEP5p^{Hyp} displayed a decreased total lateral root density, which is mainly due to a significant reduction in lateral root initiation events (**Figure 6C and Supplementary Figure S3**). Conversely, this did not occur in mCEP5p^{Hyp}-treated seedlings (**Supplementary Figure S3**). When lateral root initiation occurred, we occasionally observed regions of ectopic and/or aberrant pericycle cell divisions (observed in 10 out of 149 lateral root primordia of 8 CEP5p^{Pro}-treated seedlings, while this did not occur in untreated wild type) resulting in malformed lateral root primordia or closely spaced primordia in CEP5p$^{Pro/Hyp}$-treated seedlings, which differed from regularly spaced lateral roots in wild type (**Figure 6D-F**). Taken together, the similarities in primary and lateral root phenotypes between CEP5p treatment and *CEP5*^{OE} indicate that the chemically synthesised CEP5p^{Hyp} has the same bioactivity as the overexpressed *CEP5*. These results further support a role of CEP5p^{Hyp} in lateral root initiation.

The proposed CEP family receptor XIP1/CEPR1 regulates lateral root initiation

Recently XIP1/CEPR1 and CEPR2 were proposed to be the receptors for CEP peptides, including CEP5 (Tabata *et al.*, 2014). However, a role in lateral root initiation for XIP1/CEPR1 and/or CEPR2 was not yet explored. Therefore, we performed detailed analyses

of a previously described *pXIP1::GUS* line (Bryan *et al.*, 2012) and we showed that *XIP1/CEPR1* is expressed in the root from the basal meristem onward (**Figure 7A**), a pattern that overlaps with *CEP5* expression (**Figure 2D**). Furthermore, tissue-specific analyses showed that *XIP1/CEPR1* is expressed in the phloem pole pericycle and in the adjacent phloem (**Figure 7B**), confirming the overlap with *CEP5* expression (**Figure 2E**), and is excluded from early stages of lateral root development (**Figure 7C**), similar to *CEP5* (**Figure 2C**). This expression pattern combined with the results from Tabata *et al.* (2014) suggested that XIP1/CEPR1 could be a receptor for CEP5 in the root and therefore might take part in lateral root initiation. To further explore this, we assessed lateral root stages and density of the previously described *xip1-1* mutant (Bryan *et al.*, 2012). This revealed a reduced total lateral root density in *xip1-1* in comparison with the control, which seemed mainly due to a reduction in stage I and II lateral root primordia and – in part – to fewer emerged lateral roots (**Figure 8A and Supplementary Figure S3**), suggesting that XIP1 is a positive regulator of lateral root initiation and development.

To further evaluate an interaction between CEP5 and XIP1, we explored to what extent *xip1-1* is (in)sensitive to CEP5p^{Hyp} treatment. This revealed that, compared to the control, *xip1-1* is less or not sensitive to CEP5p^{Hyp} with respect to primary root growth (**Figure 8B**) or number of emerged lateral roots, respectively (**Figure 8C**). These data – together with the biochemical evidence from Tabata *et al.* (2014) – support that CEP5 and XIP1 are a peptide ligand−receptor kinase pair in the context of lateral and primary root development. However, in general, the mutant phenotypes of the genes encoding the peptide ligand and its receptor are very similar (Butenko *et al.*, 2009; Czyzewicz *et al.*, 2013; Kumpf *et al.*, 2013; Murphy *et al.*, 2012). But, in our case, the *xip1-1* root architecture phenotype is similar to *CEP5^{OE}* or CEP5p^{Hyp}-treated seedlings and opposite to *CEP5^{RNAi}* lines (**Figures 4 and 6**), possibly suggesting that CEP5 negatively regulates XIP1 activity (e.g. by acting as an

antagonist) in the context of lateral root initiation. In this context, the fact that CEP5p^{Hyp} had no strong impact on *xip1-1* can also be interpreted as no further CEP5-mediated inhibitory effect if XIP1 is already absent (and hence fully inhibited). Alternatively, CEP5 does not exclusively act via the XIP1 receptor (or close homologs) in regulating root architecture. Furthermore, the observed lateral root phenotypes can be obtained through various mechanisms (e.g. effect on lateral root initiation can impact development of nearby lateral root primordia), and further analyses will be required to fully unravel the developmental and biochemical mechanisms underlying CEP5 and XIP1 action.

CONCLUSION

Previously, a role for CEPs in regulating aspects of root architecture, namely nitrate-dependent lateral root elongation, was proposed. Specifically, CEPs might act as root-derived ascending N-demand signals to the shoot, where their perception by CEPRs leads to the production of a putative shoot-derived descending signal that up-regulates nitrate transporter genes in the roots (Delay *et al.*, 2013; Mohd-Radzman *et al.*, 2015b; Ohyama *et al.*, 2008; Tabata *et al.*, 2014). Here, we provide evidence that CEP5 may also act (likely together with XIP1/CEPR1) during lateral root initiation. Our gain-of-function and knock-down data suggest CEP5 to be part of a lateral root inhibitory mechanism. Faster lateral root development was observed in the *CEP5*^{RNAi} line while overexpression or treatment with the peptide resulted in fewer lateral root initiation events. The observed clustering of lateral roots in later developmental stages in the gain-of-function condition might be a secondary effect. Slowing down lateral root development can interfere with the timely development of auxin sources and therefore retard the draining of auxin from the main root. On its turn, this might

lead to higher auxin levels in the neighbourhood of existing primordia and induce ectopic and/or irregularly patterned primordia.

Finally, it is intriguing that a phloem-derived signal downstream of CEP5 and XIP1/CEPR1 has such an impact on lateral root initiation and development at the xylem pole (**Figure 9**). So far, no mutants have been reported to show lateral root initiation at the phloem poles in *Arabidopsis* (and so far we have also not observed this in loss or gain-of-function *CEP5* or *XIP1* lines) arguing for a strong and complex lateral root inhibition mechanism in this part of the root pericycle. Earlier, a cell cycle inhibitory mechanism, based on the pericycle-specific expression of *KIP-RELATED PROTEIN2* (*KRP2*), a cyclin dependent kinase inhibitor, has been proposed as essential to spatially and temporally allow for lateral root initiation by repressing cell division activity in the entire pericycle except for sites of lateral root initiation (Himanen *et al.*, 2002). In the future, it will be interesting to reveal if there is any direct interaction of CEP5-dependent signalling with the control on cell cycle regulation with respect to lateral root initiation. Additionally, it will be exciting to explore alternative mechanisms on how the phloem-expressed *CEP5* affects lateral root initiation in the xylem pole pericycle cells. At the moment, however, it is not yet possible to reliably visualize CEP5 peptide *in planta* in order to evaluate possible movement to other cells and/or tissues.

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FIGURE LEGENDS

Figure 1. Auxin effect on *CEP5* expression. (A) *CEP5* expression in 7 day old roots following indicated hours of auxin (1 μM IAA) treatment in liquid medium. **(B)** *CEP5* expression in 5 day old root tips of ~5 mm (including the basal meristem) following 2 hours auxin (NAA) treatment at indicated concentrations. *CEP5* levels were analyzed through real-time qRT-PCR. Graphs show average ± standard error of 3 biological replicates. *, $p < 0.05$ according to Student's *t*-test compared to 0 μM NAA or IAA.

Figure 2. *CEP5* expression in the *Arabidopsis* root. Representative pictures for *CEP5* expression (monitored through *GUS* expression in a *pCEP5::NLS:GFP:GUS* transgenic line) in the root: **(A)** in a complete seedling (overstained for illustrative reasons), **(B)** in a part of the root from the seedling depicted in panel A, **(C)** at the site of a lateral root primordium, **(D)** at the root apex, **(E)** in the basal meristem on a transverse section, **(F)** at a site of lateral root formation with the lateral root primordium pointing to the right (outlined with dotted red line), and **(G)** on a transverse section through a lateral root primordium (outlined with dotted red line). Seedlings are 5-6 days after germination. *, lateral root primordium; arrowheads in panel f separate individual cells; P, phloem; X, xylem; Pe, pericycle; En, endodermis; C, cortex; b.m., basal meristem.

Figure 3. Effect of reduced *CEP5* levels on root architecture. (A) Representative picture of $CEP5^{RNAi}$ line and Col-0 at 12 days after germination. **(B)** Quantification of the primary root length 12 days after germination ($n \geq 29$). **(C)** Stage I lateral root primordia at indicated seedlings age ($n = 10$). **(D)** Progression through lateral root stages at indicated hours post gravistimulus ($n \geq 14$). Graphs in B and C show average ± standard error. *, $p < 0.05$ and **, $p < 0.075$ according to Student's *t*-test compared to Col-0. Scale bar, 1 cm.

Figure 4. Effect of increased *CEP5* levels on primary root growth and lateral root development. (A) Representative picture of a *CEP5*^{OE} line and Col-0 at 12 days after germination. **(B)** Quantification of primary root length at 12 days after germination. **(C)** Lateral root stages I to VII (according to Malamy and Benfey, 1997) in Col-0 and a *CEP5*^{OE} line ($n \geq 15$) at 7 days after germination. The % reduction in total lateral root density is indicated. E, emerged lateral roots; NE, non-emerged lateral roots; Total, sum of E and NE. **(D)** Regular and adjacent positioning of lateral roots in wild type (Col-0) and *CEP5*^{OE} seedlings at 14 days after germination, respectively. Red * indicates lateral root. All graphs show average ± standard error of indicated sample numbers. *, $p < 0.05$ according to Student's *t*-test compared to Col-0.

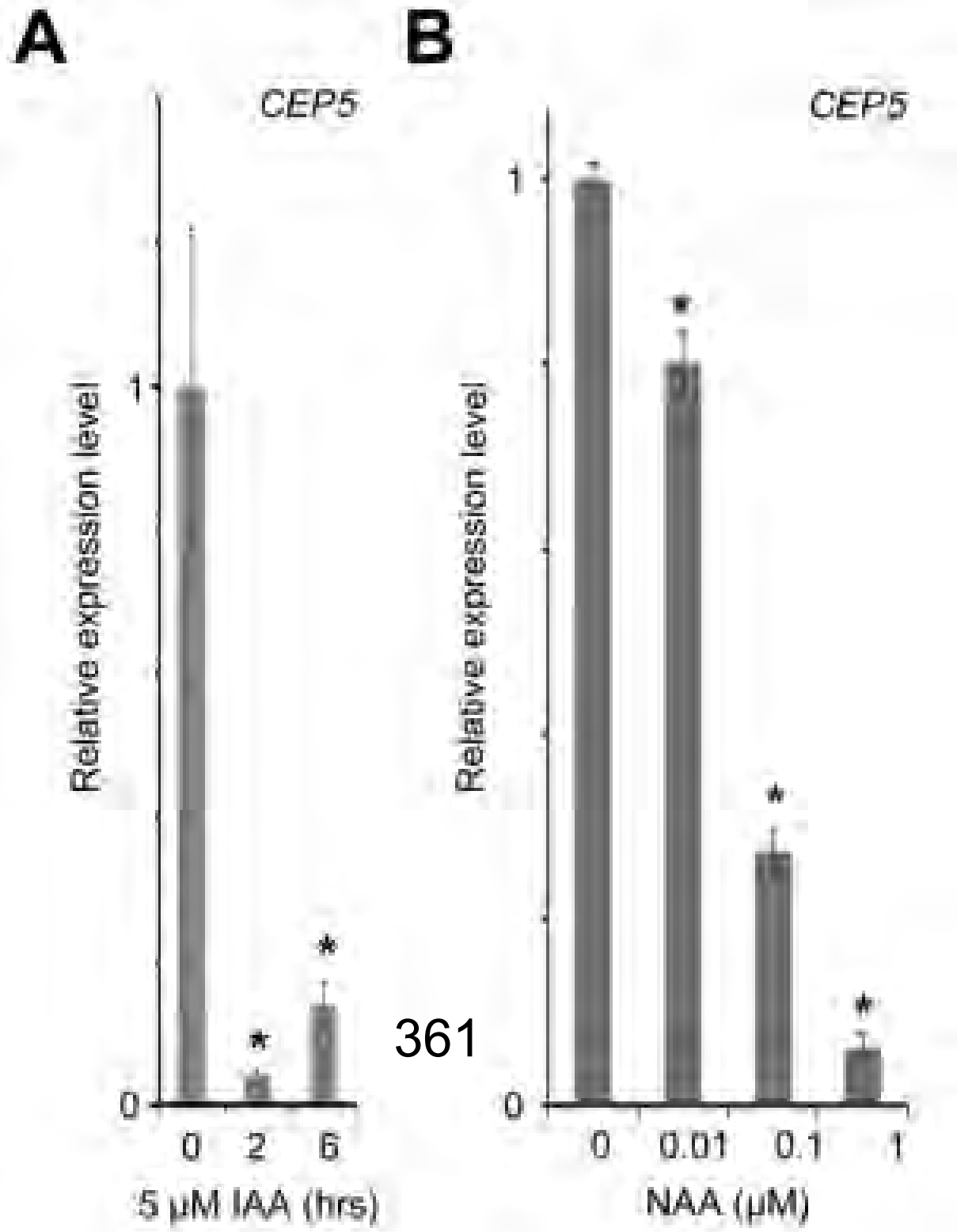
Figure 5. *In planta* CEP5 peptide. (A) Sequences for the synthetic variants of mature 15 amino acid CEP5: unmodified (CEP5p^{Pro}), with proline hydroxylation modifications on P4, P7 and P11 (CEP5p^{Hyp}), and hydroxyprolinated mutated CEP5 sequence with four residue substitutions (R3>L, T5>H, S10>V and G14>S; indicated in red) (mCEP5p^{Hyp}). **(B-E)** SRM analysis of the targeted CEP5 peptide. Characteristic y-type of fragment ions (referred to as transitions), indicated with different colors at the top of each spectrum, were monitored. As a control, the heavy CEP5p^{Hyp} alone was analyzed by SRM, and the transitions of the heavy form **(B)** and the light form **(C)** were monitored. As for the latter, no transitions could be monitored, indicating the high isotopic purity of the heavy peptide. In the *CEP5*^{OE} proteome spiked with heavy CEP5p^{Hyp}, both transitions for the heavy, spiked-in peptide **(D)** and the light, naturally occurring peptide **(E)** could be detected. Red asterisk, CEP5p^{Hyp}.

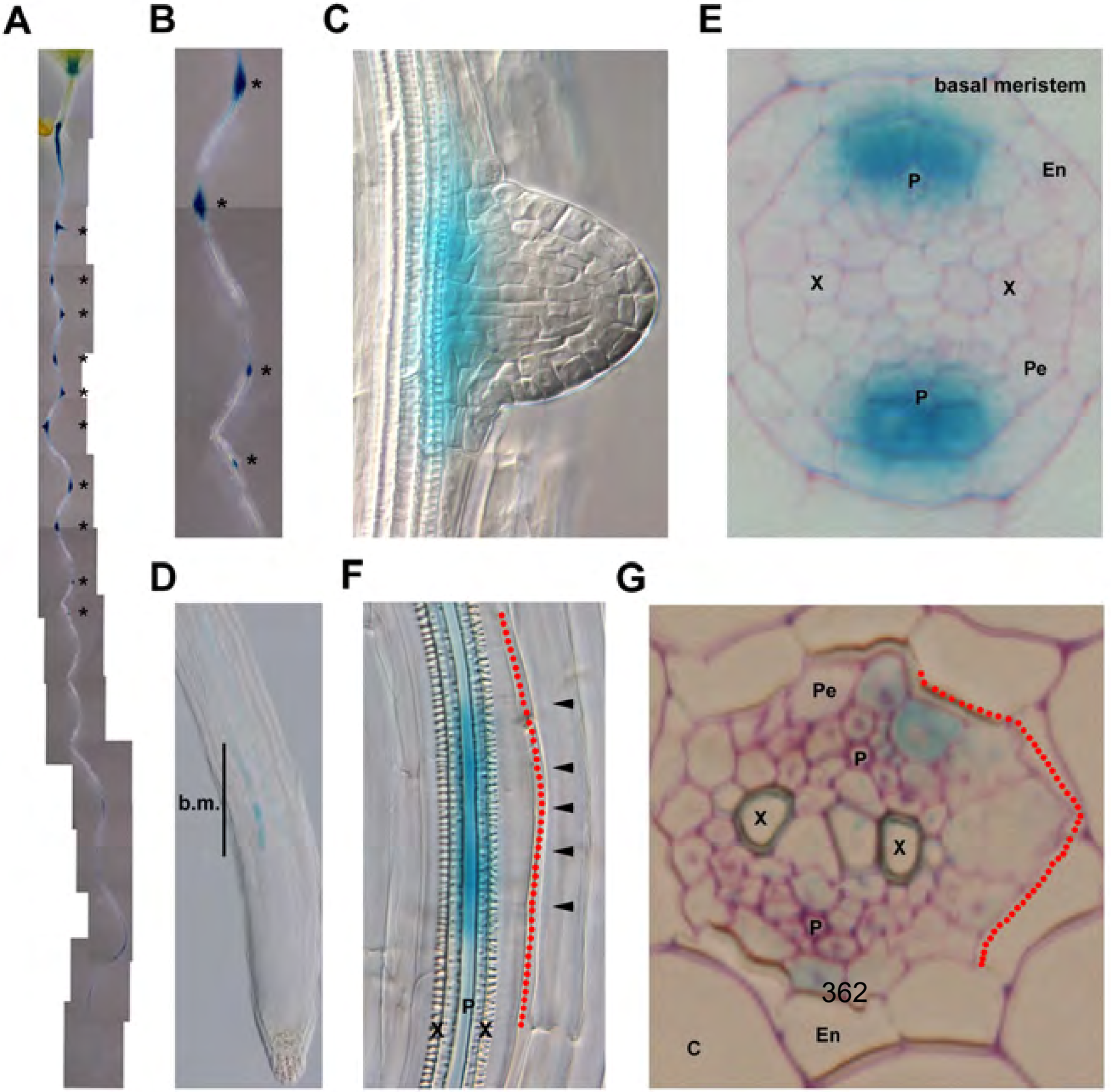
Figure 6. Effect of synthetic CEP5p on primary root growth and lateral root development. (A) Representative pictures of Col-0 *Arabidopsis* seedlings grown on indicated CEP5p^{Hyp} concentrations for 7 days after germination. Scale bar, 1 cm. **(B)** Quantification of primary root length of Col-0 seedlings treated with 5 µM mCEP5p^{Hyp} or 5 µM CEP5p^{Hyp} compared with mock treatment at 7 days after germination (n ≥ 15 per condition). The % reduction in primary root length is indicated. **(C)** Lateral root stages I to VII (according to Malamy and Benfey, 1997) upon mock or CEP5p^{Hyp} treatment at different concentrations at 9 days after germination (data from newly grown root part of 5 day old seedlings transferred to CEP5p^{Hyp} for 4 days, n ≥ 32). E, emerged lateral roots; NE, non-emerged lateral roots; Total, total lateral roots. The % reduction in total lateral root density is indicated. **(D)** Pericycle cell divisions and positioning of lateral roots in mock (left) and 1 µM CEP5p^{Pro} treated Col-0 seedlings (right) (11 days after germination) (Stage II primordia are shown). Observed in 10 out of 149 lateral root primordia (n = 8 seedlings), while this did not occur in untreated wildtype. **(E-F)** Position of lateral roots in 10 µM CEP5p^{Pro}-treated seedlings 14 days after germination (E) and in 5 µM CEP5p^{Hyp}-treated seedlings 12 days after germination (F). Scale bars, 1 cm. All graphs show average ± standard error of indicated sample numbers. *, $p < 0.05$ according to Student's *t*-test compared to Mock. In all cases, mock refers to medium with water as used to dissolve CEP5p.

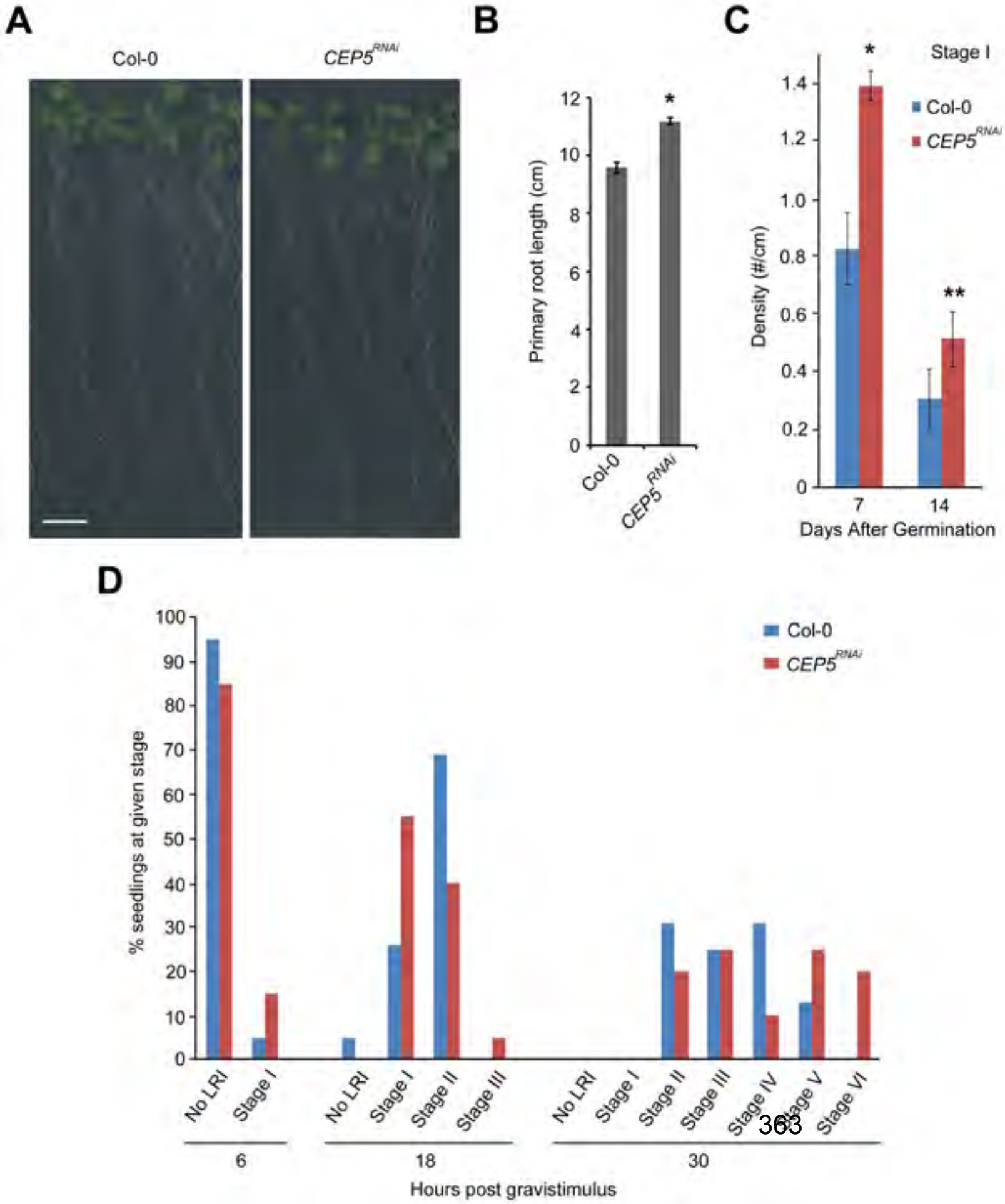
Figure 7. ***XIP1/CEPR1* expression in the root. (A)** Representative picture of *XIP1* expression in the root apex. **(B)** Transverse section through the basal meristem in a *pXIP1::GUS* transgenic reporter line. P, phloem; X, xylem; Pe, pericycle; En, endodermis; b.m., basal meristem**. (C)** Representative pictures for *XIP1* expression in different stages of lateral root development in 7 day-old seedlings. XIP1 expression was monitored through *GUS* expression in a *pXIP1::GUS* transgenic line.

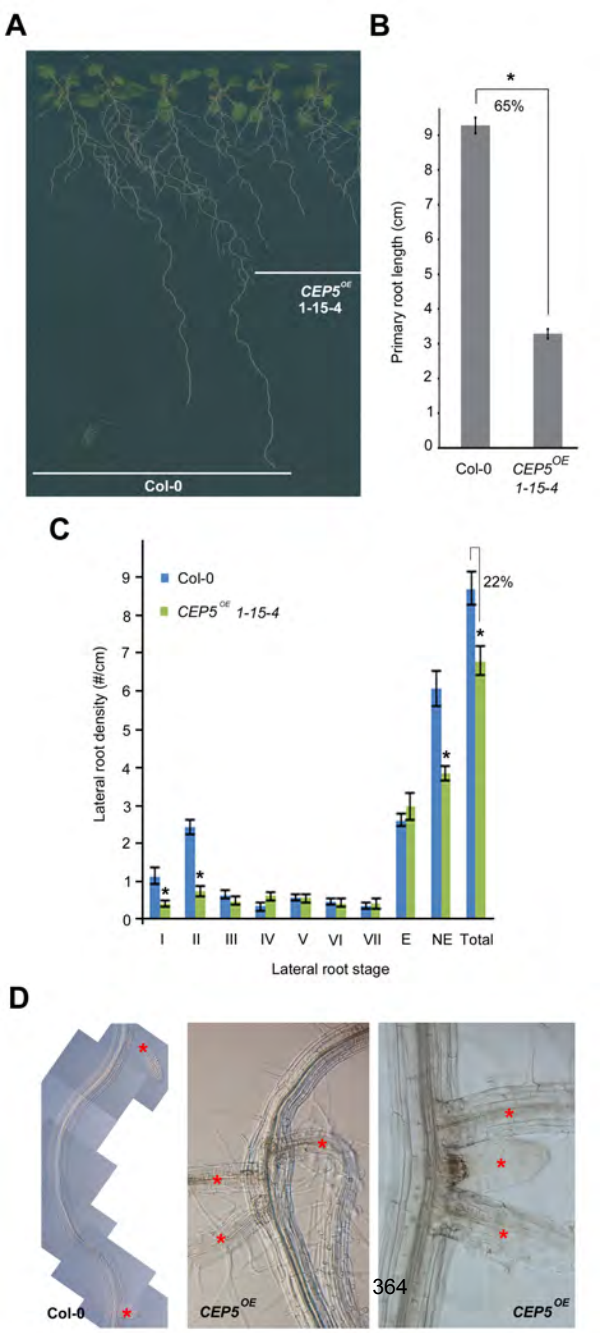
Figure 8. Lateral root phenotype in the *xip1-1* mutant. (A) Lateral root stages I to VII (according to Malamy and Benfey, 1997) in control and *xip1-1* at 5 days after germination (n ≥ 14). **(B-C)** Quantification of primary root length (B) and emerged lateral root number (C) of Col-0 and *xip1-1* seedlings treated with 1 µM CEP5p^{Hyp} or mCEP5p^{Hyp} compared with mock treatment at 10 days after germination (n ≥ 22 per condition). The % reduction in primary root length and lateral root number is indicated. E, emerged lateral roots; NE, non-emerged lateral roots; Total, total lateral roots. Graphs shows average ± standard error. * or #, $p < 0.05$ according to Student's *t*-test compared to Col-0 or mock treatment, respectively. In all cases, mock refers to medium with water as used to dissolve CEP5p.

Figure 9. The data we have – so far – suggest that *CEP5* and *XIP1/CEPR1* are expressed in the phloem pole pericycle (PPP) cells (blue cells, with the highest expression in dark blue, and domain with weaker, variable expression in light blue) associated with sites of lateral root formation and regulate lateral root initiation in the xylem pole pericycle cells (XPP) (orange cells) by a currently unknown mechanism (indicated by ?). Overall, the CEP5 peptide appears to negatively regulate XIP1 (activity). An auxin maximum in the XPP cells promotes lateral root initiation and possibly down regulates *CEP5* expression in these cells. As such, the auxin minimum in the neighbouring PPP cells likely allows *CEP5* expression.


A
CEP5
Relative expression level
1
0
0
2
6
5 μM IAA (hrs)
B
CEP5
Relative expression level
1
0
0
0.01
0.1
1
NAA (μM)


A
B
C
E
basal meristem
En
P
X
X
Pe
P
D
b.m.
F
P
X
X
G
Pe
P
X
X
P
En
C


A
Col-0
CEP5RNAi
B
Primary root length (cm)
0
2
4
6
8
10
12
*
Col-0
CEP5RNAi
C
Stage I
Col-0
CEP5RNAi
Density (#/cm)
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
*
**
7
14
Days After Germination
D
Col-0
CEP5RNAi
% seedlings at given stage
0
10
20
30
40
50
60
70
80
90
100
No LRI
Stage I
No LRI
Stage I
Stage II
Stage III
No LRI
Stage I
Stage II
Stage III
Stage IV
Stage V
Stage VI
6
18
30
Hours post gravistimulus


A
CEP5OE 1-15-4
Col-0
B
*
65%
Primary root length (cm)
0
1
2
3
4
5
6
7
8
9
Col-0
CEP5OE 1-15-4
C
Col-0
CEP5OE 1-15-4
22%
Lateral root density (#/cm)
I
II
III
IV
V
VI
VII
E
NE
Total
Lateral root stage
D
Col-0
CEP5OE
CEP5OE

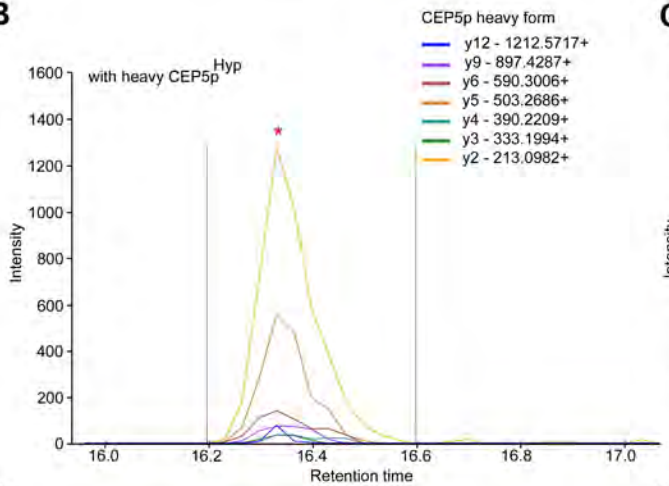
A

CEP5p^{Pro} DFRPTTPGHSPGIGH

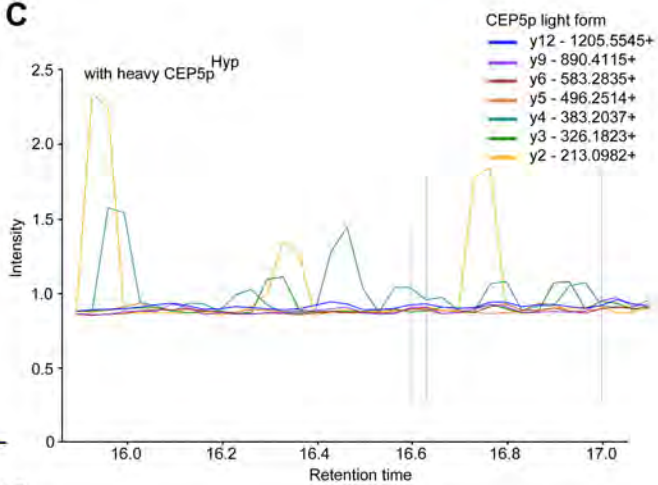
CEP5p^{Hyp} DFR{HYP}TT{HYP}GHS{HYP}GIGH

mCEP5p^{Hyp} DFL{HYP}HT{HYP}GHV{HYP}GISH

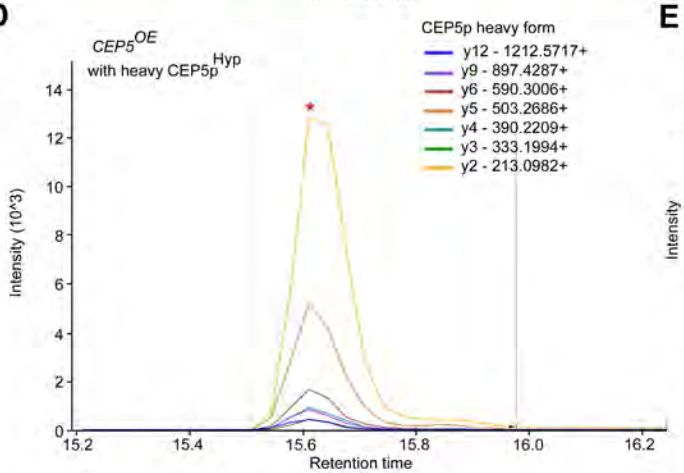
B



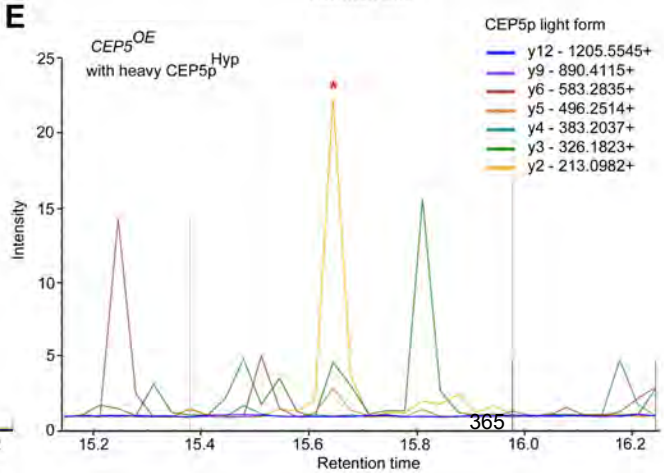
C

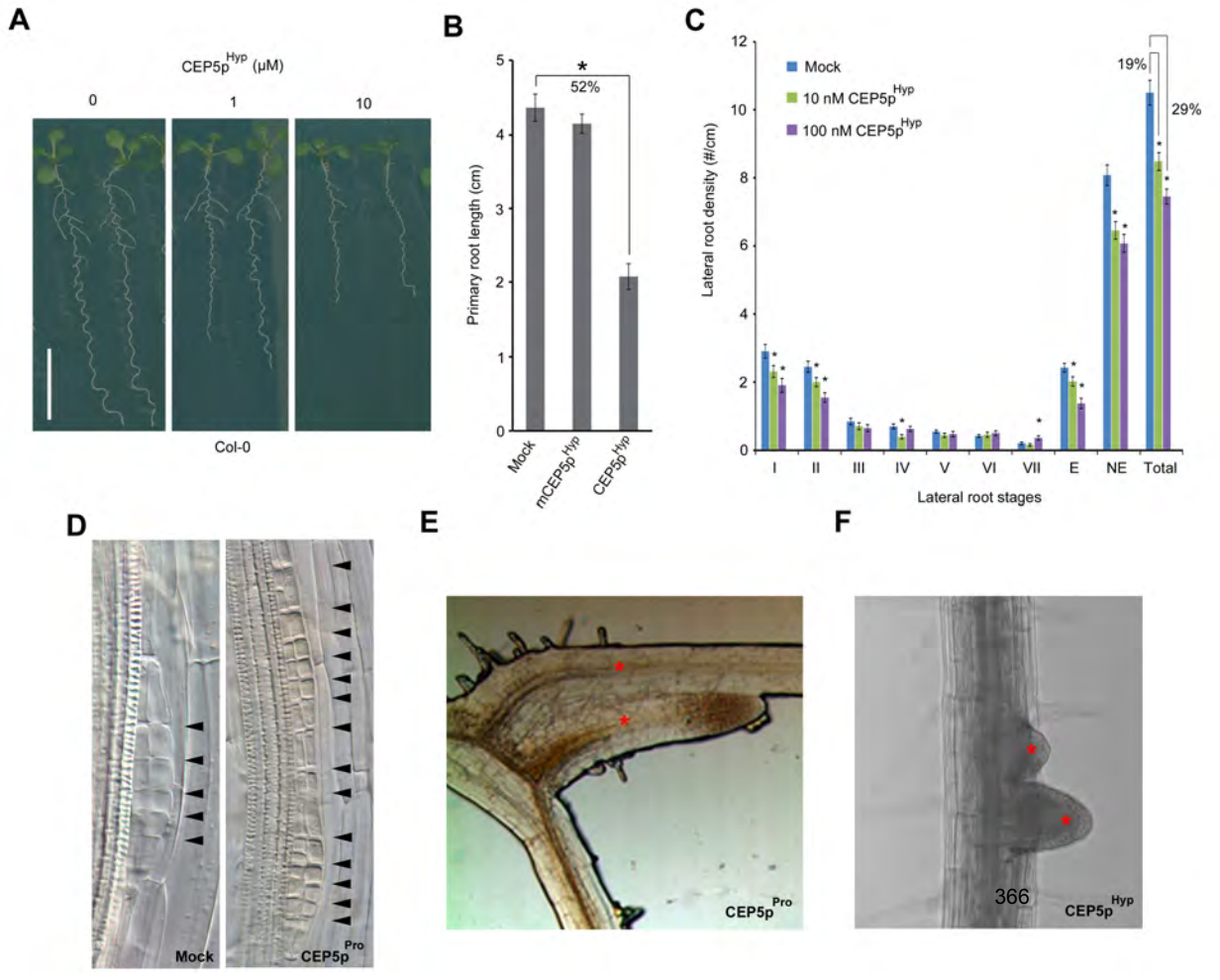


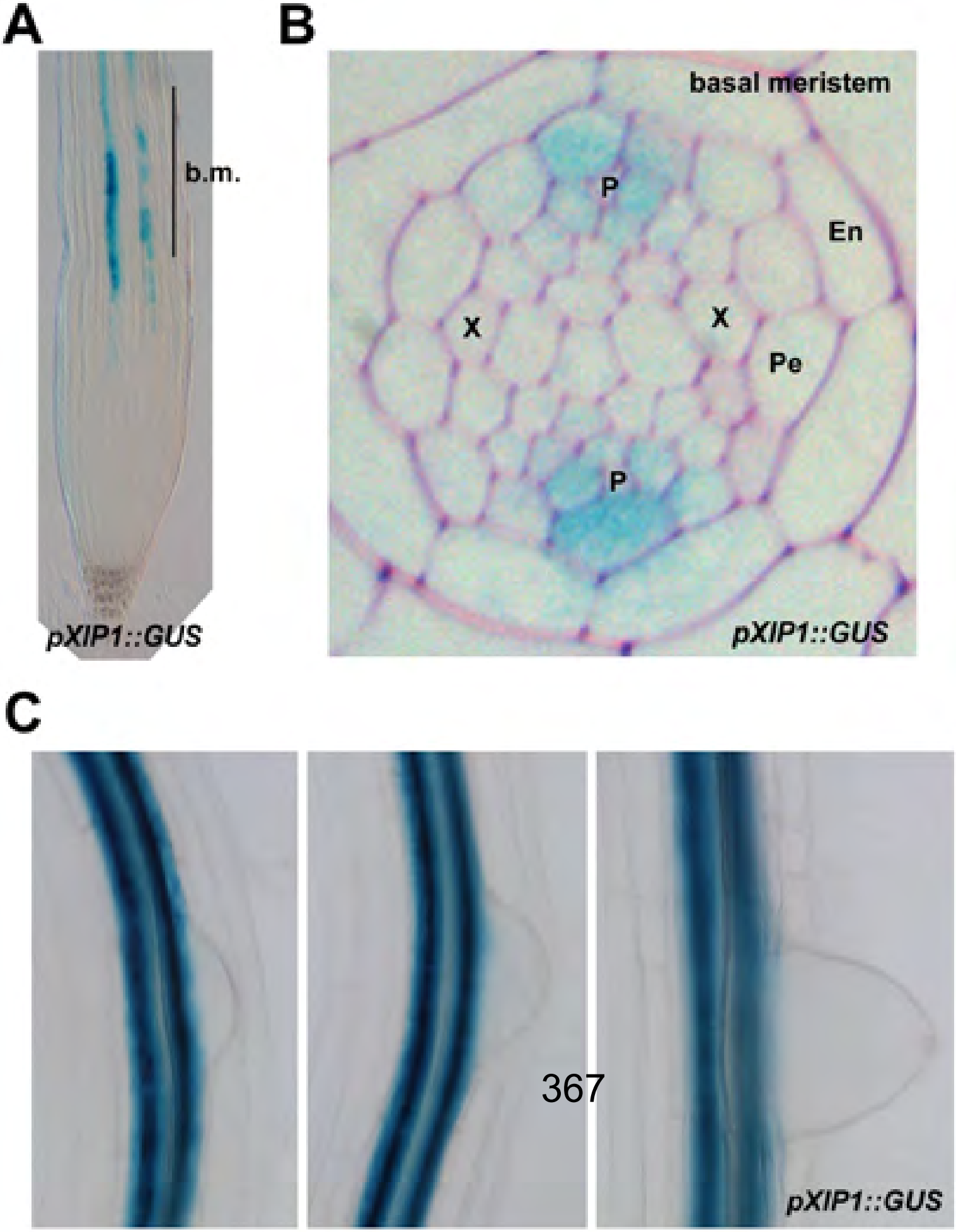
D

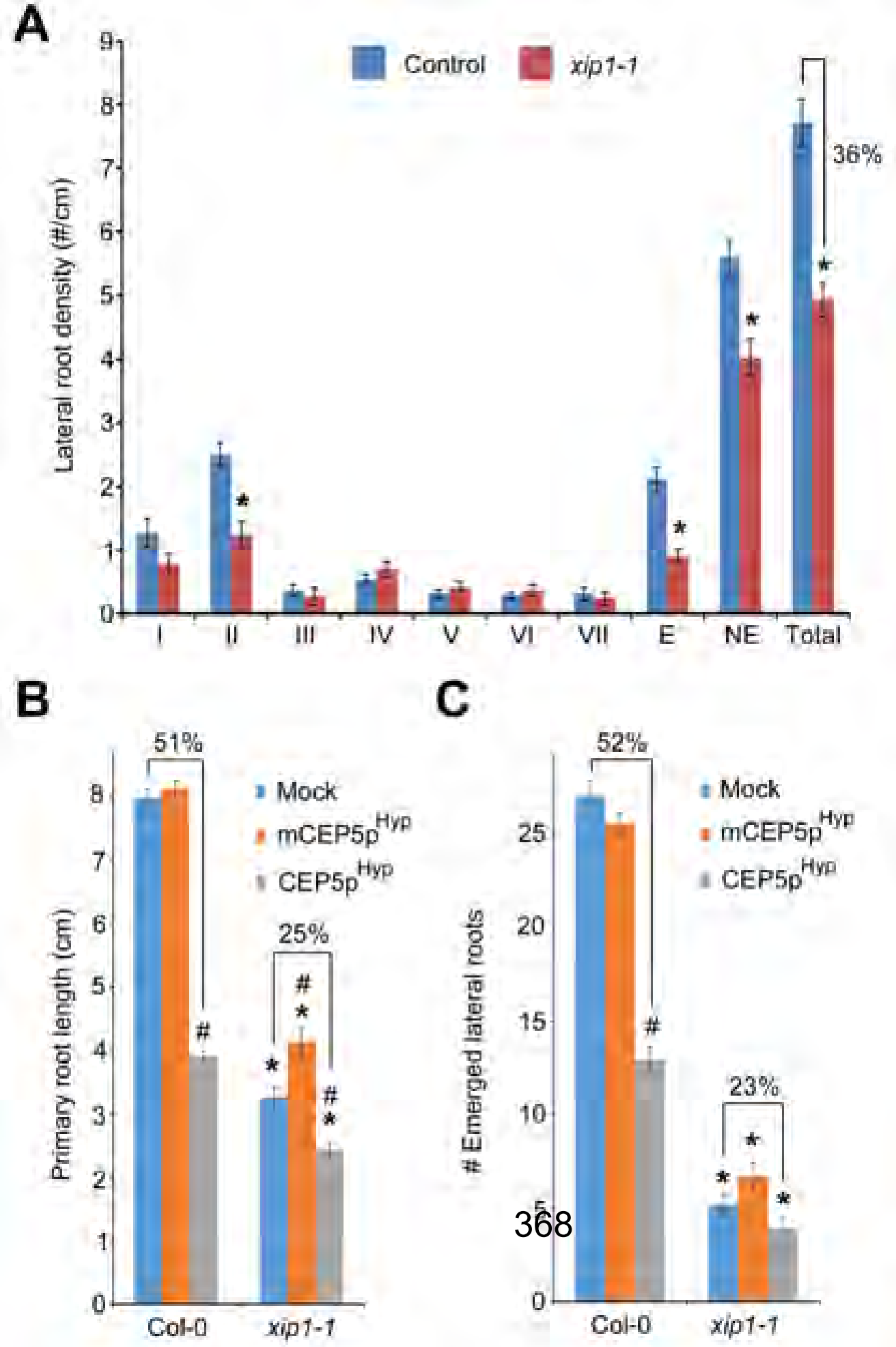


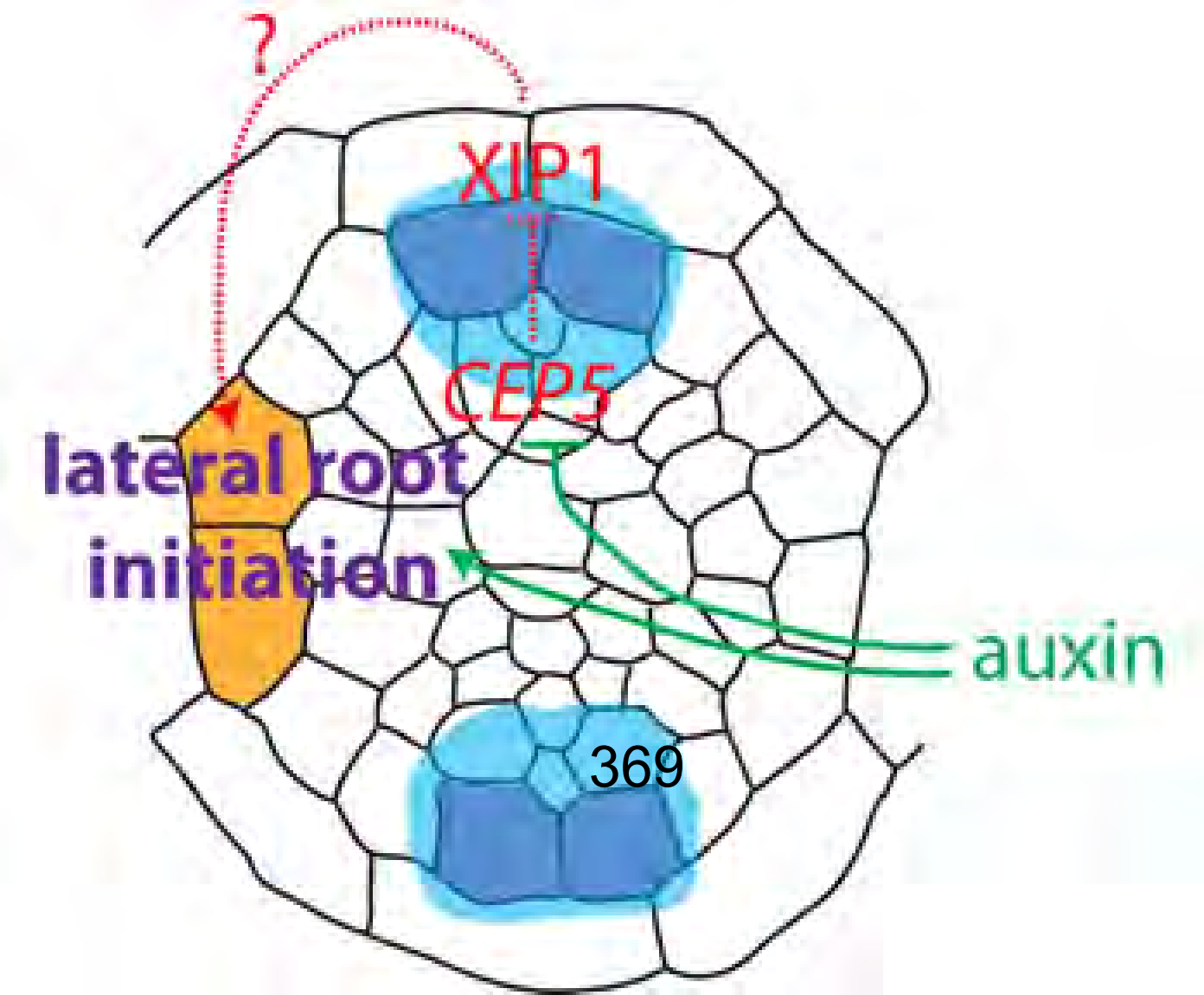
E




A
CEP5p^Hyp (μM)
0
1
10
Col-0
B
*
52%
Primary root length (cm)
Mock
mCEP5p^Hyp
CEP5p^Hyp
C
Mock
10 nM CEP5p^Hyp
100 nM CEP5p^Hyp
19%
29%
Lateral root density (#/cm)
I
II
III
IV
V
VI
VII
E
NE
Total
Lateral root stages
D
Mock
CEP5p^Pro
E
CEP5p^Pro
F
CEP5p^Hyp


A
b.m.
pXIP1::GUS
B
basal meristem
P
En
X
X
Pe
P
pXIP1::GUS
C
pXIP1::GUS


A
Control
xip1-1
Lateral root density (#/cm)
36%
I
II
III
IV
V
VI
VII
E
NE
Total
B
51%
Mock
mCEP5p^Hyp
CEP5p^Hyp
25%
Primary root length (cm)
Col-0
xip1-1
C
52%
Mock
mCEP5p^Hyp
CEP5p^Hyp
23%
Emerged lateral roots
Col-0
xip1-1


?
XIP1
CEP5
lateral root
initiation
auxin