Characterising signalling components mediating root architecture in *Arabidopsis thaliana*

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...and so like all great things, it must come to an end. A journey of great scientific discovery, I could not have arrived here without the help of many people. Unfortunately my ascent into scientific proficiency was swiftly followed by my descent into infirmity. I, unlike so many of my peers, thoroughly loved my time as a PhD student, and relished the opportunity to plan and execute experiments with each coming week. Regrettably, instances out of my control changed my path and set me on a new course. However, I made it through my trials and tribulations, both health-related and scientific, with the help of some amazing people. First and foremost, I would not have made it this far without my (now) wife, **Rachel**, who has been my encouragement and foundation these last 4 (9!) years. I might be the one doing a Doctorate, but you're the intelligent, funny, beautiful person who inspires me to be my best, so thank you! Of course, **Ive De Smet**, I can't thank you enough for your patience, encouragement, and help along the way. From the moment you gave me this opportunity, to the first beer (or two) we shared together, I knew you would be a great supervisor, and someone I would truly respect. I owe a big thank you to my **parents**, and **family** (including those I've gained along the way), for their continual support over the years, your compassion and thoughts have kept me going.

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ABSTRACT

Our planet is growing rapidly in population and with that comes a demand for resources. To address issues in food security, scientists are looking to the underground parts of plants for novel mechanisms that will eventually lead to enhanced crop traits. Scientists are examining the underlying genetic frameworks to identify which genes play key roles in specific developmental processes. In this study we examined the model plant *Arabidopsis thaliana* as the roots are easily visualised, the genome has been sequenced, and there are many tools broadly available to work with. This thesis has used a multidisciplinary approach to uncover the signalling cascades revolving around the small signalling peptide RALF34, which is significantly involved in primary and lateral root growth. We have demonstrated, in the following chapters that RALF peptides are inherent to normative lateral root initiation, potentially regulated through shoot derived auxin. Furthermore, RALF4 and 34 peptides play a strong role in restricting primary root growth, and that together these peptides have an additive effect on cell elongation. Lastly, we identify several leucine-rich repeat receptor-like proteins, kinase proteins, and cell wall remodelling enzymes, which putatively play unique and diverse roles during primary and lateral root development. Taken together, this thesis provides novel and unique insights into new signalling pathways during root growth, which may in future aid in agronomic enterprises.

PART 1 – General introduction

Chapter 1 – *Arabidopsis* root development

Plant and crop research is a field of science fundamental to providing solutions and answers to the mounting problems of an ever growing global population. Much like the food security problems the world faced in the 1960's, ultimately solved through investment in plant and crop research, deemed the 'green revolution', today we face similar hardships. The Food and Agriculture Organization estimates that approximately 805 million people were chronically undernourished in 2012-2014 and that the developing world would not meet its goal of halving the number of undernourished people by 2015 (FAO et al., 2014). The original green revolution concentrated on getting as much yield from crops as possible, in as little time as possible, focusing on breeding crops with the most favourable yield and maturation traits. Today however, food security has once again become a global priority and we must investigate beyond above ground traits and discover the underlying molecular mechanisms behind the important processes during plant development, such as the processes involved in root architecture. To this end, scientists use model species to perform fundamental, ground breaking, though often minutiae, science to first understand how the living world works.

Root development in the model plant *Arabidopsis thaliana*

Root architecture refers to the spatial configuration of a plant's root system (**Figure 1**). Root architecture is established primarily post-embryonically, as such, this thesis will focus primarily on post-embryonic developmental processes such as lateral root development (Mansfield and Briarty, 1991). However, as lateral root development does not occur until the primary root system has already developed, we will first take a brief look the events underlying primary root development.

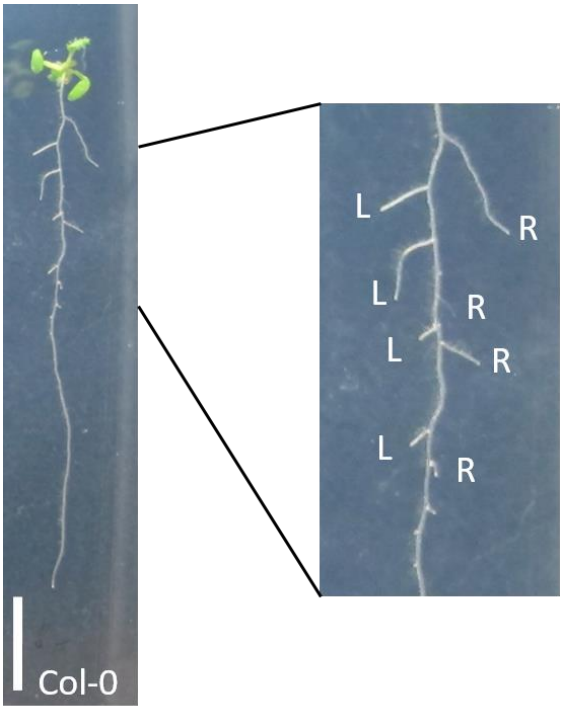


Figure 1. Representative image of root architecture in an *Arabidopsis thaliana* root, showing the typical left-to-right patterning of lateral root formation on the convex side of the growing single primary root; white bar = 1 cm.

Primary root development

In the model plant *Arabidopsis thaliana*, primary root organogenesis and tissue patterning commences with well-orchestrated asymmetric cell divisions (De Smet and Beeckman, 2011). During embryogenesis, the zygote divides asymmetrically forming an apical smaller and a larger basal cell (this later divides to become the hypophysis, which undergoes further cell divisions and has the fate of becoming the future root meristem) (Scheres et al., 1994). Thus, the root pro-meristem patterning is determined embryonically. In *Arabidopsis*, the root meristem is where the generation of post-embryonic root tissue is organised. The root apical meristem is a zone of cells consisting of a stem cell niche, pluripotent cells kept in an undifferentiated state, surrounding a 'quiescent centre' (QC), which functions to maintain the surrounding stem cells (van den Berg et al., 1995). The QC contains 4 slowly dividing cells which express the *WUSCHEL RELATED HOMEOBOX5* (*WOX5*) transcription factor, a negative regulator of cell division, which acts on the surrounding stem cell initials, regulating their rates of division (Richards et al., 2015; Stahl et al., 2009). The stem cell initials surrounding the QC give rise to the many tissue layers that make up the *Arabidopsis* root: the *Arabidopsis* root is formed of concentric layers, starting from the outer-most layer, the epidermis, then the cortex layer and endodermis (which together make up the ground tissue), the pericycle layer (from whence lateral roots are formed), and the vascular bundle formed of xylem and phloem. Specific stem cell initials give rise to each layer – ie. epidermal and lateral root cap stem cell initials give rise to the epidermal and lateral root cap tissue layers (Benitez and Alvarez-

Buylla, 2010; Kwak et al., 2015; Savage et al., 2008). Uniquely in the developing root, the vasculature is not a concentric layer, but rather a central cylinder consisting of two distinct tissues, the xylem and the phloem, responsible for transporting hydrates and photosynthates, respectively, and between these two differentiated tissues is an undifferentiated procambium (Ohashi-Ito and Fukuda, 2010; Scheres et al., 1994). The vasculature possesses bilateral symmetry, with two phloem poles perpendicular to the xylem pole, which is controlled by the LONESOME HIGHWAY (LHW) transcription factor, as seen in the *lhw* mutant which shows one pole formed for each tissue (Ohashi-Ito and Bergmann, 2007; Ohashi-Ito et al., 2013). Furthermore, there is fine balance between auxin and cytokinin crosstalk that occurs during vascular formation, with cytokinins promoting the upregulation of PIN proteins, generating the specific bisymmetric distribution of auxin which promotes the differentiation of protoxylem (Bishopp et al., 2011; De Rybel et al., 2014). The ground tissues, cortex and endodermal layers, are formed from one set of stem cell initials, the cortex/endodermis initials (CEI). Since two distinct tissue layers are formed from one stem cell pool, a tightly controlled signalling transduction is once again attached to the importance of asymmetric cell division. The CEI undergoes two asymmetric cells divisions – one *anticlinal* division, generating a second pluripotent CEI, and one CEI daughter cell, and one *periclinal* division of the daughter cell, which will later differentiate into the cortex and the endodermis. These divisions are fundamentally controlled by two transcription factors, SHORT-ROOT (SHR) and SCARECROW (SCR); mutations in either of these genes results in a meristem

with a single layer of ground tissue (Cui et al., 2007; Di Laurenzio et al., 1996; Gallagher et al., 2004; Helariutta et al., 2000). The SHR transcription factor is produced in the pericycle and vascular cylinder, and travels to the CEI where it upregulates SCR production in the initial CEI daughter cell and in the endodermal layer. In the *scr* mutant, the single aberrant tissue layer has attributes of both endodermis and cortex, suggesting SCR is needed for cell division but not differentiation, while *shr* produces a layer differentiated into cortex, suggesting SHR is necessary for differentiation (Helariutta et al., 2000). While SHR upregulates *SCR*, SCR appears to limit SHR movement as *scr* RNAi lines showed increased levels of SHR movement, causing a proliferative formation of a third tissue layer (Cui et al., 2007). These data show the importance of coordinating both the short distance (SHR and SCR within the CEI daughter cell) and long distance (movement of SHR through the vascular bundle, QC, and CEI cells) signalling within the developing root.

Lateral root development in *Arabidopsis*

The development of lateral (branching) roots, is a process that starts in the meristem and transitions over many different developmental zones of the root. In *Arabidopsis,* lateral roots form the majority of the root systems architecture, providing the basis for structural support, nutrient mining, and water uptake. Lateral roots form regularly positioned in a left-right alternating pattern, originating from asymmetrically dividing xylem pole pericycle cells deeply embedded in the root (**Figure 1**) (De Smet, 2012; De Smet et al., 2007b). These asymmetric cell divisions require the use of communication over long (shoot to

root) and short (cell to cell) distances in order to achieve the highly organised and adaptable patterning to respond to changing external environments. Plants utilise numerous signalling molecules to achieve this, including phytohormones (such as auxin and cytokinin), microRNAs, mobile transcription factors and small signalling peptides (Benkova et al., 2003; Busch and Benfey, 2010; Laplaze et al., 2007; Marin et al., 2010; Van Norman et al., 2011). Lateral root development (LRD) in *Arabidopsis* was originally characterised through phenotypic analysis on novel mutant lines such as the *aberrant lateral root formation* (*alf*) mutants, some of which fail to initiate lateral roots and can be rescued upon auxin treatments, and the *auxin response* (*axr*) mutants which are less sensitive to auxin treatment, clearly indicating the role auxin plays in the LRD process (Celenza et al., 1995; Hobbie and Estelle, 1995). Utilising the synthetic auxin reporter DR5, the PIN-FORMED (PIN) auxin transporters were shown to specifically modulate auxin distribution in a lateral root development specific process (Benkova et al., 2003), and the auxin influx carrier, AUX1, was shown to be upregulated by auxin, leading to an enhanced accumulation of auxin, leading to a localised maxima, establishing the lateral root founder cells (**Figure 2**) (Laskowski et al., 2008). Later, greater levels of auxin response were shown to oscillate in the pericycle, together with periodic waves of gene oscillations, in a zone of the root deemed the 'basal meristem', where this clock-like mechanism was shown to regulate the formation of prebranch sites (De Smet et al., 2007a; Moreno-Risueno et al., 2010; Van Norman et al., 2013). Interestingly, the plant hormone cytokinin was shown to antagonise this process, disrupting the formation of the local auxin maxima by

reducing PIN protein expression (Laplaze et al., 2007). Lateral root development begins with the establishment of prebranch sites, which are formed after zones of heightened auxin accumulation bind to the TRANSPORT INHIBITOR RESPONSE1 (TIR1)/ AUXIN SIGNALING F BOX PROTEIN (AFB) (auxin receptor) which causes the ubiquitin-ligase catalysed degradation of AUX/IAA repressor proteins. The transcription factor GATA23 is involved in regulating the founder cells, the first cells to establish lateral root primordia identity, in an AUXIN/INDOLE-3-ACETIC ACID28 (AUX/IAA28) dependant manner, as *GATA23* levels were reduced in the *iaa28* mutant background (De Rybel et al., 2010) (**Figure 2**). Lateral root development is severely disrupted in the *iaa28* mutant background, and nuclear migration (part of lateral root asymmetric cell division) is also abnormal, clearly indicating the role these two proteins play during the initial prebranch stages of lateral root formation (De Rybel et al., 2010). De-repression of IAA14/ SOLITARY ROOT (SLR), allows AUXIN RESPONSE FACTORs (ARFs) such as ARF7 and ARF19 to modulate the synchronous migration of the two founder cell nuclei to the common cell wall (**Figure 2**) (De Rybel et al., 2010; De Smet et al., 2007a; Dubrovsky et al., 2008; Fukaki et al., 2002; Goh et al., 2012; Kepinski and Leyser, 2005; Tan et al., 2007).

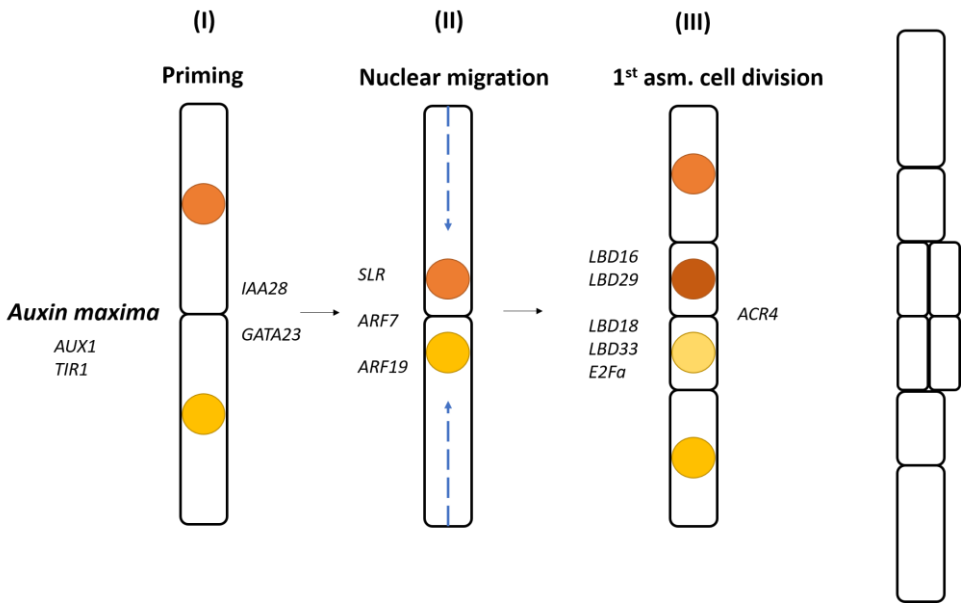


Figure 2. Early events in lateral root development. The auxin transporter *AUX1* is upregulated by auxin, increasing auxin transport to specific founder cells in the pericycle. This auxin maximum leads to the binding of auxin to the TIR1 auxin receptor, leading to the degradation of IAA28, upregulation of *GATA23*, and the priming of the founder cells (I). The consequential degradation of SLR leads to the activation of ARF7 and ARF19, leading to nuclear migration (II), and the further activation of downstream auxin regulated *LBD* transcription factors (III). LBD upregulates cell cycle machinery such as *E2Fa* leading to the first round of asymmetric cell division, and the expression of the negative regulator of cell division *ACR4* (III); coloured nucleus represents clonal origin.

Gain-of-function mutations in SLR or the loss-of-function *arf7 arf19* double mutant show strong lateral rootless phenotypes, suggesting these are necessary for patterning lateral root initiation (Fukaki et al., 2002; Okushima et al., 2007). The *arf7 arf19* mutant shows a lack of auxin induced pericycle cell division, since the LATERAL ORGAN BOUNDARIES-

DOMAIN/ASYMMETRIC LEAVES-LIKE (LBD/ASL) are endogenously controlled by the ARF7/ARF19 dimer (Goh et al., 2012; Okushima et al., 2007; Wilmoth et al., 2005). The transcription factor heterodimer LBD18 and LBD33 regulates the expression of the cell cycle *E2Fa* transcription factor, which promotes asymmetric cell division (Berckmans et al., 2011; De Smet et al., 2010). Lateral root xylem pole pericycle founder cells undergo asymmetric cell division giving rise to two larger flanking and two smaller daughter cells (**Figure 2**) (for a detailed look at the later stages of lateral root development, see Chapter 3 – RALFs in lateral root development). After many rounds cell division moving through stages 1 to 7 of lateral root development, stage 7 to 8 is lateral root emergence (Malamy and Benfey, 1997). This process is facilitated by the hormone auxin through the influx carrier LAX3, as shown through *lax3* mutants which had a 40% reduction in emerged lateral roots (Swarup et al., 2008). The influx carrier, thus influx of auxin, was shown to specifically promote the expression of cell wall remodelling enzymes ahead of the developing lateral root to aid in the cell separation and emergence process (Swarup et al., 2008).

Lateral root development and the environment

Unlike the promeristem which is laid out during embryonic development, the post-embryonic root system can develop and respond dynamically to the changing environment around it. These external influences include stimuli such as salinity (Sun et al., 2008; Wang et al., 2009), water (Kobayashi et al., 2007; Miyazawa et al., 2012), pathogen response (Contesto et al., 2010), and nutrient composition such as nitrogen, potassium, and phosphorus (Contesto et al., 2010;

Krouk et al., 2010; Lopez-Bucio et al., 2002; Perez-Torres et al., 2008; Remans et al., 2006; Rigas et al., 2001; Rigas et al., 2013; Vicente-Agullo et al., 2004). Under salt stress, auxin homeostasis is altered through to PIN2 and AUX1 transporters, as shown by *pin2* and *aux1* sensitivity to salt stress, generating more lateral roots than wild type (Wang et al., 2009). Furthermore, *PIN2* expression is inhibited under salt stress, reducing auxin signalling, and thus, the capacity to generate lateral roots is diminished (Sun et al., 2008). Under water stress, the hydrotropic response leads to roots moving toward areas of higher moisture. Plants overexpressing *MIZU-KUSSEI1* (*MIZ1*) show enhanced hydrotropic curving toward moisture, with an overall reduction in primary and lateral root development, suggesting the two processes are linked, potentially through auxin signalling (Kobayashi et al., 2007; Miyazawa et al., 2012). Interestingly, soil borne bacteria such as *Phyllobacterium brassicacearum*, or *Azospirillum brasilense*, actually produce auxins perceptible by *Arabidopsis*, which result in a strong increase in lateral root production, however, the plants own auxin transport machinery must be functional in order to transport the pathogen derived auxin, as shown through *aux1* and *axr1* mutants which produced no proliferative lateral roots (Contesto et al., 2010). Lastly, lateral root production is also highly linked to nutrient availability in the surrounding environment. The NITRATE TRANSPORTER1.1 (NRT1.1) protein has been linked to lateral root production, as the *nrt1.1* mutant produces fewer and shorter lateral roots under high nitrogen conditions (Remans et al., 2006). Oppositely, at lower nitrogen concentrations, *nrt1.1* produces a higher density of lateral roots; thus, under low nitrogen

conditions, NRT1.1 natively acts to aid in the basipetal transport of auxin (visualised through *DR5::GUS*) to reduce the proliferation of lateral roots, and under high nitrogen, NRT1.1 transport is inhibited, leading to an increase in lateral root generation (Krouk et al., 2010). Under varied potassium levels, a KT/KUP/HAK protein, encoded by *TINY ROOT HAIR1* (*TRH1*), is putatively involved in potassium transport, as shown through the *trh1* mutant, which had a reduced ability to transport potassium, as well as defects in root hair development and response to gravity (Rigas et al., 2001). These phenotypes were acting partially through PIN1 mislocalisation, and were recovered upon exogenous treatment with auxin, linking potassium response to auxin signalling (Rigas et al., 2013). Finally, phosphorus creates a large change in the lateral root architecture, stimulating lateral root organogenesis while inhibiting primary root growth (Lopez-Bucio et al., 2002). Interestingly, *axr* mutants had normal responses to low phosphate, but pericycle cells showed increased sensitivity to exogenously applied auxin, suggesting lower external phosphate levels also lowers the required auxin maxima needed to induce lateral root initiation (Lopez-Bucio et al., 2002; Perez-Torres et al., 2008). Indeed, in the auxin receptor, *tir1*, mutant background lateral root generation was significantly reduced in low phosphate conditions, and the *TIR1* expression was observed to be upregulated specifically in the basal meristem and 'central cylinder' of the root under low phosphate conditions, confirming that phosphate conditions modify lateral root organogenesis through altered auxin response (Perez-Torres et al., 2008).

Lateral root development and non-auxin hormones

Clearly, root architecture is a highly adaptable process and auxin plays a large role, especially during the formative stages in lateral root development, however, auxin is not the only phytohormone to play a role in lateral root development, as there are many other phytohormones that function during lateral root development, such as the previously mentioned cytokinin (Chang et al., 2013; Laplaze et al., 2007), ethylene (Lewis et al., 2011; Negi et al., 2008), brassinosteroids (Bao et al., 2004; Li et al., 2005), and abscisic acid (Himanen et al., 2002; Shkolnik-Inbar and Bar-Zvi, 2010; Tan et al., 2003). Lateral root formation was shown previously to be reduced by exogenously applied cytokinin (Laplaze et al., 2007). Utilising the cell cycle marker *CYCB1:GUS*, it was observed that exogenous cytokinin disrupts the cells in the pericycle undergoing formative division (Chang et al., 2013). The lateral root formation process was linked to the ARABIDOPSIS HISTIDINE KINASE (AHK) family of cytokinin receptors, as *ahk2*, *ahk3*, and *ahk4* mutants caused increases to lateral root density (Chang et al., 2013). Ethylene is a hormone that upon exogenous application, represses lateral root development by upregulating the auxin transporters *PIN3*, *PIN7* and *AUX1* above the site of lateral root formation, creating a basipetal shift in the flow of auxin away from the root tip (Lewis et al., 2011; Negi et al., 2008). This shift in auxin signalling is absent in *ethylene response1* (*etr1*) and *ethylene insensitive2* (*ein2*) mutants, ethylene transduction genes, correlating it is an ethylene dependant response, and interestingly, this ethylene induced lateral root reduction is still observed in the *tir1* auxin receptor mutant, suggesting ethylene

acts independently of TIR1-mediated auxin signalling (Lewis et al., 2011). Brassinosteroids have been associated with lateral root development by stimulating acropetal auxin transport, enhancing auxin accumulation in the root tip (Bao et al., 2004; Li et al., 2005). Specifically, the auxin transporter *PIN2* was upregulated upon brassinolide treatment, clearly showing crosstalk between brassinosteroids and auxin (Li et al., 2005). Abscisic acid (ABA) has been shown to block lateral root development, potentially by inhibiting pericycle cell division through the upregulation of the negative cell cycle regulator *INTERACTOR OF CDC2 KINASE1* (*ICK1*)/*KIP RELATED PROTEIN1* (*KRP1*) (De Smet et al., 2003; Himanen et al., 2002; Wang et al., 1998; Zhang et al., 2010). ABA is potentially involved in the spatial-regulation of new primordia as the biosynthesis of ABA is induced at the base of young primordia, possibly generating ABA gradients and decreasing proliferative formative cell division in neighbouring cells (Tan et al., 2003). ABA has also been shown to reduce the ability for lateral roots to develop post-emergence, as ABA causes the lateral root meristem to enter an induced quiescence state (De Smet et al., 2003; Zhang et al., 2010) Furthermore, utilising an overexpression construct of *ABSCISIC ACID INSENSITIVE4* (*ABI4*) which encodes an ABA-regulated transcription factor, it was observed that *PIN1* expression was specifically reduced, and alternatively, enhanced in the *abi4* mutant, showing a direct mechanism through which ABA alters lateral root development (Shkolnik-Inbar and Bar-Zvi, 2010).

Lateral root development and other signals

Clearly, there are many processes beyond simply auxin signalling involved during lateral root development, and indeed, among the other small molecules involved in lateral root development, microRNAs - small non-coding RNAs which specifically inhibit the expression of genes by non-reversibly cleaving target mRNA transcripts – have also been shown to regulate ARFs (Marin et al., 2010). A trans-acting short interfering RNA (tasiRNA) *TAS3a* was shown to reduce the activity of *ARF2*, *ARF3*, and *ARF4*, mediated by the microRNA, miR390 (Marin et al., 2010). Utilising 35S::*TAS3a*, it was observed that plants had longer lateral roots, which was also observed in 35S::*miR390* plants, suggesting these two act in concert; furthermore, *arf2*, *arf3*, and *arf4* mutant plants also displayed this growth, correlating all 3 phenotypes and demonstrating these molecules act in a regulating pathway during lateral root development (Marin et al., 2010)

Lastly, my work specifically investigates the role of a small signalling peptide on primary and lateral root development (Chapters 3 & 4 – RALFs in lateral root development, and RALFs in primary root development). The following chapter will present a summary of the literature on the roles of small signalling peptides during primary root growth, lateral root development, and several other important aspects, such as receptor-ligand identification.

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Chapter 2 – Small Signaling Peptides in *Arabidopsis* Development: How Cells Communicate Over a Short Distance

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SUMMARY

In this chapter we provide a review, which represents an accumulation of knowledge on the various aspects of signalling in plant development. With a focus on key areas like peptide identification, processing, followed by the roles peptides play during cellular signalling, this chapter gives a thorough overview on our current understanding of peptides during *Arabidopsis* development.

Author contribution: I contributed significantly to the writing of this review, primarily on the introduction, and sections on development and peptide-receptor interaction, discussion, and conclusions. Furthermore, I contributed to the creation of all figures (except Figure 1).

Chapter 2

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, lipophilic 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).

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 REGIONRELATED (CLE), TRACHEARY ELEMENT DIFFERENTIATION INHIBITORY FACTOR (TDIF), PHYTOSULFOKINE (PSK), PLANT PEPTIDE CONTAINING SULFATED TYROSINE1 (PSY1), CTERMINALLY 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-a (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 repressing 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, 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).

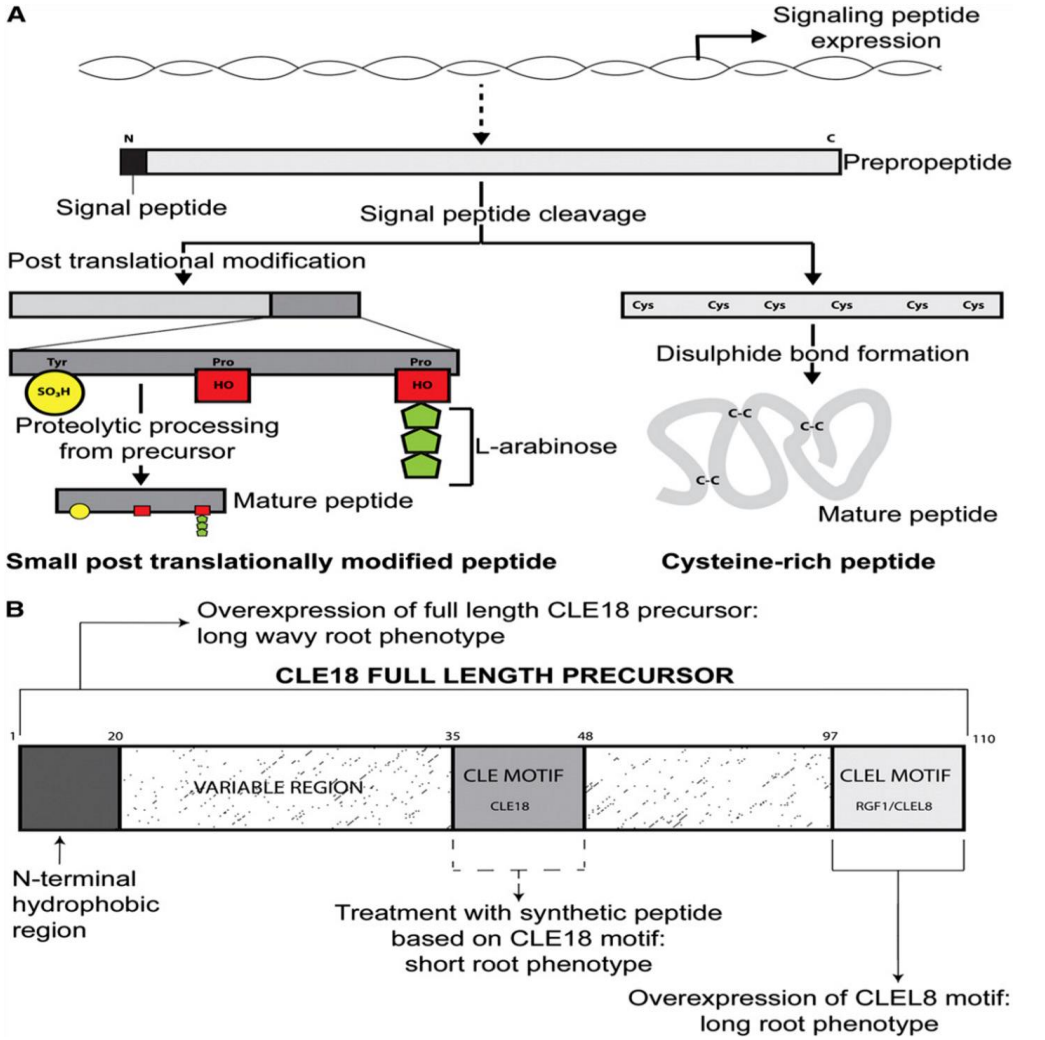


Figure 1. **Posttranslational Modifications and Processing of Small Signaling Peptides.** (A) Following entry of the full-length mRNA-encoded prepropeptide to the secretory pathway, the N-terminal hydrophobic signal peptide is cleaved by a signal peptidase. Small posttranslationally modified peptides 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 Cysrich 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).

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 aberrant 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 Nterminal 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 Cysrich 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 co-expressed 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 39-phosphoadenosine 59-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 b-linkage between the hydroxyl group of Hyp to an arabinose, and an arabinosyltransferase mediates the b1, 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 1 B).

Amino acid sequencing of purified PSK-a 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-a peptide was later confirmed through synthesis and subsequent sulfation of the peptide using arylsulfotransferase. Solid-phase peptide synthesis and sulfation of PSK-a and 12 analogs with an adimethylformamide-sulfurtrioxide complex confirmed the active peptide core of PSK as Tyr(SO_3H)-Ile-Tyr(SO_3H)-ThrGln. The second peptide identified, PSK-b, was found to be a C-terminal truncated tetrapeptide of PSK-a, 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-a is proteolytically processed from an 80–amino acid precursor, preprophytosulfokine-a (the exact precursor length varies according to species and PSK paralog). Preprophytosulfokine-a 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 Cterminal 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 (MALDI-TOF 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).

Table 1. Posttranslational Modifications and Proteolytic Processing of Small signalling 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.

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 2 A).

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).

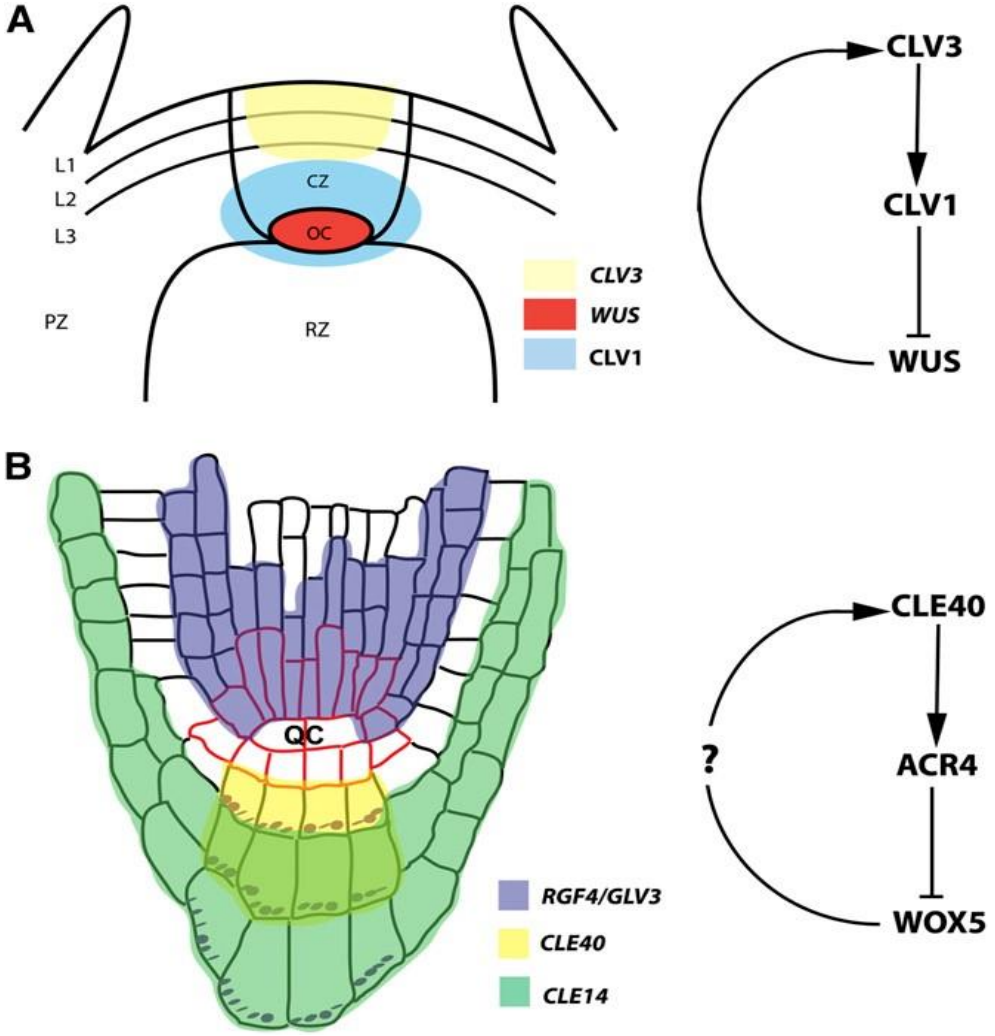


Figure 2. SAM and RAM. (A) CLV3 (yellow) is expressed in the central zone (CZ) of the SAM and inhibits expression of WUS (red) in the OC. WUS itself promotes CLV3 expression. The CLV1 receptor is expressed below layers one and two of the SAM (L1 and L2). On the right is the proposed CLV3-CLV1-WUS signaling 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 auxinindependent 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* 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 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).

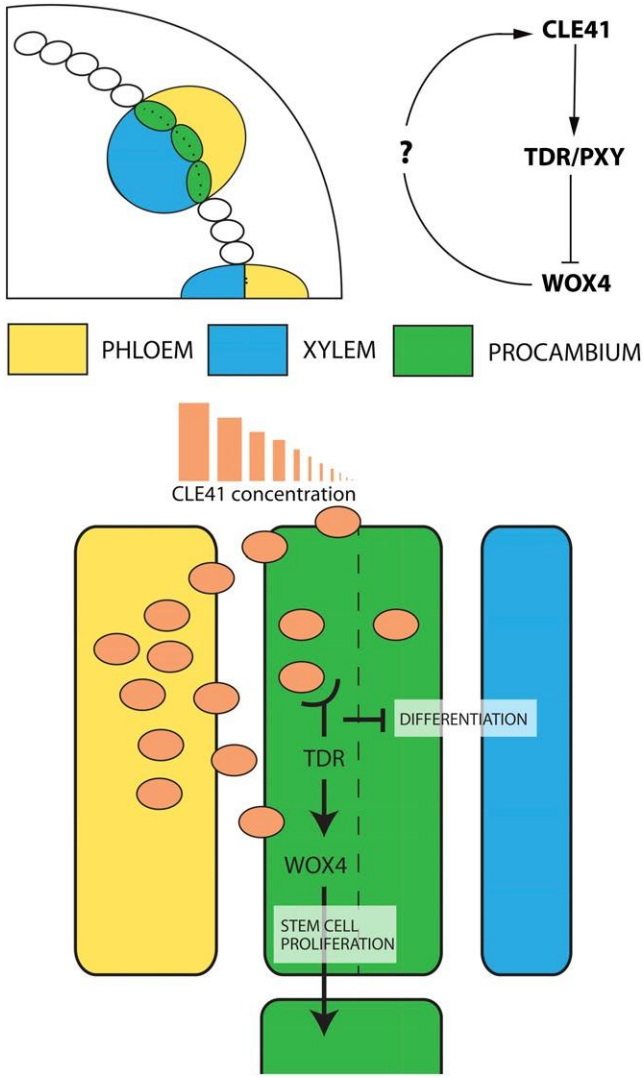


Figure 3. Vascular Differentiation. Schematic of a stem section highlighting the vascular bundles (top left). Phloem cells (yellow) generate and secrete CLE41 peptides (peach) into procambial cells (green) where they are bound by membrane-localized TDR/PXY. TDR/PXY inhibits differentiation of procambium 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.

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

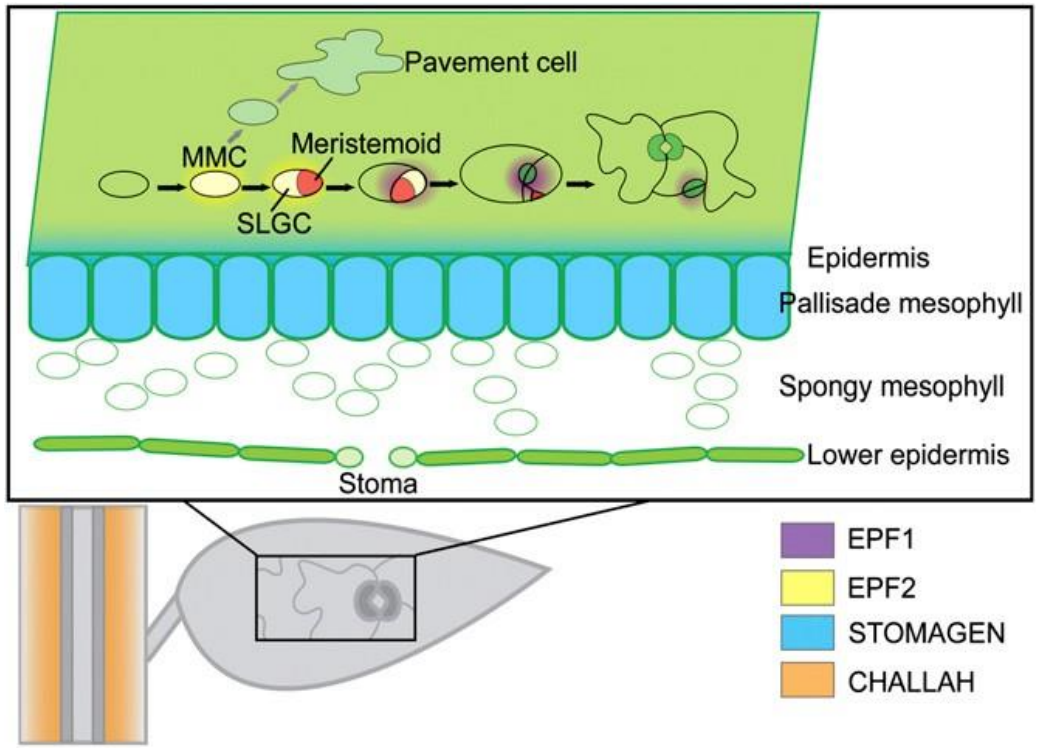


Figure 4. Stomatal Development. Stomatal development begins when protodermal cells differentiate into MMCs. Protodermal cells that do not differentiate into MMCs instead differentiate into epidermal pavement cells. The MMC then divides asymmetrically to form a small triangular meristemoid cell (red) 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 (SERK 1) 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 flagellinderived 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 organspecific 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 (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-cellautonomous 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 procambium 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). CHAL peptides have also been shown to act through the TMM receptor (Abrash et al., 2011). In a *tmm* background, *CHAL* overexpression leads to a striking reduction in stomatal density, suggesting that TMM binds CHAL to buffer signaling and block transmission into stomatal lineage cells. CHAL overexpression phenotypes are also mediated by ER family mutations, suggesting an additional pathway through which CHAL 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

All authors contributed to writing the article.

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PART 2 – RALF peptides in *Arabidopsis thaliana* growth and development

Chapter 3 – Understanding the RALF family: a tale of many species

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SUMMARY

In this chapter we provide a review, which is comprised of a comprehensive overview on the current knowledge of the RALF family. The RALF peptide was originally discovered in leaf cells of Tobacco during a novel peptide identifying assay, and has since been identified in Poplar, Tomato, *Arabidopsis*, and many other species. Interestingly, RALF peptides show conserved properties across species (ie. the presence of a cysteine rich mature peptide sequence), however, the mature peptide has been shown to have various and stratified effects between the various species. This section will convey the multifaceted nature of this large family of signalling peptides and will set the foundations for Chapters 4, 5, and 6.

Author contribution: I wrote this review and generated all figures, with editing and direction from Ive De Smet.

Understanding the RALF family:

a tale of many species

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Small secreted peptides are gaining importance as signalling molecules in plants. Among the 1000 open reading frames (ORFs) in the Arabidopsis (Arabidopsis thaliana) genome potentially encoding small secreted peptides, the members of the RAPID ALKALINIZATION FACTOR (RALF) family of peptides have been linked to several physiological and developmental processes. Here, we provide a comprehensive overview of current knowledge on the RALF family. Discovered in tobacco (Nicotiana tabacum), the role of RALF peptides has been investigated in numerous plant species. Together, these observations suggest that RALF peptides impact on acidification and cell expansion during growth and development. Although few components of the signalling pathway have been revealed, the recent identification of FERONIA (FER) as a RALF receptor and plasma membrane H -ATPase 2 as a downstream target provide a major step forward.

Small signalling peptides: a brief introduction

Cell-to-cell communication in plants has canonically revolved around phytohormones, such as auxin [1]. However, over the past few decades, a focus on short distance communication has highlighted the role of small signalling peptides during tissue patterning and development. These peptides have been implicated in almost all developmental processes [2,3]. Among the many signalling peptides discovered to date, these small proteins can be grouped into two broad categories: small post-translationally modified peptides and cysteine-rich peptides. The first class, small post-translationally modified peptides, currently outnumber the known cysteine-rich peptides in Arabidopsis, and are involved in many developmental processes [2,4–8]. Although smaller than cysteine-rich peptides (less than 20 amino acids), they are subject to numerous post-translational modifications, such as tyrosine sulfation, proline hydroxylation, and hydroxyproline arabinosylation, which may conformationally alter the peptides into an active or inactive state [2,6,7,9–12]. Small post-translationally modified peptides are often grouped in highly conserved families, including C-TERMINALLY ENCODED PEPTIDE (CEP), CLAVATA3/EMBRYO SURROUNDING REGIONRELATED (CLE), and ROOT GROWTH FACTOR (RGF)/ GOLVEN (GLV)/CLE-LIKE (CLEL)

peptides, with great developmental redundancy within the families [2,7,13–16]. The second class of peptides, cysteine-rich peptides, have many structural features in common despite their greater diversity in amino acid sequences. Cysteine-rich peptides are usually much larger than small post-translationally modified peptides (less than 160 amino acids), are positively charged, and generally contain 4–16 cysteine residues. These residues appear to be integral to the correct conformational folding of the mature peptide, but, unlike small post-translationally modified peptides, have not yet been shown to be post-translationally modified [17]. Similar to small post-translationally modified peptides, they contain a conserved N-terminal signal peptide, which directs the peptide to the correct cellular location via the secretory pathway [18]. To date, the known cysteine-rich peptides family of peptides includes RALF, EPIDERMAL PATTERNING FACTOR1 (EPF1), EPF2, TAPETUM DETERMINANT1 (TPD1), STOMAGEN/EPF-LIKE9 (EPFL9), and EARLY NODULIN [2,17,19–22].

Here, we provide a comprehensive overview of our current knowledge on the RALF family. Based on observations in numerous plant species, it appears that RALF peptides impact on acidification and cell expansion during growth and development. Although few components of the signalling pathway have been revealed, the recent identification of FERONIA as a RALF receptor and plasma membrane H+-ATPase 2 as a downstream target provides a major step forward.

RALF function: a many species saga

The RALF peptide family was discovered just over a decade ago and current knowledge on their function is limited. Here, we summarise the RALF-related research from different species, which exposes a general trend in the function of RALF peptides (**Figure 1** and **Table 1**).

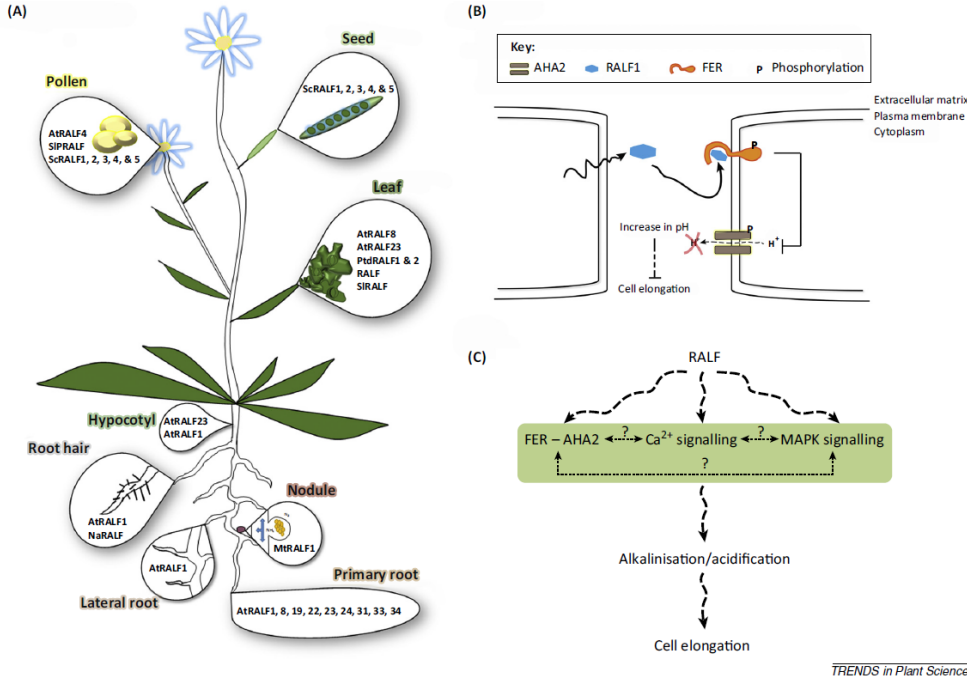


Figure 1. RAPID ALKALINIZATION FACTOR (RALF) peptides in plant growth and development. **(A)** Schematic of a plant highlighting where RALFs have a role (combined observations from various species). **(B)** RALF binding to the FERONIA (FER) receptor and regulating H^+-ATPase 2 (AHA2) activity. **(C)** Summary of events downstream of RALF, without any specific order of events or interactions and indicating possible connections (broken line and '?').

Nicotiana tabacum

RALFs were discovered during a peptide screen for systemin proteins (defence-related, alkalinisation-inducing peptides) in Nicotiana tabacum leaf cells,

utilising an assay developed to identify peptides that cause an increase in pH of the medium [17]. Having caused a stronger and more rapid alkalinisation of the medium compared with two systemin peptides, the then unknown peptide was investigated further. Through MALDI-MS on HPLC purified leaf tissue samples, RALF was identified to be a 5-kDa polypeptide, with a 115 amino acid preproprotein. The new RALF peptide was dismissed as being a systemin because it was shown to induce MITOGEN-ACTIVATED PROTEIN (MAP) KINASE activity peaking earlier than previously identified systemin proteins. Furthermore, the systemin family of peptides induces the synthesis of tobacco trypsin inhibitors as a defensive wound signal; however, when exogenously applied to tobacco leaves, RALF did not induce this wound response, thus firmly dissociating this novel peptide from the systemin peptide family and beginning the journey to discovering the function of these novel peptides [17].

Hybrid poplar (Populus trichocarpa x Populus deltoides)

The difference in RALF compared with the systemin peptide family was reinforced when novel poplar-derived RALF peptides also did not induce a defence response. Using similar methodology to [23], poplar RALFs (specifically PtdRALF1 and PtdRALF2) were isolated from hybrid poplar leaf cells, and were shown to alkalinise strongly the culture medium, be nonresponsive to fungal treatment (no change in expression), and to not elicit a defence response (in this case, no production of PHENYLALANINE AMMONIA LYASE, which is often associated with a defence response [24]),

suggesting that indeed a novel, nondefence-related family of peptides had been discovered [25].

Nicotiana attenuata

To explore RALF functionality in a N. tabacum- related species, *NaRALF* was silenced in Nicotiana attenuata with RNA interference (RNAi), which showed increased primary root length and produced trichoblasts that formed abnormal root hairs [26]. In these *NaRALF* RNAi lines, slow apoplastic pH oscillations were observed with increased pH at the trichoblast tip. This phenotype was partially restored when the inverted-repeat RALF construct (*irRALF*) plants were grown in low pH-buffered medium, indicating the first regulatory role for RALF peptides in modulating root hair pH and pH-related growth [26].

Solanum lycopersicum (teaming up with A. thaliana)

The physiological role of RALFs was investigated further by developing a synthetic RALF peptide based on tomato derived endogenous RALF [17]. Exogenously applying synthetic tomato RALF peptide to germinating tomato seeds caused a significant arrest in root elongation of the newly emerged primary roots. This synthetic peptide was then applied to germinating Arabidopsis seeds and a similar striking reduction in root growth was observed. Root meristems and elongation zones appeared to have arrested growth, while having larger cell sizes. Furthermore, root hairs were prevented from growing on the RALF-treated roots. These effects were reversible when seedlings were transferred on

to nonRALF-containing media. These results strongly suggest a regulatory role in root growth and development for the RALF peptides.

The pollen-specific SlPRALF also appears to have a role in cell elongation, because synthetically generated SlPRALF inhibited pollen tube elongation [27]. Interestingly, addition of SlPRALF peptide inhibited growth of pollen tubes until they were between 40 and 60 mm, suggesting a specific developmental window of activity, without having an effect on pollen hydration or viability.

[a]*Populus trichocarpa x Populus deltoids* [b]*Application of peptide* [c]*Overexpression* [d]*Downregulation*

Intriguingly, SlPRALF did not induce a strong alkalinisation in Solanum peruvianum cells, whereas another tomato (SlRALF) and tobacco RALF did.

However, SlPRALF did induce a strong alkalinising response in Arabidopsis, suggesting that there is a regulatory mechanism that varies between species during the alkalinisation process [27].

Solanum chacoense

RALFs also have a role during sexual development in Solanum chacoense. Novel *RALF* genes were identified through a BLAST search on an expressed sequence tag (EST) database generated from Solanum chacoense ovary tissues. Five *RALF* genes (*ScRALF1, 2, 3, 4*, and *5*) were observed to be expressed during sexual reproduction and development, and were once again shown to not be involved in wounding or defence response [28]. One of those five peptides, *ScRALF3*, was shown to be expressed specifically in the sporophytic tissue yet

Table 1. Known RALF peptides in various plant species and their physiological and developmental roles.

Species	GenGenee	Tissues examined	Activity	Refs
Arabidopsis thaliana	AtRALF1	Aerial, root, root hairs, microcalli, and hypocotyl	Arrests emerging primary root growth of germinating seedlings[b] , inhibits root hair growth[b] , induces increase of cytoplasmic Ca^{2+} [b], dwarf-like phenotype[c] , inhibits cell elongation of microcalli[b], inhibits H^+ ATPase activity[b], positively[d] or negatively [c] affects primary root length, hypocotyl length, and lateral root development	[17,31 ,33, 37 , 38,42]
	AtRALF4	Pollen	Inhibits pollen germination [b]	[32]
	AtRALF8	Aerial and root	c Dwarfism in aerial tissues and in root tissue c and confers decrease in drought tolerance	[39]
	AtRALF19, AtRALF22 AtRALF23, AtRALF24 AtRALF31, AtRALF33 AtRALF34	Hypocotyl, root, and pollen	Inhibits cell elongation in hypotcotyl and root cells[b], increases Ca^{2+} signalling[b] , and inhibits pollen germination (except RALF34) [b]	[32,39]
	AtRALF23	Aerial, hypocotyl, and root	Inhibits hypocotyl elongation after brassinolide treatment[c] , shorter and bushier (dwarfed) plants with roots unable to acidify the medium [c]	[34]
Hybrid poplar[a]	PtdRALF1, PtdRALF2	Leaf	Strong alkalinisation of medium, with no defence response elicited[b]	[25]
Medicago trunculata	MtRALF1	Root	Decrease in nodulation and abnormal nodule development, regulated by Nod factors[c]	[30]
Nicotiana attenuata	NaRALF	Primary root and root hairs	Causes longer primary roots and increases pH at the tips of root hairs[b]	[26]
Nicotiana tabacum	RALF	Leaf	Rapidly alkalinises the medium b, induces MAP KINASE signalling b , and does not induce a defence response b	[17]
Saccharum spp	SacRALF1	Microcalli	b Inhibits cell elongation in microcalli	[31]
Solanum chacoense	ScRALF1, ScRALF2, ScRALF3, ScRALF4, ScRALF5	Ovaries	Produces smaller fruits with fewer seeds, disruption of the embryo sac (for ScRALF3)[d]	[28,29]
Solanum lycopersicum	SlRALF	Leaf	Arrests emerging primary root growth of b germinating seedlings	[17]
	SlPRALF	Pollen	b Inhibits pollen tube elongation	[27]

to have an effect only on embryo sac development [29]. Utilising RNAi against *ScRALF3* led to improper embryo sac development and a resultant smaller fruit with fewer seeds. The embryo sacs lost polar localisation of the nuclei, had premature embryo sac development arrest, and those that matured were aberrant and unhealthy. A *ScRALF3:GFP* construct showed the movement of the peptide through the secretory pathway, suggesting that the peptide is generated in the sporophytic tissue, is secreted, and has an effect on the surrounding female gametophyte during ovule development [29]. This provides evidencethat RALF peptides are secreted and can act on neighbouring tissues; in this case, possibly independent of pH regulation.

Medicago truncatula

In Medicago truncatula, *MtRALF1* was shown to be upregulated during Nod factor response [30]. Nod factors are signalling molecules elicited by bacteria to facilitate the nodulation process in the symbiotic relationship between rhizobia (nitrogen-fixing bacteria) and leguminous plants. Overexpression of *MtRALF1* resulted in a significant decrease in nodulation and an increase in aberrant nodules when they did form. Interestingly, overexpression of *MtRALF1* showed no apparent effect on primary root length, architecture, or root hair development, again suggesting that the RALF family of peptides has diverse roles both within and between species [30].

Sugarcane (Saccharum spp.)

In sugarcane, utilising the alkalinisation assay, two RALF peptides were isolated from leaf cultures [31]. Application of the purified SacRALF1 peptide to

microcalli cell suspensions inhibited microcalli growth by arresting cell elongation. Similarly, application of AtRALF1 (with over 80% similarity to SacRALF1) also resulted in an arrest of growth and reduction of cell elongation. These microcalli resumed growth and expansion when transferred to nonpeptide-containing media. To further analyse the developmental role of *SacRALFs* in cell elongation, gene expression analysis was performed on the elongation zones of sugarcane leaves. Through real-time quantitative PCR analyses on tissue extracts, it was shown that *SacRALF1* showed over 20 times higher expression in the elongation zone than in meristematic cells. Following this trend, expression was highest in young and growing leaves, and extremely low in old, mature leaves. These results strongly suggest a role for RALF peptides during cell expansion, which correlates well with the previously mentioned inhibition of primary root, root hair, and pollen tube elongation [31].

Arabidopsis thaliana

To assess the effect on cell elongation, Arabidopsis seedlings were germinated in the dark to induce the elongation of the hypocotyls. In seedlings grown in the presence of RALF1 peptide, hypocotyl elongation was inhibited. Hypocotyl elongation resumed when the seedlings were removed from the RALF-containing medium [31].

Inhibition of hypocotyl elongation was also observed using synthetic RALF19, 22, 23, 24, 31, 33, and 34, or by overexpressing *RALF1*. The latter also displayed a similar inhibition of cell elongation for primary root growth and a reduced lateral root density. By contrast, *RALF1* knockdown lines displayed the opposite

phenotype to overexpressing lines with respect to hypocotyl and primary root growth and lateral root density. Interestingly, RALF4 did not have any effect on cell elongation in either hypocotyl or primary root growth [31–33]. These observations strongly support the hypothesis that RALFs repress cell elongation throughout Arabidopsis growth and development.

Similar to overexpression of *RALF1*, *RALF23*-overexpressing plants displayed reduction in growth, producing shorter, bushier plants [33,34]. Interestingly, *RALF23* is downregulated in presence of brassinolide [34], and *RALF23*-overexpressing plants were resistant to treatment with brassinolide (a brassinosteroid phytohormone involved in promoting growth [35]) that normally induces hypocotyl elongation in wild type plants [34]. Likewise, exogenous brassinolide had a reduced efficacy on *RALF1*-overexpressing plants. In agreement with this, *RALF1* induced two negatively regulated brassinosteroidrelated genes, *CONSTITUTIVE PHOTOMORPHOGENIC DWARF* (*CPD*) and *DWARF4* (*DWF4*), further suggesting that *RALF1* acts antagonistically during brassinosteroid signalling. In addition, *RALF1* upregulated expression of cell wall-remodelling enzymes, such as *PROLINE RICH PEPTIDE1* (*PRP1*), *PROLINE RICH PEPTIDE2* (*PRP2*), *HYDROXYPROLINE-RICH GLYCOPROTEIN2* (*HRPG2*), and *XYLOGLUCAN ENDOTRANSGLUCOSYLASE*/ *HYDROLASE22* (*XTH22*)/*TOUCH4* (*TCH4*), but upon simultaneous treatment with RALF1 peptide and brassinolide, these genes displayed attenuated expression levels when compared with RALF1 application alone [33]. These results suggest that RALF1 and brassinolide act through shared signalling components.

Interestingly, the root system of *RALF23* overexpressing plants was unable to acidify effectively the surrounding medium. These data suggest that RALF23 (and other RALFs) counteract cell wall acidification, which in turn reduces cell growth and the ability of the roots to acidify the rhizosphere [34,36]. In this context, it has been shown that (at least) AtRALF1 has an effect on plasma membrane H^+-ATPase2 (AHA2) [37]. Application of RALF peptide significantly increased the phosphopeptide abundance of AHA2, inhibiting the H^+-ATPase activity, which in turn would increase the apoplastic pH, resulting in a reduction in cell elongation [37] (Figure 1B). However, it is possible that not all RALFs are directly affecting proton pumps, but pH alteration may also be a secondary response down-stream of Ca^{2+} and/or MAP kinase signalling (see below).

RALFs have also been implicated in Ca^{2+} signalling in Arabidopsis root cells [38]. Calcium is thought to act as a secondary messenger in growth and developmental processes and in environmental responses. Various fractions of Arabidopsis tissue extracts were added to single seedlings and were monitored for activity using the fluorescent marker protein Aequorin to identify Ca^{2+} signalling spikes.

Among the substances eliciting large Ca^{2+} signalling responses, AtRALF1 was identified. Both purified and synthesised AtRALF1 were applied to Aequorin-expressing seedlings, and both elicited strong Ca^{2+} signalling responses (Figure 1C). Interestingly, of the known peptides, only systemin has been shown to also elicit a downstream response in Ca^{2+} signalling. Although *AtRALF1* is primarily expressed in the root, calcium signalling has been shown to be needed for pollen tube growth, a process in which RALF peptides have been

implicated [27,38]. It was shown that RALFs induce MAP kinase activation in tobacco, which is another form of intercellular signalling, within minutes of RALF peptide treatment [17] (Figure 1C). However, in Arabidopsis, Ca^{2+} signalling was activated within 40 s of peptide treatment [38]. This indicates that Ca may be the primary response to RALF induction and that MAP kinase signalling and inevitably alkalinisation may bedownstream targets of various signalling cascades. RALF19, 22, 23, 24, 31, 33, and 34 were also shown to induce increases in calcium signalling, with RALF1 and 22 producing significantly higher Ca^{2+} levels than the others. Although application of RALF4 showed little effect on calcium levels, RALF4 had the most significant inhibitory effect on pollen germination. RALF1 and 34 had little to no effect on pollen germination [32].

Lastly, RALFs have been implicated in the response to simultaneous biotic and abiotic stresses in Arabidopsis [39]. Unlike the initial experiment identifying RALF in tobacco, which concluded that RALF was not involved in defence response, recent evidence has shown that RALFs may be involved in response to multiple stresses. Plants respond to individual stresses with a linear program of gene expression. However, the response of a plant to simultaneous stresses requires a more adaptive and dynamic range of gene expression and the combination of, for example, biotic (nematode) and abiotic (drought) stress, will lead to a novel gene expression profile. Indeed, RALF8, RALF23, RALF33, and RALF34 were all induced by the aforementioned joint stresses, and less so by the individual ones. In the absence of a loss-of-function mutant, a strong *RALF8* overexpression line displayed significantly stunted growth; specifically, aerial tissues were stunted, with rosette diameter and biomass accumulation

significantly reduced, root architecture was 80% smaller than wild type plants, with a shorter primary root, increased root hair density, and reduced lateral root density. The *RALF8* overexpression line was highly intolerant to drought, with survival rates 91% less than those of wild type plants. These plants also showed a greatly increased susceptibility to nematode infections, 2.6 times greater than wild type plants [39]. These results suggest that RALF8 (and potentially numerous RALFs) is utilised as a signalling molecule in response to biotic and abiotic stresses, as a fine-tuning mechanism balancing growth rates to prioritise more urgent requirements. With prominent roles in essential processes, such as cell expansion and environmental stress response, it seems evident why the large RALF family is so highly conserved within the plant kingdom (see below).

A large and widespread RALF family

In addition to tobacco, Arabidopsis, tomato, and the previously mentioned species, RALF peptides have been identified in a large number of plant species, from dicots to monocots and gymnosperms [17]. Evolutionarily, RALF numbers remain similar in lower plants, Selaginella moellendorffii (two) and Physcomitrella patens (three); however, in higher plants, their numbers vary significantly. This is believed to be due to tandem duplication events having occurred in some species, whereas segmental duplication events occurred in others. In Arabidopsis, the RALF peptides are a family of at least 34 *RALF* genes, of which 50% are believed to have occurred through tandem duplication. In rice, 43.7% (seven) of the 16 *RALF* genes are arranged in tandem repeats [40]. In addition, the number of *RALF* genes in poplar, for example, is 30% less than

the number of *RALF* genes in Arabidopsis even though the genome is 67% larger [41]. This supports recent genome duplication events allowing for the greater expansion of the RALF family in Arabidopsis.

Through phylogenetic and in silico analysis of RALF peptides in Arabidopsis, poplar, maize, and rice, RALF peptides were identified to have evolved six distinct conserved amino acid motifs within the family, creating ten distinct groups or classifications for individual genes (Figure 2A). Many of the Arabidopsis genes cluster together in these groups (especially group 1, where 15 of the 16 genes come from Arabidopsis). In Arabidopsis, 26 (13 pairs) of the 34 genes are highly related (between 30% and 97 % similarity), further suggesting that these RALFs have undergone repeated tandem duplication and the RALF family has experienced significant gene duplication [41].

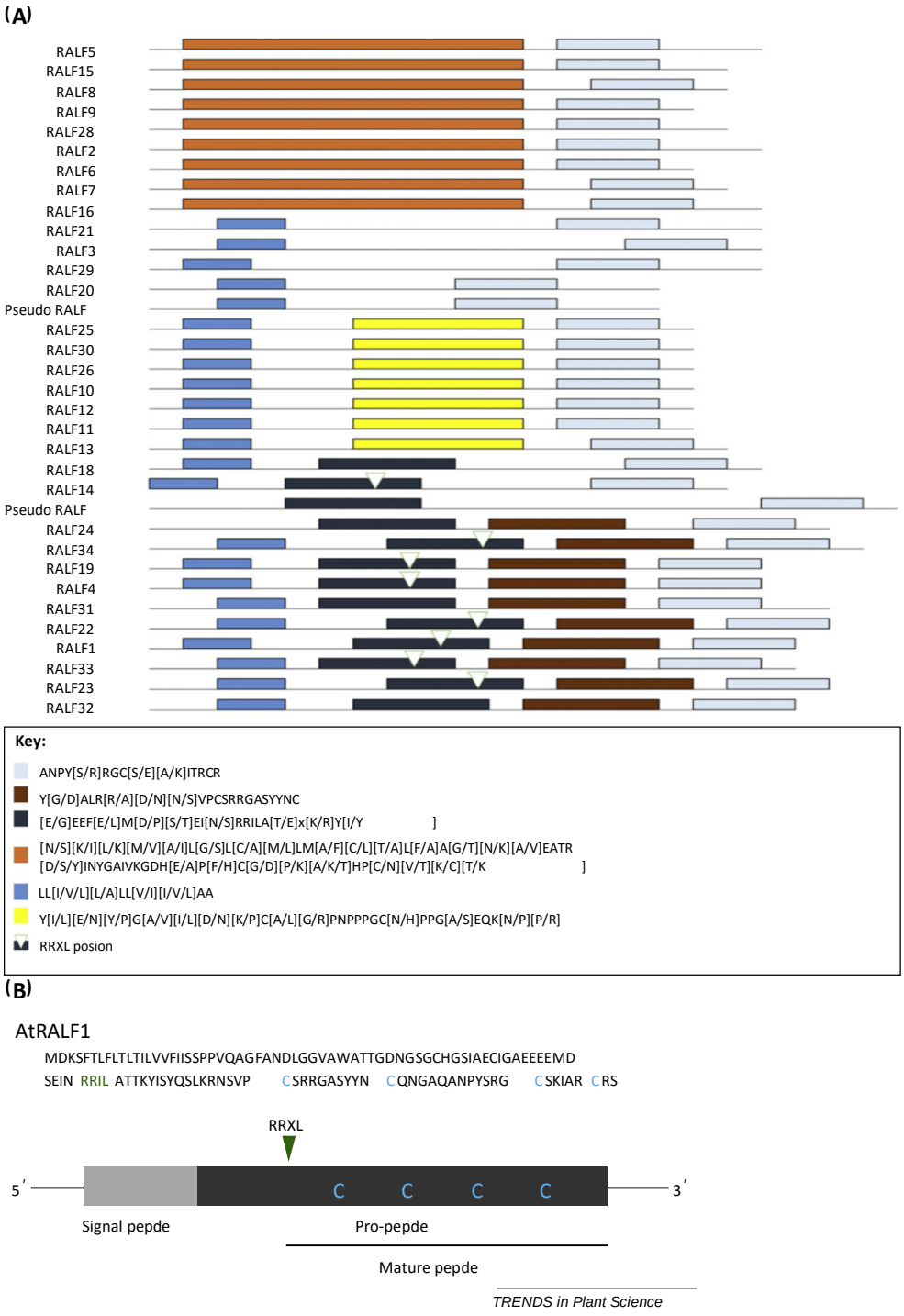


Figure 2. (A) Schematic representation of members of the RALF family in Arabidopsis (full-length protein) with the six conserved amino acid motifs (based on [41]). (B) Sequence and schematic of AtRALF1 sequence with canonical RRXL cleavage site (green arrowhead), and four conserved cysteines within the mature peptide (light-blue 'C').

Processing of RALF to the mature peptide

Among the conserved motifs found within the RALF family is an arginine-arginine (RR) motif identified in the original tobacco RALF peptide (Figure 2B). This RR motif was suggested as a potential cleavage site, often found in animal and yeast peptides. At the time, it was the first indication of an RR motif being used as a putative cleavage site for a proteolytic enzyme in plants [17]. It was later confirmed that this dibasic site was indeed a cleavage site for a proteolytic enzyme (AtS1P, SITE-1-PROTEASE) separating the propeptide from the mature active RALF peptide [34,42]. This canonical subtilase site with dibasic amino acid motif RR (RRXL) occurs in ten of the 34 Arabidopsis RALF peptides. This motif was previously shown to be the protease recognition site of the AtS1P cleavage enzyme in AtbZIP17 [43]. AtS1P is a Golgi-localised enzyme, so it is likely that RALF peptides get processed in the Golgi during the journey of the peptide through the secretory pathway [44]. Alternatively, AtS1P may also be shipped in the secretory pathway to the apoplast, where RALFs have been shown to localise, and process the RALF peptides extracellularly when the pH conditions are right [34,45]. Following processing by AtS1P, the mature peptide is predicted to be the active region of the protein. This was shown by mutating the RRIL motif in AtRALF23 to GGIL. In plants overexpressing *AtRALF23* (*35S::AtRALF23*), a significant dwarf phenotype is evident compared with wild type plants. However, in plants overexpressing *AtRALF23* with the mutated GGIL motif, no phenotype was evident. This shows that the RRIL motif is essential for AtRALF23 processing, and that the mature peptide is the active domain of the protein [34]. RALFs have a predicted mature peptide of approximately 50 amino acids that is highly conserved at the C terminus

throughout the family. The mature RALF peptide contains four conserved cysteines, which have been shown to be involved in forming disulfide bridges and are important for protein conformation [17]. Furthermore, the mature peptide also contains a YISY motif near the N terminus, a GASYY motif (found between the first two cysteines), and a PYXRGCS motif that contains another conserved cysteine. Given the nature of the mature peptide, and lack of evidence of post-translational modification, the RALF family of peptides can be classified as cysteine-rich peptides [46].

The search for a RALF receptor

Several of the cysteine-rich peptides receptors have been identified, such as TOO MANY MOUTHS (TMM), ERECTA (ER), and ERECTA-LIKE (ERL), which are receptor-like proteins and receptor-like kinases that strongly bind to the EPIDERMAL PATTERNING FACTOR (EPF) peptides [47]. There are over 600 potential receptor-like kinases and over 1000 ORFs potentially encoding small secreted peptides in the Arabidopsis genome; however, few interactions between peptide ligand and receptor have been shown [2,37,48–55]. Identification of ligand–receptor pairs is technically challenging and requires the combination of strategies to overcome limitations when dealing with potentially minor, weak, and fleeting interactions.

It was shown that RALFs act through receptor binding, as shown by the use of suramin, a broad inhibitor of receptor–ligand interactions. Suramin added to the cell suspension medium containing tomato RALF strongly inhibited the alkalinisation process [56,57]. A synthetic photoaffinity analogue of tomato

RALF (called LeRALF) was added to tomato suspension cells in a photoaffinity crosslinking assay, to identify any binding proteins. Using UVB light, the LeRALF radiolabelled two proteins in the cell suspension, one 120-kDa protein and one 25- kDa protein, which was more strongly labelled. The binding locality of the RALFs was tested by acid washing the cell suspension, completely dissociating the RALFs from the receptor. This suggested that the peptides were acting on the cell surface and were not internalised after binding. Through fractionation, solubilisation, application of salt and pH extremes (which release peripheral proteins), the 25- and 120-kDa proteins were shown to not solubilise and, thus, are integral membrane proteins [56]. These two proteins have not yet been investigated further. In addition, a yeast two-hybrid screen utilising the recognition domain of the tomato pollen-specific cell wall-localised Leu-rich repeat extensin chimera (LRX) protein identified SlPRALF as an interacting partner [27]. However, additional experiments to confirm and characterise pollen LRX-RALF interactions are still in progress.

Recently, an Arabidopsis interacting partner to RALF (AtRALF1) was identified as FER, a ubiquitously expressed member of the malectin receptor kinase family [37] (Figure 1B). A phosphoproteomic approach using Escherichia coli synthesised, biologically active His-tagged RALF peptide, was used to identify phosphopeptides that were significantly differentially regulated upon treatment. Five proteins had a significant change in abundance post treatment: a plasma membrane H^+-ATPase, a calcium-dependent protein kinase 9, an ABCG36 transporter, and the receptor-like kinase FER were positively regulated, and a FER-related RLK deemed ERULUS (ERU) was negatively regulated. The *fer4* T-DNA insertion knockout mutant was shown

to be insensitive to RALF treatment, failing to elicit an increase in Ca^{2+} signalling or inhibition to cell elongation. The *eru* mutants remained sensitive to treatment, suggesting a role outside of RALF ligand binding. RALF binding with FER was confirmed through co-immunoprecipitation from Nicotiana benthamiana samples, and also through in vitro interactions. In both cases, the biologically active HisRALF peptide preferentially bound to FER, whereas an inactive RALF analogue showed little to no interaction. Interestingly, FER has a molecular mass of approximately 140 kDa, which is different from the previously mentioned unknown 25- and 120-kDa proteins shown to bind synthetic RALF peptide. This would suggest that RALF acts on multiple receptors, possibly in a tissue-specific manner. Furthermore, *fer4* is not completely insensitive to high concentrations of RALF peptide, further suggesting there are weak interactions with other receptor proteins.

To assess the possibility of multiple ligand–receptor combinations, saturating doses of RALF peptides were applied in succession to suspension cells. It was shown that after RALF23 peptide application induced media alkalinisation, no secondary alkalinisation of the medium could be elicited by application of any other RALF peptide. Additionally, application of RALF23 induced further alkalinisation of the medium regardless of which RALF peptide was applied first. This suggests preferential binding of the RALF23 peptide to a specific receptor over all the other investigated peptides. This assay identified a cluster of RALF1, 22, 24, 31, and 33 as having similar binding properties. RALF19 and 34 were grouped in a separate cluster, and RALF4 was once again separate from all other RALF peptides [32]. To further assess receptor specificity, the chemical agent suramin was used to inhibit ligand–

receptor interactions. Interestingly, the RALF peptides clustered in to different binding groups than before. RALF1, 19, 22, 24, 31, and 33 all had their maximal activity reduced by half, whereas RALF23 and 34 were less susceptible. Intriguingly, RALF4 had the opposite reaction, with ligand–receptor activity increasing after suramin application. These results illustrate how this diverse family of peptides must act on multiple receptor-like proteins, involved in numerous developmental processes.

Concluding remarks: piecing together the puzzle The RALF family has been classified as cysteine-rich peptides, containing four or more conserved cysteine residues responsible for the mature peptide conformation. Within the family, six conserved motifs can be found within the mature peptide, dividing the family into possible functional clades [41]. It has been shown that the processing enzyme AtS1P is responsible for cleaving at least one RALF peptide from its propeptide precursor into its mature form [34].

Several RALF family members have been linked to a developmental or physiological process, with many of the peptides performing similar functions. Cell elongation and growth appear to be primary mechanisms that the RALF family affects, as seen through synthetic peptide application and overexpression [17,39]. The peptides affect cell and organ growth through seemingly three processes: Ca^{2+} signalling, MAP kinase signalling, and pH modulation [17,26,34,38]. However, the specific mechanisms, order of events, and interactions through which RALFs modulate pH, activate MAP kinases, and elicit Ca^{2+} responses are still unknown. The identification of AHA2 as a downstream target regulating H^+ flux is an important first step toward the

unravelling of the molecular pathway(s) that mediate the physiological and developmental output of RALF activity

Importantly, identifying FER as one receptor of RALF(s) opened research possibilities into both upstream and downstream effectors that, in turn, will place these peptides into specific developmental pathways. However, it is likely that other receptors can bind RALFs, and advancements in the field of protein–protein interactions, along with more intricate computational modelling and peptidomics to allow the identification of receptor–ligand pairs [58,59], will enable these to be identified.

In future, it will be crucial to investigate further how the RALF family of small signalling peptides is integrated in plant hormone responses, such as auxin and brassinosteroids, that also affect cell elongation.

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Chapter 4 - RALF34 regulates formative cell divisions in Arabidopsis pericycle during lateral root initiation

RESEARCH MANUSCRIPT

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SUMMARY

In plants, root growth is driven by the coordination of many signalling molecules: phytohormones, micro RNAs, transcription factors, and small signalling peptides. Lateral root development is an auxin driven process that requires the precise regulation of dose responsive auxin machinery, which targets responsive genes and cell cycle activation. Our work focuses on identifying and quantifying the underlying genetic processes involved in early lateral root development.

We describe the analysis of the phenotype of the small signalling peptide *ralf34* mutant during lateral root development and show how it is fundamental during the early formative stages of lateral root progression. Furthermore, we show that a shoot derived source of auxin alone is not enough to drive the advancement of lateral root development, and that the small signalling peptide is also required. We present novel data suggesting a small signalling peptide affects lateral root development through an early expressed, auxin regulated transcription factor, specific to asymmetrically dividing xylem pole pericycle cells known as founder cells.

INTRODUCTION

Root plasticity is one of the main adaptive traits enabling plants to cope with an ever changing environment. Root architecture in the model plant *Arabidopsis thaliana* is composed of the primary root, lateral roots, and root hairs, each of which plays a vital role in the plants ability to coordinate specific developmental processes such as nutrient acquisition and water uptake. Lateral roots are generated post-embryonically from deep within the root, from the pericycle cells adjacent to the xylem poles, known as founder cells (Dubrovsky et al., 2001; Malamy and Benfey, 1997). Lateral roots are initiated regularly and this is regulated by the plant hormone auxin in an acropetal direction, triggering tightly regulated asymmetric cell divisions, leading to characteristically sized and shaped lateral root primordia (Beeckman et al., 2001; Casimiro et al., 2001; Laskowski et al., 1995; Malamy and Benfey, 1997). The divisions of lateral root primordia have been categorised into 8 stages: Stage 1- the first initial asymmetric cell divisions of two pericycle founder cells, forming two smaller daughter cells with distinct cell fates. Stage 1 continues with a second round of anticlinal division finishing with the pericycle cells enlarging in a radial direction. Stage 2- a rotation in the plane of division occurs; the cells divide periclinally toward the outer tissues forming an outer layer and an inner layer. This division normally occurs first in the two most central cells, followed by the adjacent cells. The most peripheral cells do not divide periclinally, so as the central cells expand radially, the establishment of the lateral root primordia

dome shape materialises. In Stages 3 through 7, numerous rounds of well organised anticlinal and periclinal cell divisions occur establishing distinct tissue layers eventually mimicking the organisation of the primary root tip. Stage 8 involves few cell divisions, however, rapid cell expansion results in penetration of the overlying tissue, emerging from the primary root (Malamy and Benfey, 1997). Several of the underlying genes and proteins that are involved in priming, founding, initiating, and advancing lateral root development have been identified through transcript profiling and the use of gain-of-function and loss-of-function mutants, such as SOLITARY-ROOT/IAA14, ARF7/ARF19, LBD16/29, GATA23, ACR4 (De Rybel et al., 2010; De Smet et al., 2008; Fukaki et al., 2002; Lavenus et al., 2013; Okushima et al., 2007; Okushima et al., 2005; Tatematsu et al., 2004), though less information is known on cellular communication during lateral root development, specifically, through the relatively recently discovered small signalling peptides.

Small signalling peptides have been shown to play a wide variety of roles in the plant (Delay et al., 2013b; Murphy et al., 2012), with recent evidence showing: CLEL (CLE-like)/GLV (GOLVEN)/RGF (ROOT GROWTH FACTOR) peptides inhibiting pericycle cell divisions when overexpressed (Fernandez et al., 2013; Matsuzaki et al., 2010; Meng et al., 2012; Whitford et al., 2012); IDA and its receptors HAE (HAESA) and HSL2 (HAESA-Like2) were shown to play a role in lateral root emergence (Kumpf et al., 2013); the various CEPs (C-TERMINALLY ENCODED PEPTIDEs) appear to reduce emerged lateral root density when synthetic peptide is exogenously applied, or endogenously overexpressed (Delay et al., 2013a); CLE (CLAVATA3/EMBRYO

SURROUNDING REGION (ESR)) peptides inhibit lateral root emergence when overexpressed (Araya et al., 2014) and also regulate lateral roots through transcriptional regulation of ARF7 andARF19 (Cho et al., 2014); a recently discovered peptide, AREP1 (AUXIN-RESPONSIVE ENDOGENOUS POLYPEPTIDE 1), was shown to have a significant effect promoting lateral root organogenesis in presence of auxin (Yang et al., 2014); and finally, RALF (RAPID ALKALINIZATION FACTOR) peptides in *Arabidopsis* have canonically been shown to regulate cell elongation processes (such as cell size, pollen tube elongation, microcalli elongation, and root hair growth) as well as controlling developmental processes such as pollen germination, and drought tolerance (Atkinson et al., 2013; Bergonci et al., 2014; Mingossi et al., 2010; Morato do Canto et al., 2014; Pearce et al., 2001; Srivastava et al., 2009). However, several RALF peptides (RALF1,19,23) have also been have been shown to increase the emerged lateral root densities in *RALF*-silenced transgenic lines and decrease densities in 35S::*AtRALF1/8* overexpressing lines (Atkinson et al., 2013; Bergonci et al., 2014).

Recently, the RALF1 receptor was identified as FERONIA (FER), a member of the highly expressed malectin receptor-like kinase family (Haruta et al., 2014). It was shown that RALF1 binds to FER, causing downstream phosphorylation on a plasma membrane associated H^+-ATPase (AHA2), resulting in an increased apoplastic pH and a decrease in cell wall elongation. AHA2 (*aha2*) has since been shown to also cause a decrease in lateral root density, correlating a downstream mechanism as to why 35S::*RALF1* lines have decreased lateral root

density (Mlodzinska et al., 2015). However, beside FERONIA no other RALF peptide receptor has yet to be identified.

Using a top-down approach, we investigated the transcriptome dataset that also yielded the already functionally characterised receptor-like kinase ACR4 inherent to restricting proliferative cell divisions during lateral root development (De Smet et al., 2008). The dataset comprising 1920 differentially regulated genes during lateral root initiation was broken down into a core of 15 highly upregulated lateral root primordia genes: *ACR4* being one, and the small signalling peptide *RALF34* being another. Thus, we began the first in-depth analysis of a RALF signalling peptide on the early stages of lateral root development.

RESULTS AND DISCUSSION

RALF34 regulates cell division patterns in the pericycle

In a transcriptome analysis focusing on early stages of lateral root initiation, *RALFL34* (AT5G67070) was identified as differentially expressed (De Smet et al., 2008) (**Figure 1A**). *RALF34* encodes a member of a family of cysteine-rich small signalling peptides (Murphy and De Smet, 2014) and likely gives rise to a 56 amino acid mature peptide (**Figure 1B**). While members of the RALF family have been functionally characterized (Pearce et al., 2001; Olsen et al., 2002; Matos et al., 2008; Haruta et al., 2008; Srivastava et al., 2009; Atkinson et al., 2013; Haruta et al., 2014; Bergonci et al., 2014; Morato do Canto et al., 2014;

Li et al., 2015), no data are so far available for RALFs in the context of lateral root initiation.

To validate a role for RALF34 in lateral root initiation, we identified 2 T-DNA lines, namely *ralf34-1* (in Col-0) and *ralf34-2* (in Ler), with strongly reduced *RALF34* expression (**Figure 1B-C**). Both *ralf34-1* and *ralf34-2* were analysed with respect to overall emerged lateral root density, lateral root stages and division patterns in the pericycle. Overall, *ralf34-1* displayed an increase (+25%) in total lateral root density compared to the Col-0 control (**Figure 2A**) and *ralf34-2* displayed the same phenotype (+24%) over Ler. A more detailed analysis quantifying the different stages of lateral root development revealed that this is likely due to an enrichment of stage I lateral root primordia in *ralf34-1* compared to the control (**Figure 2B**). Furthermore, we observed a two-fold increase (2.3x) in aberrant pericycle division patterns or abnormal lateral root primordia in *ralf34-1*, which occurred at a lower frequency in the control (**Figure 2C-D**), and this trend was present in the *ralf34-2* allele as well (2.4x increase over Ler, Based on these observations, we concluded that RALF34 plays a role during lateral root initiation and likely acts to restrict (proliferative) cell division in the pericycle during lateral root initiation.

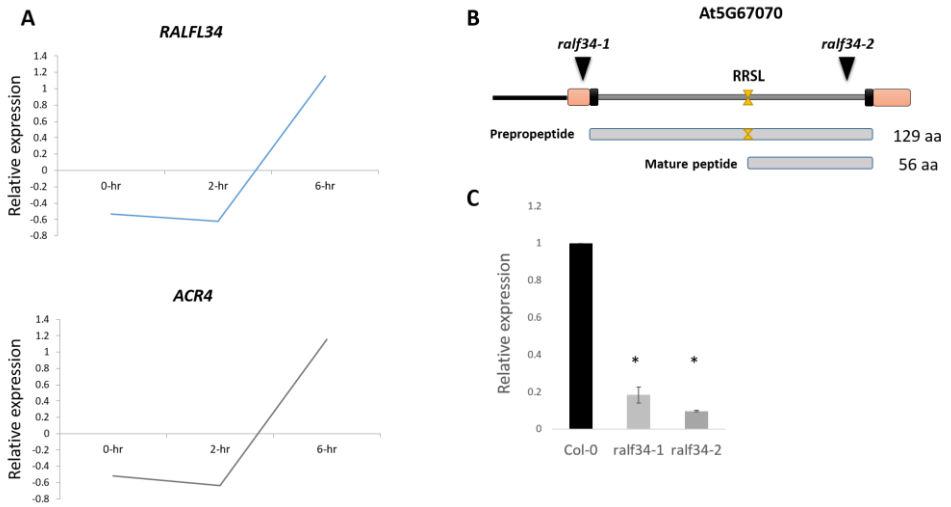


Figure 1. (A) Transcriptome data profiling (top) *RALF34* and (bottom) *ACR4* expression specifically during lateral root initiation (De Smet et al., 2008) ; (B) Characterisation of the *RALF34* gene (*At5G67070*) showing the *ralf34-1* T-DNA insert in the 5' UTR and the *ralf34-2* insert in the EXON in the mature peptide coding region, RRSL identifying a potential cleavage site, light grey box showing the putative sizes of the prepropeptide and the mature peptide; red box – UTR, black box – start/stop codon, black bar – PROMOTER, dark grey bar – EXON, blue box – INTRON; (C) Relative expression levels of the *ralf34-1* and *ralf34-2* insert lines, significantly lower than wild type ($P < 0.05$).

RALF34 is expressed before asymmetric cell division in the pericycle

To investigate at which stage during lateral root development *RALF34* is expressed, we generated a *pRALF34*$_{869bp}$*::n3xRFP* line. This revealed that *RALF34* is already expressed before any visible sign of asymmetric cell division (**Figure 3A**). At a later stage, *RALF34* is expressed in the lateral root initiation site (**Figure 3A**), and continues to be expressed during lateral root primordium development (**Figure 3A**). Interestingly, *RALF34* also displays a 'tail' of expression that extends from the lateral root initiation site shootward

and/or rootward, which we explored in more depth later (see below, **Figure 4**).

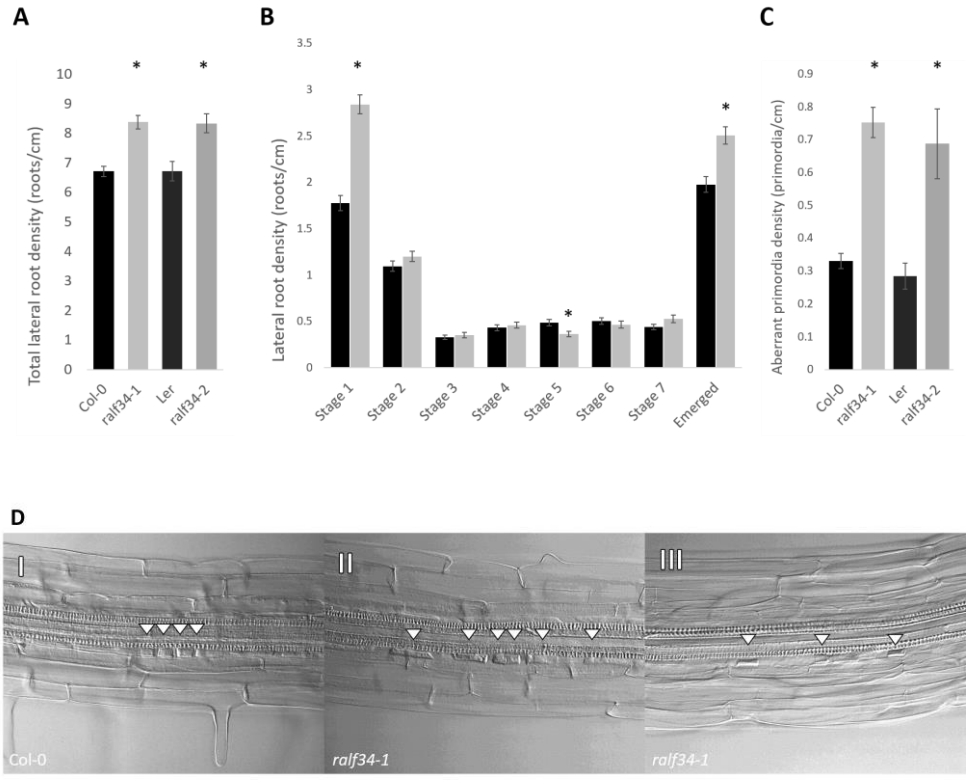


Figure 2. (A) Total lateral root density for Col-0, Ler, *ralf34-1*, and *ralf34-2*; (B) Lateral root densities for individual stages in Col-0 and *ralf34-1*; (C) Density of aberrantly formed primordia for Col-0, Ler, *ralf34-1*, and *ralf34-2*; (D) Representative DIC images of (I) Col-0 seedling with a normal stage 1 primordia, (II) *ralf34-1* seedling with an aberrant stage 1 primordia surrounded by shortened pericycle cells, (III) *ralf34-1* showing small intermittent cells divisions atypical of normal stage 1 development; Graphs show average of 3 biological replicates ± standard error, asterisks indicate statistical significance (two tailed student's *t*-test, $P < 0.05$).

Given that lateral root development is controlled by auxin at various levels (Lavenus et al., 2013), we explored if *RALF34* expression is regulated by auxin in the root using qPCR. While a mild, concentration-dependent transcriptional up-regulation was observed following a 24 hour NAA treatment, a shorter 6 hour treatment did not result in any significant change (**Figure 3B**). Likely, the up-

regulation at 24 hours is due to an increased number of lateral root primordia expressing *RALF34* (**Figure 3C**). To position *RALF34* expression with respect to transcriptional auxin response during lateral root initiation, *pRALF34::n3xRFP* was crossed with a *pDR5::VENUS* reporter. The expression of *RALF34* begins at the same time as *DR5* expression (**Figure 3D**) and then carries on concurrently throughout lateral root development until the latter stages.

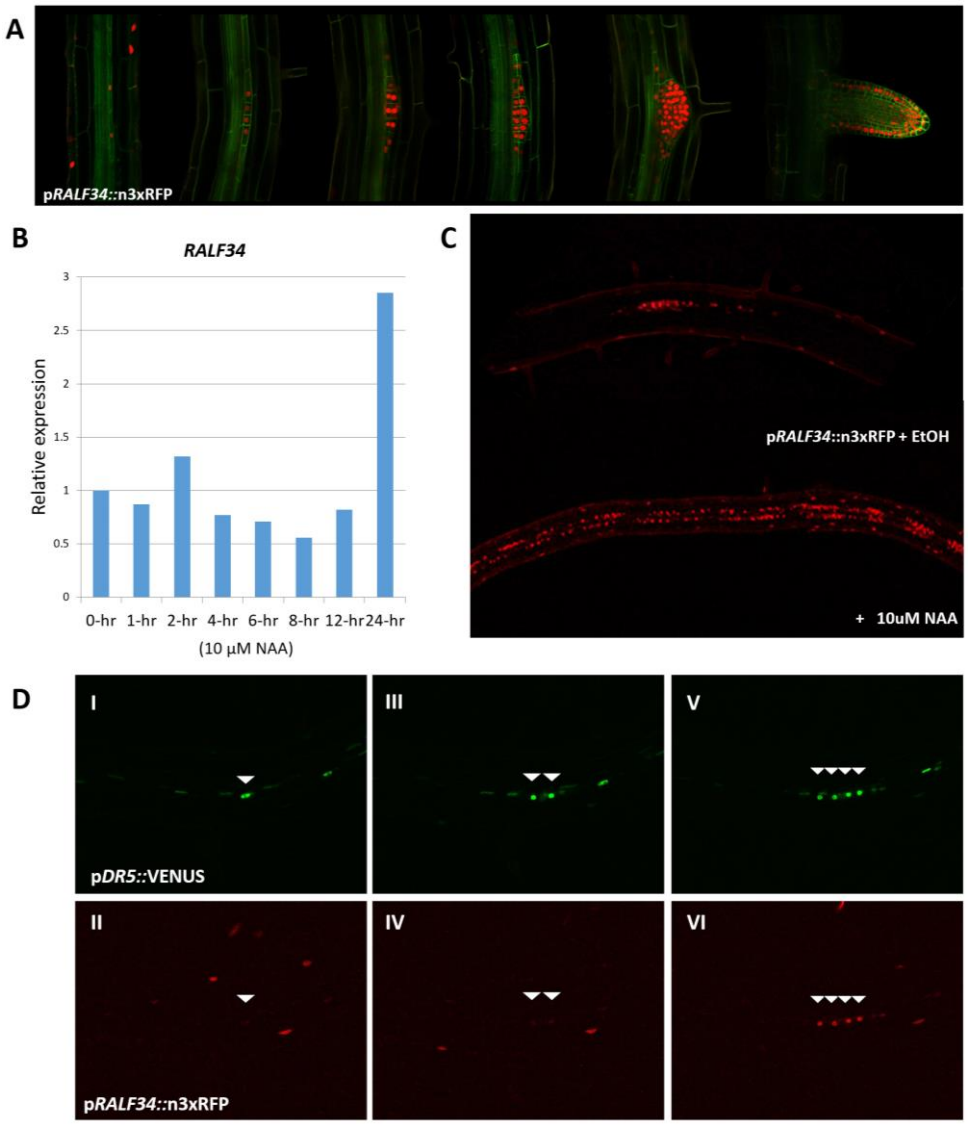


Figure 3. (A) Lateral root expression of p*RALF34*::n3xRFP progression from – pre-asymmetric cell division (founder cells), post-first asymmetric cell division (stage 1), first periclinal cell divisions (stage 2), second round periclinal cell divisions (stage 3), developing primordia (stage 7) reaching epidermal layer, and a developed, emerged lateral root primordia (stage 8+); (B) Relative gene expression of *RALF34* over 24 hours after treatment with 10 µM NAA; (C) Expression profile of p*RALF34*::n3xRFP (top) in control, and (bottom) treatment with 10 µM NAA; (D) Expression of p*DR5*::VENUS and p*RALF34*::n3xRFP during the formative asymmetric cell division of lateral root organogenesis: (I,II) *DR5* and (to a lesser extent) *RALF34* are expressed before the first asymmetric cell division, (III, IV) expression of both reporters after the first asymmetric cell division, (V, VI) expression of both reporters after the second round of asymmetric cell division.

RALF34 is expressed during lateral root initiation in cells flanking the developing primordia

Interestingly, the p*RALF34*::n3xRFP expression around the lateral root initiation site often showed a "tailing' (25/30 lateral root primordia) (**Figure 4**).

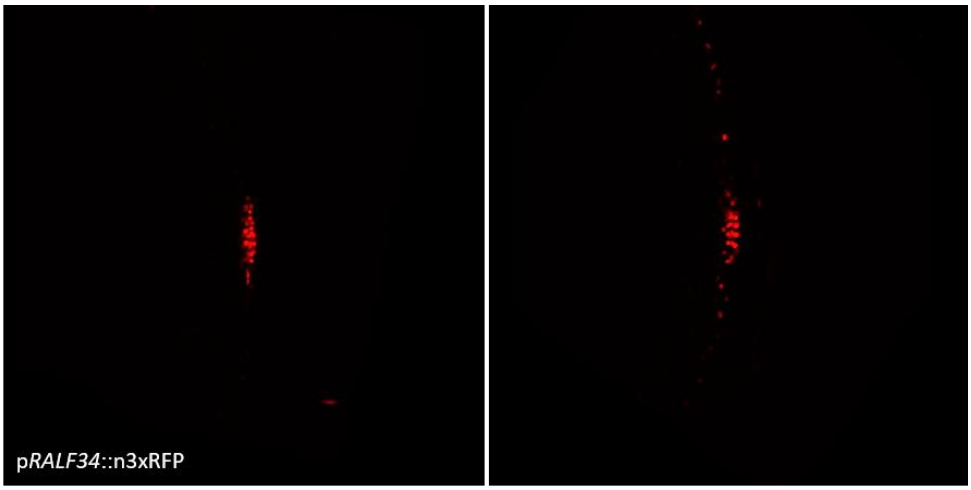


Figure 4. Representative confocal images of p*RALF34*::n3xRFP 'tailing'. Expression of p*RALF34*::n3xRFP 20 hours after gravitropic bend stimulus was applied, p*RALF34*::n3xRFP (left) shows a normal stage 3 primordia with no 'tailing', with expression contain solely within the primordia; p*RALF34*::n3xRFP (right) shows expression within every cell of the developing primordia and several upstream and downstream flanking cells.

What regulates *RALF34* expression?

In addition to the previously shown minor auxin regulation (**Figure 3B**), we explored to what extent *RALF34* expression is altered in the lateral rootless mutants *arf7arf19* and *slr-1* (Fukaki et al., 2002; Okushima et al., 2007). This revealed – using qPCR – a surprising difference between the two mutants, as *RALF34* was significantly upregulated in the *slr* mutant but not in the *arf7*, *arf19*, or *arf7 arf19* double mutant (**Figure 5A**).

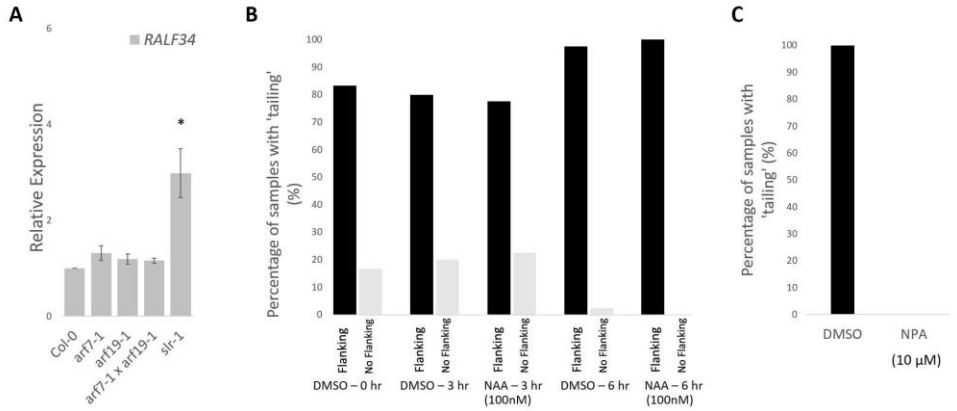


Figure 5. (A) Relative gene expression of *RALF34* in key auxin related lateral root mutants, *arf7-1*, *arf19-1*, *arf7-1 arf19-1*, and *slr-1*, graph show average of 3 biological replicates ± standard error, asterisks indicate statistical significance (two tailed student's *t*-test, $P < 0.05$); (B) Percentage of lateral root primordia in root samples, 5 days post germination, surrounded by additional p*RALF34*::n3xRFP expression over time, in either control 0 hr (DMSO, n= 30), 3hr (DMSO n= 40, NAA n= 39), and 6hr (DMSO n= 40, NAA n= 55) treated conditions (graph represents one biological replicate); (C) Flanking expression of p*RALF34*::n3xRFP shown as a percentage in mock treated (DMSO, n= 8) and 10 µM NPA (n= 8) treated samples 20 hours after the mechanical bend assay, 5 days post germination; result from one biological replicate.

To observe whether this 'tailing/flanking' expression was an auxin regulated process we applied the synthetic plant hormone NAA to 5 day post germination *Arabidopsis* Col-0 background seedlings and observed the change in p*RALF34*::n3xRFP expression over time (**Figure 5B**). These data show that the application of auxin does not directly induce the increase in p*RALF34*::n3xRFP flanking expression within 3 hours of 100 nM NAA treatment (**Figure 5B**). Statistically, 6 hours post 100 nm NAA treatment (P= 0.02), flanking expression increased over time 0, however, it did not differ from the DMSO control (P= 0.32) (**Figure 5B**). Thus, caution must be noted as the mock DMSO treatment increased flanking nearly 20% over the control time 0

(P= 0.06) as well, suggesting that a secondary mechanism may also be inducing p*RALF34*::n3xRFP induction. Potentially, p*RALF34*::n3xRFP could be mechanically induced and it is the transfer from the control plate to the DMSO plate, forcing the root to bend and unbend, causing the increase in p*RALF34*::n3xRFP flanking expression over the 0 hr control. Interestingly, mechanical stimulation of roots causes increases in Ca^{2+} in epidermal, cortical, endodermal, and pericycle cells of roots (potentially through stretch activated Ca^{2+} channels); RALF peptides have been implicated numerous times in Ca^{2+} mediated signalling as application of several RALF peptides elicits a rapid and often strong Ca^{2+} response (Haruta et al., 2008; Haruta et al., 2014; Morato do Canto et al., 2014; Richter et al., 2009). Further investigation needs to be performed into whether RALF34 is involved in Ca^{2+} mediated signalling. This could potentially be observed through crossing p*RALF34*::n3xRFP into the fluorescent marker aequorin-expressing seedlings (which specifically highlight strong Ca^{2+} signalling responses), which could illustrate a mechanism during mechanical stimulation.

To further correlate or dissociate the p*RALF34*::n3xRFP flanking expression from auxin mediated signalling, we performed the mechanical bend assay, inducing the lateral root formation machinery, however, we performed this on NPA media – which blocks auxin transport (**Figure 5C**). These data show once again that p*RALF34*::n3xRFP tailing expression can be induced through mechanical stimulation of lateral root initiation, and that it is a lateral root initiation specific expression pattern (**Figure 5C**). In this data set, 100% of mechanically bent roots, generating lateral root stage 2 primordia 20 hours post stimulus, formed flanking RFP on control media; oppositely, 0% of the NPA

treated roots formed lateral root primordia, and concurrently did not form flanking RFP expression. However, it has already been established that the DMSO control could generate flanking expression in the majority of cases due to (a) – lateral root primordia initiation, and (b) – potential mechanical stimulation; thus, since we know NPA will inhibit the formation of a lateral root initiation, but cannot rule out the effects of transferring seedlings, another approach must be utilised.

Therefore, to further established whether mechanically inducing bends forces an increase in the flanking expression, we needed to investigate expression of p*RALF34*::n3xRFP in a background that would not normally form lateral root primordia through mechanical stimulation, without transferring seedlings. Thus, to connect our small signalling peptide flanking expression specifically to a lateral root specific developmental process, we crossed the fluorescent marker line into the lateral root-less gain-of-function mutant SOLITARY-ROOT/IAA14/SLR (*slr-1*) (Fukaki et al., 2002) (**Figure 6**). Performing the mechanical bend assay to induce a lateral root in wild type background, we observed normal *pRALF34::n3xRFP* flanking expression (**Figure 6-I**); however, in *slr-1* x p*RALF34*::n3xRFP we observed no such flanking expression (no lateral root formation) (**Figure 6-II**). Interestingly however, *RALF34* expression appears to be significantly enhanced throughout

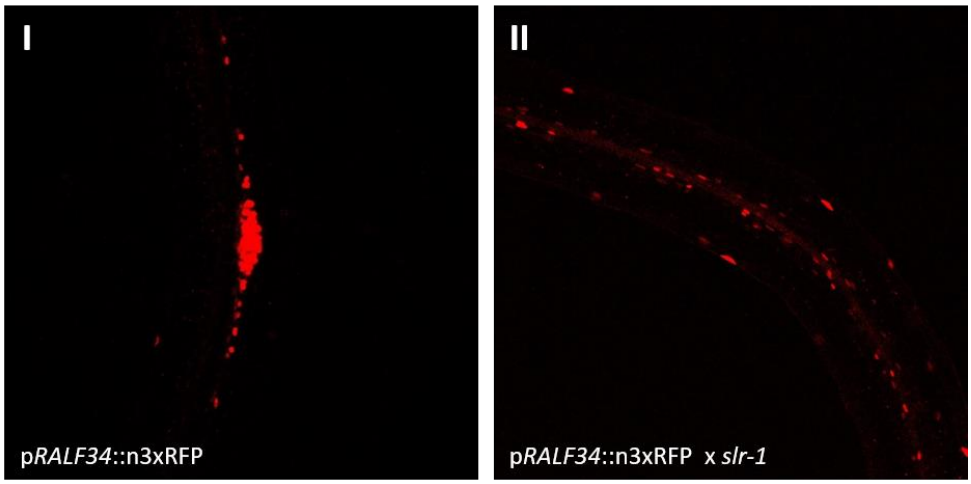


Figure 6. Confocal images of p*RALF34*::n3xRFP (n= 10) and p*RALF34*::n3xRFP x *slr-1* F3 homozygous seedlings (n= 10) 22 hours after mechanical bend stimulus was applied. (I) p*RALF34*::n3xRFP shows a normal stage 3 primordia with upstream and downstream 'tailing' expression outside of the primordia; (II) p*RALF34*::n3xRFP x *slr-1* shows epidermal expression and further expression all throughout the vascular tissue; result from one biological replicate.

the stele tissues, in cell layers *RALF34* isn't normally expressed (**Figure 6-II**). These data confirm the transcriptional regulation shown in (**Figure 5A**) revealing *RALF34* transcripts are upregulated in the *slr-1* background. However, we must be cautious interpreting mechanical bending assay data as the lateral roots developed through manipulation (gravitropic stimulus or especially mechanical bending) are subject to simulated growth conditions, with high levels of auxin generated through altered PIN protein localisations (Ditengou et al., 2008). This could potentially be activating/repressing genes in a manner different from normal conditions, which could lead to incorrect interpretations of the underlying genetic framework. However, it is still a powerful tool in inducing lateral roots in those mutants which do not normally respond to gravity (such as *slr-1*), or in inducing lateral roots to observe relative genetic changes within a specific timeframe.

Root bending induces* pRALF34::n3xRFP *in lateral roots in an auxin dependent manner

After its inferred role on the expression of p*RALF34*::n3xRFP, we used the gravitropic bending assay to induce the generation of a lateral root to further investigate the role of auxin in the flanking expression pattern of p*RALF34*::n3xRFP. We then excised the aerial tissues (hypocotyl and upward) to remove the shoot derived sources of auxin to ascertain what role they play on the p*RALF34*::n3xRFP flanking expression. Furthermore, we also removed the root tip, or the auxin 'sink', to observe any potential opposite effects (**Figure 7**).

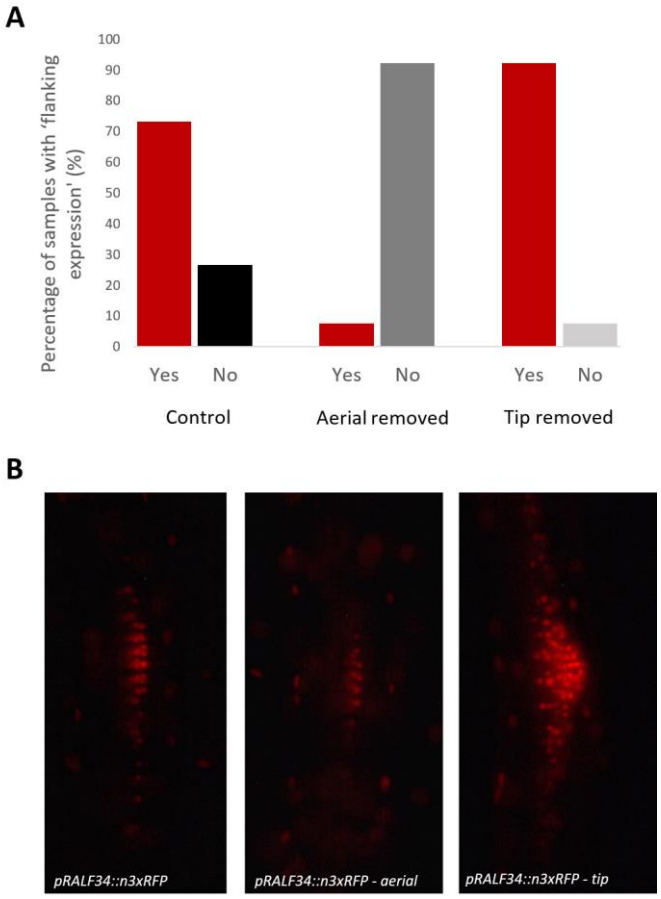


Figure 7. (A) Percentage of samples with 'flanking expression' of p*RALF34*::n3xRFP in roots 20 hours post gravitropic stimulation, 5 days post germination: control (n= 15); aerial tissues removed (P= 0.0001, n= 13); and root apical meristem removed (P= 0.1913, n= 13); graph represents result from one biological replicate; (B) Representative images of (left) *pRALF34::n3xRFP*, (middle) *pRALF34::n3xRFP* – aerial tissue, and (right) *pRALF34::n3xRFP* – root tip.

To test whether a signal from the shoot was necessary for p*RALF34*::n3 x RFP flanking expression, we subjected p*RALF34*::n3 x RFP plants to mechanical root tip bending for 20 hours after the removal of their aerial tissues. Furthermore, to see whether a root tip derived signal was required, we removed the root apical meristem (**Figure 7**). Seedling samples grown under control conditions (no tissues removed) showed over 70% p*RALF34*::n3xRFP flanking expression after 20 hours gravistimulation (**Figure 7A**). Seedlings with aerial

tissues removed had over 90% without p*RALF34*::n3xRFP flanking expression (**Figure 7A**). These data strongly suggest that a shoot derived source of auxin is required for p*RALF34*::n3xRFP 'tailing' expression. Removing the root tip, which sped up the primordia development, did not significantly impact the tailing expression pattern compared to control (**Figure 7B**). However, since tailing expression is already high in the control, it may be easier to detect a reduction in expression. It is difficult to interpret auxin induced changes, as removing the root tip causes aberrant divisions within many of the primordia owing to the higher accumulated auxin content, potentially restricting signals to flanking cells. Furthermore, the increased auxin content in the root tip causes proliferative divisions, generating many closely spaced lateral root primordia, making it difficult to ascertain where the 'flanking' expression is. Lastly, as high auxin primes cells to become lateral root primordia, it may be difficult to interpret what is a primed cell (which would express *RALF34*) and what is an extension of an already initiated primordia. Thus, these results prove that not only is auxin necessary for p*RALF34*::n3xRFP flanking expression, but specifically it is shoot derived auxin and/or a novel shoot transported signal that is required.

To precisely observe the impact of removing shoot derived auxin on RALF peptides *in planta* we utilised the gravitropic bend assay (Ditengou et al., 2008) on RALF T-DNA insertion mutants, and excised the aerial tissues (hypocotyl and upward) to observe the cellular formations during lateral root development. Since p*RALF34*::n3xRFP flanking expression is significantly reduced when the aerial tissue, ie. shoot derived auxin, is removed we assumed we might observe a potential restriction in cell division (restriction in lateral root

primordia length) after performing the bend assay. In our gravitropic bend assay, 20 hours post bend, without removing aerial tissues, there was no significant differences between any of the controls or mutant lines (**Figure 8A**). However, 20 hours post bend and having removed the aerial tissues – 100% of Col-0 and 93% Ler control seedlings generated stage 1 primordia, whereas, only 64%, and 43% of *ralf34-1*, and *ralf34-2* mutant seedlings initiated stage 1 primordia, respectively (**Figure 8A-B**).Clearly, this suggests RALF34 is required for lateral root initiation in addition to a shoot derived signal, likely auxin.

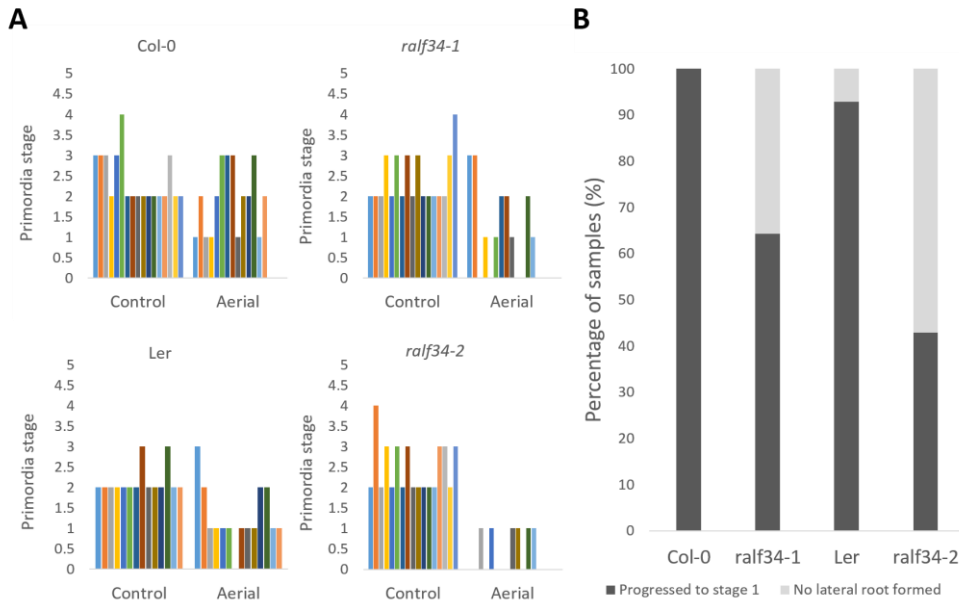


Figure 8. Roots observed for lateral root primordia stages 20 hours post gravitropic stimulation after aerial tissue removal, 5 days post germination (A) Primordia stages after 20 hours gravistimulation of wild types (Col-0 and Ler) and two *RALF34* alleles (*ralf34-1* and *ralf34-2*) with and without aerial tissues; (B) Percentage of samples, with aerial tissues removed, which progressed to stage 1 (dark grey) or failed to form a lateral root (light grey), in wild types (Col-0 and Ler, n= 14 each) and *RALF34* mutant alleles (*ralf34-1* (P= 0.043073575, n= 14) and *ralf34-2* (P= 0.001459954, n= 14)), graphs represents result from one biological replicate.

RALF34 acts upstream of GATA23

One of the earliest markers for lateral root initiation is GATA23 (De Rybel et al., 2010). First, we observed – using qPCR – that *GATA23* expression is significantly lower in *ralf34-1* roots (**Figure 9A**). Second, we evaluated this using the *pGATA23::nGFP* line (De Rybel et al., 2010) in the *ralf34-1* background. This also revealed less GFP positive nuclei in the root tip (**Figure 9B**). In addition, the location of the first GFP positive nucleus, with respect to the QC, was higher up the root (**Figure 9C, E**). Interestingly, GATA23 signal is often present even in cells not destined for lateral root development (in the pericycle and vasculature), and this signal is also significantly reduced in the *ralf34-1* background in the mature tissues of the root (**Figure 9D**). Based on these results, we conclude that RALF34 likely acts upstream of GATA23.

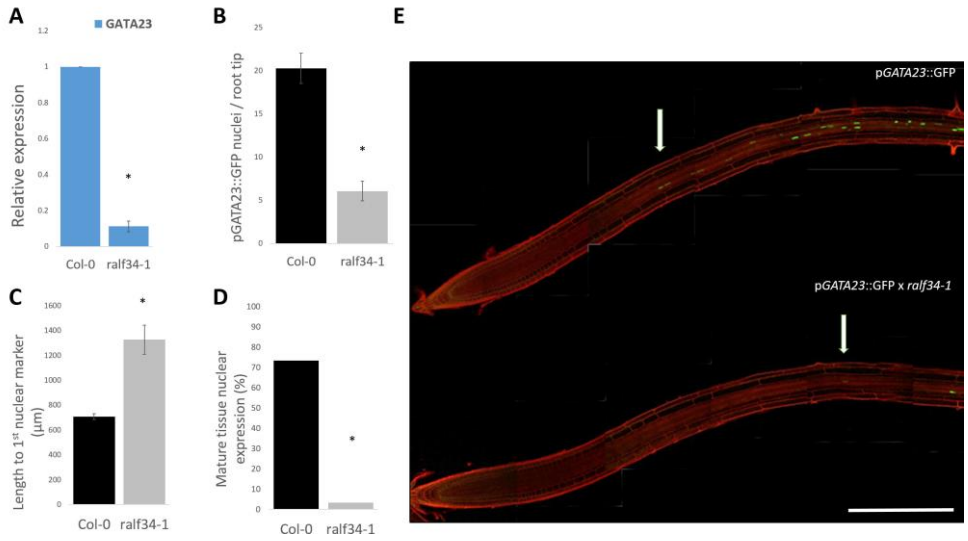


Figure 9. (A) Relative expression of *GATA23* in Col-0 and *ralf34-1* mutant; (B) Average number of p*GATA23*::GFP expressing nuclei in root tips of Col-0 and *ralf34-1* background plants; (C) The average length from the QC to the first nucleus expressing p*GATA23*::GFP in Col-0 and *ralf34-1* background seedlings; (D) The average percent of samples that expressed p*GATA23*::GFP in mature pericycle and vascular tissues in Col-0 and *ralf34-1* background seedlings; (E) Representative images of Col-0 and *ralf34-1* seedlings expressing p*GATA23*::GFP fluorescent marker proteins, white arrows indicating the first nucleus expressing GFP, white bar = 300 µm ; Graphs show average of 3 biological replicates ± standard error, asterisks indicate statistical significance (two tailed student's *t*-test, $P < 0.05$).

CONCLUSIONS

The results presented in this paper indicate a strong developmental role for the small signalling peptide RALF34 during the early stages of lateral root development. Our data show us two important details: 1) shoot derived auxin is required for the normal progression of lateral root development from stage 1, as all lines resulted in slowed primordial development with aerial tissues removed, agreeing with the literature (Bhalerao et al., 2002; Ditengou et al., 2008; Marchant et al., 2002); and 2) shoot derived auxin alone is not enough to drive

the progression from founder cell to stage 1 primordia, as the RALF34 peptide appears to be required to develop a primordia in roughly 50% of our samples. Functional redundancy within the RALF family may explain why the lateral root primordia were still able to form 40-60% of the time in the T-DNA mutant lines, (inspection in to the closely related *ralf4* homologue showed the mutant failed to develop primordia in 57% of samples ($P < 0.05$), data not shown) suggesting higher-order mutants (such as the *ralf4-1 ralf34-1* double mutant) be used to further elucidate their role in this pathway. While our transcriptomic data shows only small changes in *RALF34* expression through auxin application, clearly we've observed strong phenotypes during auxin regulated processes, suggesting *RALF34* may not be directly responsive to auxin, but acts in an auxin mediated pathway, potentially upstream of *GATA23*. These data revealed an as yet unreported role for RALF peptides in the (potential initiation and) progression of primordia development through an auxin driven process. How these data connect to auxin related genes, potentially through the aforementioned GATA23 transcription factor, or the negative regulator of ARF7 and ARF19, SLR, still needs to be shown.

MATERIALS AND METHODS

Growth of *Arabidopsis thaliana* seedlings: Seeds were surface sterilised (70% ethanol – 2 minutes, 10% bleach - 15 minutes, $_dH_2O$ – 5 washes) then stratified at 4°C for 2 days. Seeds were then plated on ½ Murashige and Skoog (MS) agar plates (2.154gL^{-1} MS, 0.1gL^{-1} myo-inositol, 0.5gL^{-1} MES, 10gL^{-1} bacteriological agar, pH 5.7 with 1 M KOH) and germinated vertically under constant white light at 21°C.

Phenotyping of T-DNA insertion lines

The following T-DNA insertion mutant lines were used in our investigations: *At5G67070*/*ralf34-1*/*SALK_004441*, genotyping primers Fp: TGACTAACCAAAAAGTCCACG, Rp: ACGGGACCTCTAGCTCTGAAG, T-DNAFp: ATTTTGCCGATTTCGGAAC), (*ralf34-2*/*JIC_SGT4223*, genotyping primers Fp: ATGGCAGCTTCGTCTCTC, Rp: CTATCTCCGGCATCGAGT, T-DNAFp: ACggTCgggAAACTAgCTCTAC:

Microscopic analysis: Phenotyping was analysed under a Leica stereo dissection microscope at varying magnifications to observe and count the emerged lateral roots. Lateral roots were counted, marked, and then photographed. Root lengths were measured from the bottom of the hypocotyl to the root tip, using ImageJ software (http://imagej.nih.gov/ij/). Lateral root staging of the *ralf34-1* and *-2* mutants was performed on a Leica DMRB microsystem using differential interference contrast (DIC). Fluorescent seedlings were analysed on Nikon confocal microscope utilising both an Argon 488 laser and a 514 HeNe laser at 20x and 40x magnification.

***RALF34* auxin response quantification:** Arabidopsis (ecotype Columbia) seeds were surface sterilised, stratified at 4°C for 2 days, then plated on ½ MS media and grown vertically under constant white light at 21°C for 4 days post germination. Seedlings were transferred to ½ MS liquid media on the 4th day to acclimate overnight. Seedlings were transferred to liquid ½ MS containing NAA at a concentration of 10 µM (or ethanol control) and were grown for a further 0, 1, 2, 4, 6, 8, 12, and 24 hours; at each time point the roots of 10 seedlings were excised, frozen in liquid nitrogen and then total RNA extracted as below.

Quantitative RT-PCR: Total RNA was extracted from lines of interest using an RNeasy kit (Qiagen) as per manufacturer's instructions. Using Superscript II (Invitrogen), cDNA was generated from total RNA (200 ng) via oligo(dT) primers as per instructions. Quantitative PCR reactions were performed in 4 technical repeats, using the SYBR Green QPCR Master Mix (Quanta Biosciences), in our modified reaction protocol: Master mix - 7 µL/reaction (forward primer [100 µM] – 0.1 µL/reaction, reverse primer [100 µM] – 0.1 µL/reaction, SYBR(2X) – 6 µL/reaction, $_{d}H_2O$ – 0.8 µL/reaction) and template cDNA [diluted 1/100 $_{d}H_2O$] – 5 µL/reaction. Gene specific quantification was performed with intron spanning primer pairs (where possible) designed using Primer3 software (http://bioinfo.ut.ee/primer3/). Expression was normalised to *EEF1α4* (At5g60390). Primers used: qRALF34_Fp (CGATGCCGGAGATAGATGAAG), qRALF34_Rp (CAATCCACCCCTCACGACT), qARF7_Fp (GCTCATATGCATGCTCCACA), qARF7_Rp (GCAATGCATCTCTGTCATATTTG), qARF19_Fp (CACCGATCACGAAAACGATA), qARF19_Rp (TGTTCTGCACGCAGTTCAC), qIAA14_Fp (ATGACTCGACAAACATCGGCCAGG), qIAA14_Rp (ACGAGGACAAAGATGGTGACTGGA), qGATA23_Fp (AGTGAGAATGAAAGAAGAGAAGGG), qGATA23_Rp (GTGGCTGCGAATAATATGAATACC), qEEF1α4_Fp (ctggaggttttgaggctggtat), and qEEF1α4_Rp (ccaagggtgaaagcaagaaga). All qRT-PCR experiments were performed in three biological replicates (unless

otherwise specified) and the data presented represent means ± standard deviation.

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Chapter 5 – RALFs role in primary root growth

RESEARCH MANUSCRIPT

Evan MURPHY and Ive DE SMET

SUMMARY

During plant development, two main processes underlie growth – cell division and cell elongation. Each process occurs in distinct regions within the root – the root apical meristem (RAM) controls cell division and the elongation zone is the area where meristematic activity terminates and rapid cell elongation takes over. Many factors such as plant hormones, such as brassinosteroids, control the transition from the meristematic region to the elongation zone. Recently implicated in brassinosteroid mediated cell elongation was a RALF small signalling peptide.

Here we provide novel evidence on the role of RALF34 and RALF4 peptides in cell elongation in the *Arabidopsis* primary root. We generated a *RALF34* transcriptional marker which showed very specific expression profiles and taken together with root phenotypes, suggests specific functions for these peptides within the root tip and specifically, within cell elongation. Identifying novel peptides involved in root mediated cell elongation may prove fruitful in the future, specifically through agronomic applications.

INTRODUCTION

For plants to grow, in both size and maturity, they generally utilise two mechanisms: cell division and cell elongation. In the plant root, these processes originate from the root apical meristem (RAM), an epicentre of slowly dividing stem cells, from which all other tissues of the root are formed. The central core of the RAM consists of 2-4 slowly diving cells known as the quiescent centre (QC) which diffuses developmental signals (WUSCHEL-RELATED HOMEOBOX5 (WOX5)) to surrounding stem cells, keeping their pluripotent identities (Sarkar et al., 2007). Moving away from the QC, the stems cells divide, forming daughter cells. The distal stem cell loses its quiescent signal from the QC and differentiates into the various tissues of the root (epidermis, cortex, endodermis, pericycle, and vascular bundle) due to various (sometimes mobile) transcription factors, SHORT ROOT (SHR), SCARECROW (SCR), PLETHORA1-3 (PLT1-3) (Aida et al., 2004; Di Laurenzio et al., 1996; Drisch and Stahl, 2015; Helariutta et al., 2000). These cells then undergo proliferative divisions within the meristems, multiplying in identity and generate the various

tissue layers. As cell division is generally costly, once the tissue layers have established their identities following the meristematic activity, cell elongation is the growth process that contributes to the majority of a primary roots overall length. Cell elongation is caused by the loosening of the many diverse components within the cell wall structure, closely followed by the synthesis, transport, and re-uptake of new cell wall components (Carpita et al., 1982; Cosgrove, 2005; Cosgrove and Li, 1993). Cell elongation is a dynamic process that is affected by many factors including phytohormones (auxins, brassinosteroids, and ethylene) (Clouse et al., 1996; Guzman and Ecker, 1990; Lincoln et al., 1990), environmental conditions (water, nutrients, and light) (van der Weele et al., 2000; Wu and Spalding, 2007; Zhang and Forde, 2000) and recently, many small signalling peptides have been shown to have significant roles in primary root elongation, including PHYTOSULFOKINE (PSK), PLANT PEPTIDE CONTAINING SULFATED TYROSINE 1 (PSY1) (Matsubayashi et al., 2006) (Ladwig et al., 2015; Matsuzaki et al., 2010), many of the CLE family of peptides (Casamitjana-Martinez et al., 2003; Fiers et al., 2005; Fiers et al., 2004; Jun et al., 2010; Meng and Feldman, 2010; Strabala et al., 2006; Strabala et al., 2014), and lastly, several of the RAPID ALKALINIZATION FACTOR (RALF) family of peptides.

RALFs have been identified to play a role in primary root growth in a variety of species, from *NaRALF* in tobacco (Wu et al., 2007), *SlRALF* in tomato (Pearce et al., 2001), to many of the known *RALFs* in *Arabidopsis thaliana* (Atkinson et al., 2013; Bergonci et al., 2014; Morato do Canto et al., 2014; Srivastava et al., 2009). Interestingly, *MtRALF1* (*Medicago trucatula*), which is

expressed in the root and functions during nodulation, has no apparent function in primary root development or elongation (when overexpressed), showing these peptides carry both a conserved and diverse set of developmental roles (Combier et al., 2008). Little is known about the crosstalk between the diverse phytohormone signals and RALF peptides. In poplar, *PtdRALF2* responds negatively to methyl jasmonate, however, auxin and cytokinin have no apparent transcriptional affect (Haruta and Constabel, 2003). Recently, it has been shown that RALF1/RALF23 peptides may be mediated through brassinosteroid signalling during primary root growth, since both an *irAtRALF1* knockdown line and (oppositely) a *35S::RALF23* line were less sensitive to brassinolide (a biologically active brassinosteroid) than wild type (Bergonci et al., 2014; Srivastava et al., 2009). Little is known of the role of RALFs and the phytohormone auxin in *Arabidopsis*, a signal known to govern numerous plant processes, including gravity response (Sato et al., 2015), lateral root development (Band et al., 2014), and cell elongation (Muraro et al., 2013). Previous research has connected the small signalling peptides RALF34 and RALF4 with lateral root development, connecting them to the auxin responsive transcription factor GATA23, suggesting these genes may have a role in auxin mediated development (see Chapter 4 – RALFs in lateral root development). This led us to explore how RALF34 and RALF4 affect another auxin mediated pathway, cell elongation, in the primary root.

In this study, we identified *RALF34* as a highly expressed gene in the primary root. We showed knockdown/out levels of the *RALF4* and *RALF34* genes in *Arabidopsis* plants increased cell elongation significantly and played a

small role in increasing cell division. Additionally, we showed that generating a higher order mutant, *ralf4 ralf34*, enhanced these phenotypes and may suggest that multiple RALFs play roles of cell elongation and division, through separate pathways, simultaneously. To correlate the effects at the transcriptional level, we generated a fluorescent marker line to report on the expression pattern and localisation of the *RALF34* gene, which is strongly expressed in the root apical meristem, and in every epidermal cell. Lastly, we showed that *ralf4/34* T-DNA plants were less sensitive to auxin induced root growth inhibition. Our data suggests that RALF4/34 act in the negative regulation of cell expansion, likely under the secondary control of the phytohormone auxin.

RESULTS AND DISCUSSION

RALF34 is strongly expressed in the root tip

To identify whether *RALF4* and *RALF34* were genes potentially involved in primary root growth, we performed an *in silico* analysis of high-resolution expression profiling using *Arabidopsis* eFP browser and compared these data to the previously characterised *RALF1* gene, known for playing a role in primary root cell elongation (Bergonci et al., 2014; Brady et al., 2007; Winter et al., 2007). Interestingly, *RALF4* was observed to be equally lowly expressed throughout the majority of tissues as *RALF1*, however, it was slightly higher in the meristematic region (**Figure 1A**). *RALF1* is more highly expressed in mature vascular tissue, whereas *RALF34* was observed to be highly expressed in the xylem pole pericycle, and importantly, all throughout the root tip (**Figure 1A**). We confirmed this expression pattern *in vivo* for *RALF34* by utilising a

transcriptional reporter, where we observed strong expressed throughout the entire root meristem, root cap, and epidermal tissues (**Figure 1B**) (*RALF4* reporter in progress at time of writing). Since genes that are expressed in the meristematic region can have a profound effect on primary root growth, we investigated *RALF4* and *RALF34* loss-of-function phenotypes in more detail (Sabatini et al., 2003).

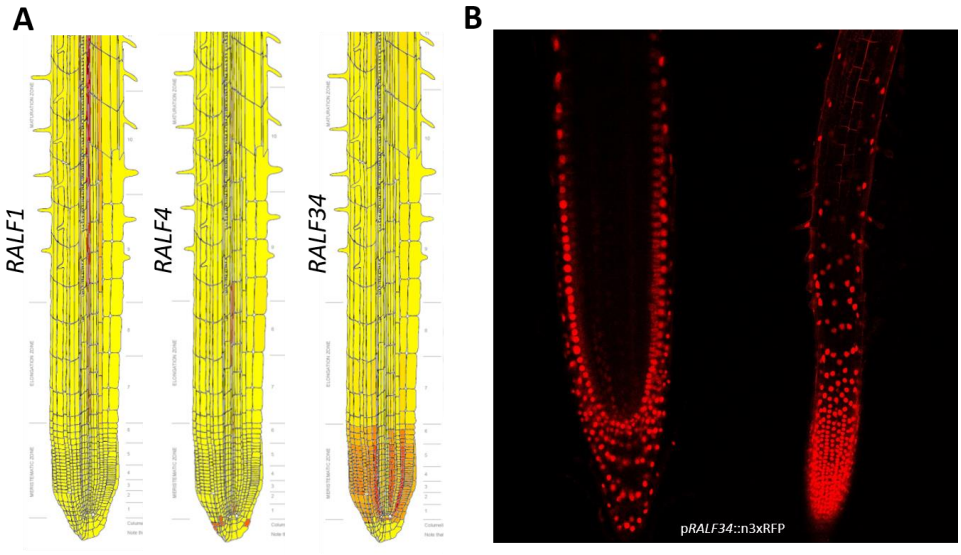


Figure 1. (A) Schematic heat map representation of expression profiles of *RALF1* (left), *RALF4* (middle), and *RALF34* (right), deeper colour indicates higher potential expression; (B) Expression of p*RALF34*::n3xRFP in *Arabidopsis* primary root - (left) strong expression throughout the meristematic tissues, including root cap and epidermal layer (40x magnification), (right) expression continues outwardly from the meristem and continues significantly throughout the epidermal layer (20x magnification; image is two pictures stitched into one larger image).

RALF34 and RALF4 regulate primary root growth

To explore the role of *RALF* peptides in primary root growth, we utilised T-DNA insertion lines in *RALF4* (*ralf4*-1) and *RALF34* (*ralf34-1*, *ralf34-2*)

genes to investigate the effects of downregulating the levels of these peptides in *Arabidopsis* plants (**Figure 2A**). A plant line with a single T-DNA insertion in *RALF4* or *RALF34* shows a significant increase in root length over wild type of at least 10% (**Figure 2B, C**). These data are consistent with previous research showing a knockout line (*irAtralf1*) for RALF1 producing significantly longer primary roots (Bergonci et al., 2014). This suggests that numerous RALF peptides natively function to restrict cell expansion, or potentially, the irRALF line was not highly specific and may have targeted other closely related family members. However, to isolate whether these two closely related peptides function in correlation or in a redundant fashion, to further elucidate the role these two may play in root elongation, a higher order double mutant (*ralf4-1* x *ralf34-1*) was generated. Interestingly, this produced an additive phenotype increasing primary root length to around 20% greater root length than wild type (**Figure 2B, C**). Thus, RALFs can cooperatively contribute to primary root growth, potentially acting through multiple receptor-like kinases, such as the recently identified RALF1 receptor, FERONIA (Haruta et al., 2014).

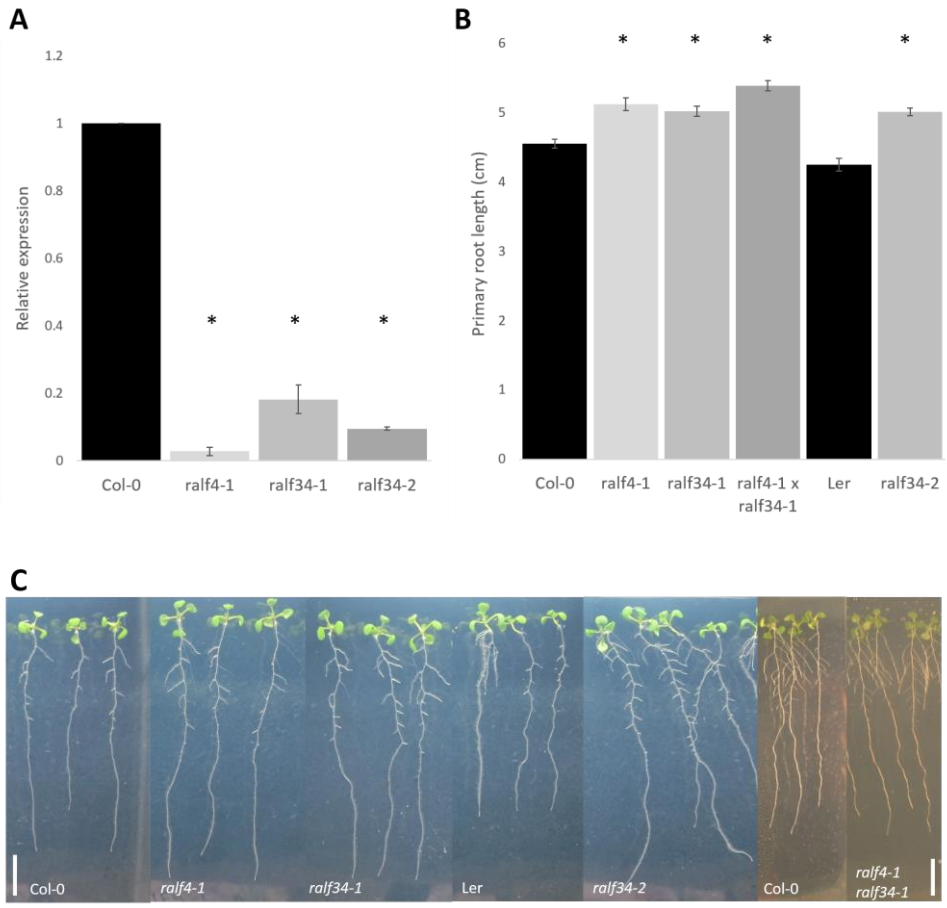


Figure 2. (A) Relative gene expression levels through quantitative real-time PCR of genes *RALF4* and *RALF34* in the T-DNA insertion mutant lines *ralf4-1*, and *ralf34-1* and *ralf34-2*, respectively; (B) Primary root lengths (cm) of 7 days post germination seedlings in wild type (Col-0 (n=133)), Ler (n=40)), and RALF T-DNA insertion mutants (*ralf4-1*, P= 1.49E-06 (n=67); *ralf34-1*, P= 1.92E-07 (n=104); *ralf34-2*, P= 1.22E-09 (n=120); and *ralf4-1* x *ralf34*-1, P= 2.60E-21 (n=136)); (C) Representative growth phenotypes of (from left to right) Col-0, *ralf4-1*, *ralf34-1*, Ler, *ralf34-2*, and Col-0 with the *ralf4-1 ralf34-1* double mutant, all grown for 7 days post germination; white bars represent 1 cm each, respectively. Graphs show average of 3 biological replicates ± standard error, asterisks indicate statistical significance ($P < 0.001$).

RALF34 and RALF4 control meristematic cell number and size

To elucidate the mechanism behind these RALFs role in primary root elongation we performed cortical cell length measurements. In *Arabidopsis* the cortex is derived from cortex/endodermis initials (CEIs) located in the root apical meristem. Through formative asymmetric cell divisions of the CEIs, regulated largely by SHR and SCR, gives rise to the mature endodermis and cortex, together known as the ground tissue (Cui et al., 2007; Gallagher et al., 2004; Helariutta et al., 2000). The cortical cell file is easily visualised with the

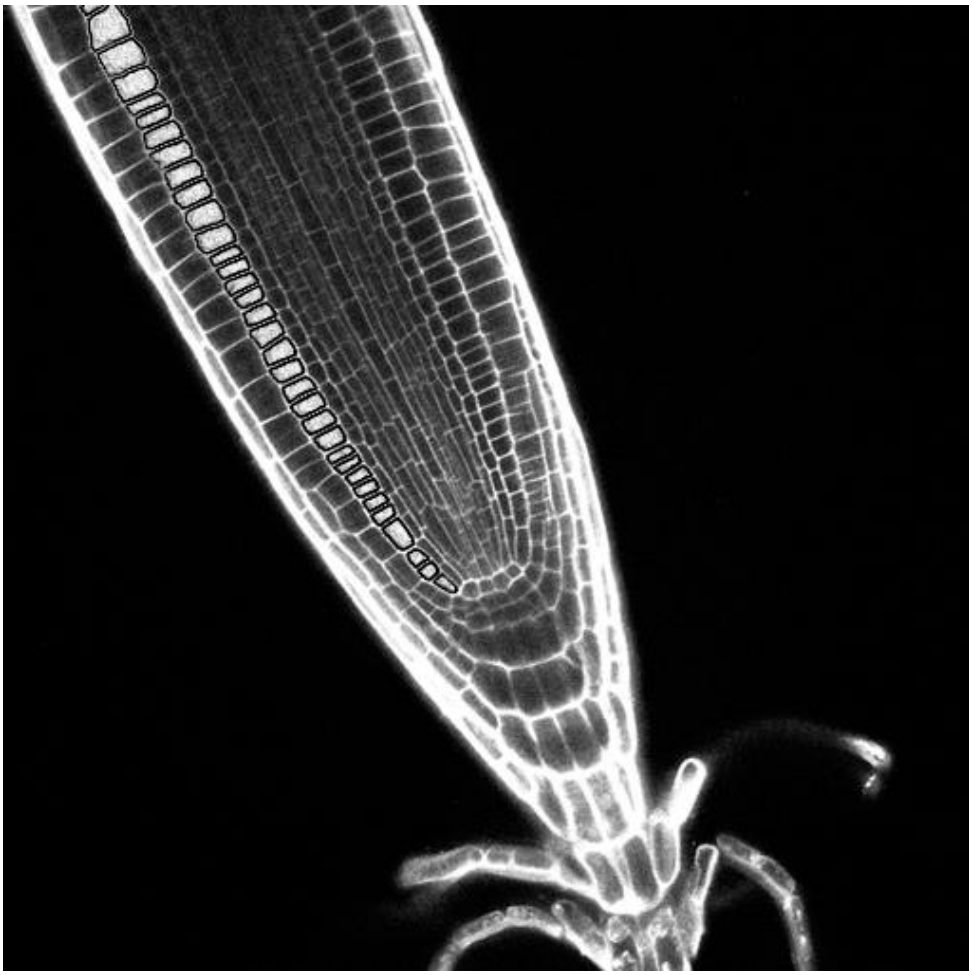


Figure 3. Confocal image of a propidium iodide stained root tip of a wild type (Col-0) seedling; highlighted in grey are the cortical cells used to measure and quantify the transition from root apical meristem to transition zone; 40X magnification.

use of propidium iodide stain, here used as a cell wall marker, and can help measure and quantify the ratios and distances between key areas of the developing root, such as the meristematic region, the transition zone, and the elongation zone (**Figures 3 & 4**).

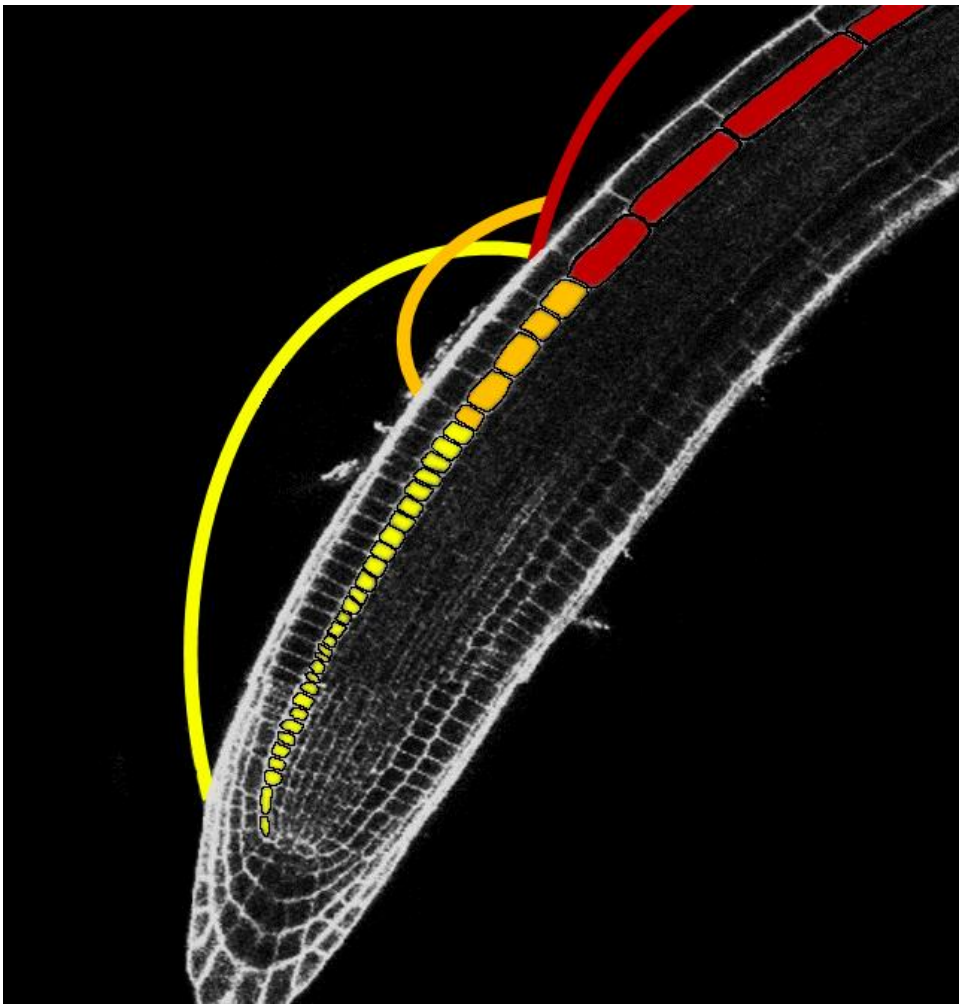


Figure 4. Confocal image of a propidium iodide stained root tip of a wild type (Col-0) seedling showing the switch from meristematic region (yellow) to the transition zone (orange) to elongation zone (red); 20X magnification.

Cortical cell length measurements show that the average meristem length in the *ralf4-1* and *ralf34-1* mutant backgrounds are significantly longer than wild type (roughly 13% and 6% increases, respectively) (**Figure 5A**). Furthermore, this phenotype is additive in the *ralf4-1 ralf34-1* double mutant background (increase of 22% over wild type), suggesting these peptides have an additive effect and may participate in the same or similar regulatory role. Interestingly, the number of cells within the meristem region is increased in the *ralf4-1* mutant (approximately 10%), but not in the *ralf34-1* mutant (**Figure 5B, 6**), potentially

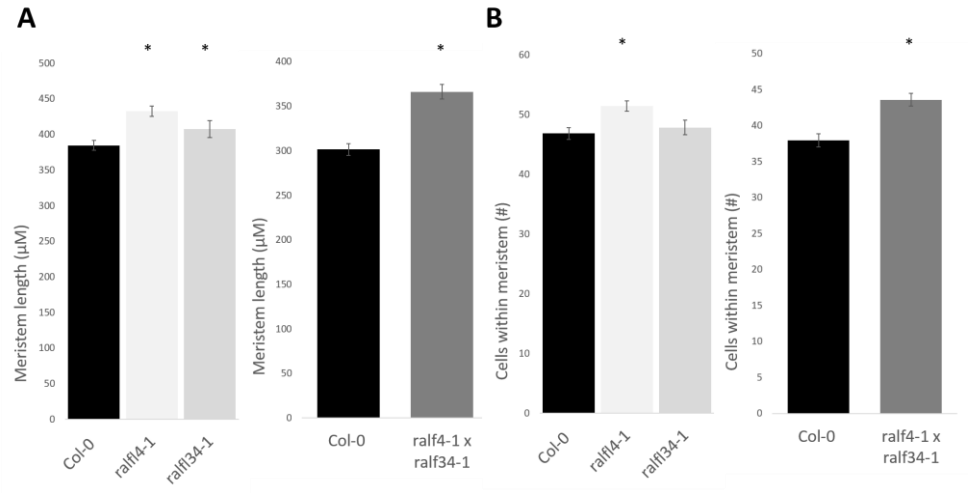


Figure 5. (A) Average primary root meristem length (µM) of 6 days post germination seedlings in wild type (Col-0 (n=46), (n=45)), and RALF T-DNA insertion mutants (*ralf4-1*, P= 0.0007 (n=36); *ralf34-1, P*= 0.0452 (n=44); and *ralf4-1* x *ralf34*-1 F3 homozygous cross, P = 2.10E-08 (n=45)); (B) Average number of cells within the root apical meristem of 6 days post germination seedlings in wild type (Col-0 (n=46), (n=45)), and RALF T-DNA insertion mutants (*ralf4-1*, P= 0.0040 (n=36); *ralf34-1*, P= 0.4986 (n=44); and *ralf4-1* x *ralf34*-1, P = 1.66E-05 (n=45)). The length of the meristem is defined here as the length from the quiescent centre cells in the root tip to the last cortical cell <30 µM; Graphs show average of 3 biological replicates ± standard error, asterisks indicate statistical significance (P < 0.05).

identifying a divergence in function between these peptides. This phenotype is still present in the *ralf4-1 ralf34-1* double mutant, with the number of cells within the meristem being higher than wild type, and interestingly, is enhanced further (15% over wild type) than the single *ralf4-1* mutant alone (**Figure 5B, 6**). This suggests there may be a small amount of functional redundancy within this processes between these two closely related genes.

To lastly correlate the RALF peptides to cell elongation through cell image analysis, we observed the lengths of mature cortical cells (**Figure 7A, B**). These data showed that *ralf4* and *ralf34* increased mature cortical cell length by 9% and 11% respectively. This phenotype was increased further in the *ralf4 ralf34* mutant background, increasing mature cortical cell length 20% over wild type (**Figure 7B**). These data correlate with the primary root length data (**Figure 2B**), suggesting cell expansion is likely the cause for the primary root growth phenotype, and furthermore, RALF4 and RALF34 natively act to restrict cell length, potentially in concert with each other in similar signalling cascades. As RALF4 and RALF34 are the most closely related homologues, this is not unusual for some functional redundancy to remain between these peptides, as also observed within the CLE family of peptides (Czyzewicz et al., 2015).

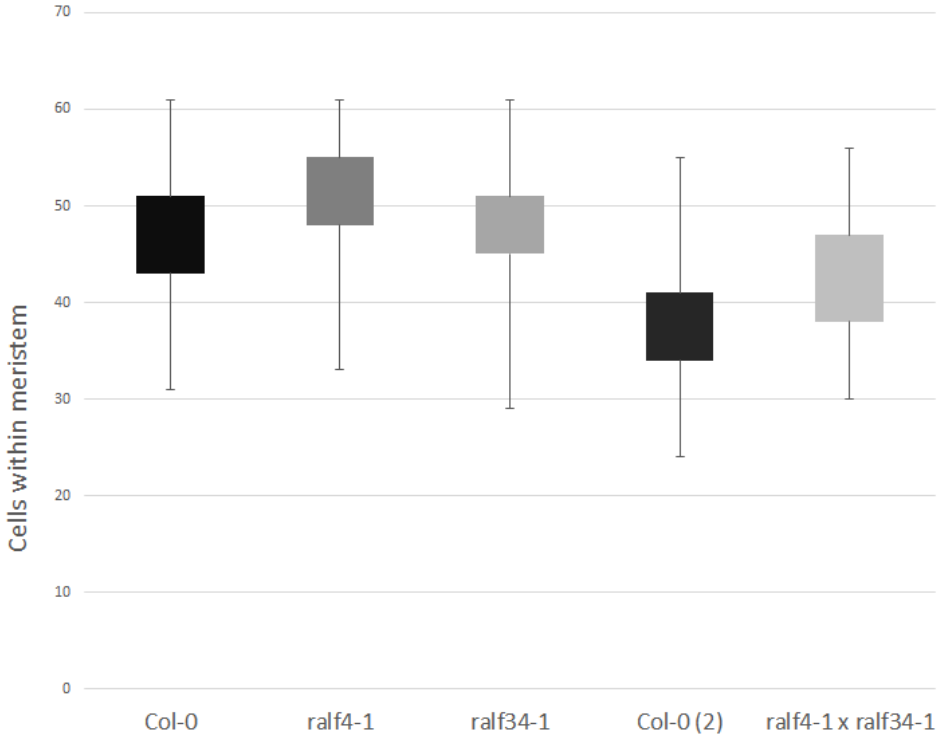


Figure 6. Cortical cell measurements revealing the average number of cells within the root apical meristem of 6 days post germination seedlings in wild type: Col-0 (n=46); *ralf4-1* (n=36); *ralf34-1* (n=44); Col-0(2) (n=45); and *ralf4-1 ralf34*-1 (n=45). The length of the meristem is defined here as the length from the quiescent centre cells in the root tip to the last cortical cell ≤ 30 μM; Graphs show average of 3 biological replicates.

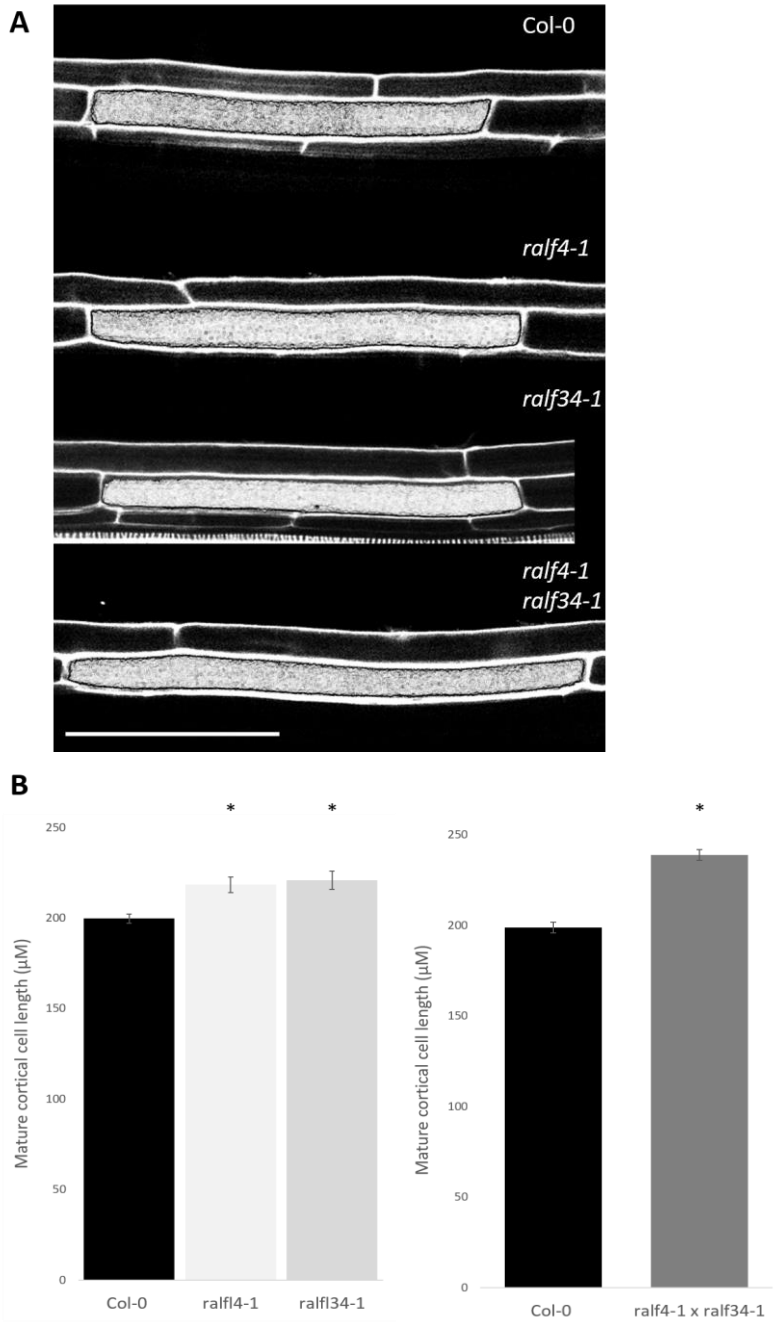


Figure 7. (A) Representative images of cortical cells stained with propidium iodide, from top to bottom – wild type (Col-0), *ralf4-1*, *ralf34-1*, and *ralf4-1 ralf34-1*, cortical cells have been enhanced for representative purposes, white bar = 100 µm; (B) Average mature cortical cell size of 6 days post germination seedlings in wild type (Col-0 (n=46), (n=45)), and RALF T-DNA insertion mutants (*ralf4-1*, P= 0.0016 (n=36); *ralf34-1*, P= 5.03E-05 (n=44); and *ralf4-1* x *ralf34*-1, P = 4.22E-15 (n=45)); Graphs show average of 3 biological replicates ± standard error, asterisks indicate statistical significance ($P < 0.05$).

Down regulation of* RALF34 *and* RALF4 *affects auxin responsiveness

A reduction in primary root elongation and an increase in lateral root density are well established effects of high quantities of exogenously applied auxin (NAA) in *Arabidopsis* roots (Davies, 1995). To establish the responsiveness of RALF4 and RALF34 to auxin, we exogenously applied the synthetic phytohormone NAA to the transgenic T-DNA insertion mutants and observed their sensitivity (**Figure 8-9**).

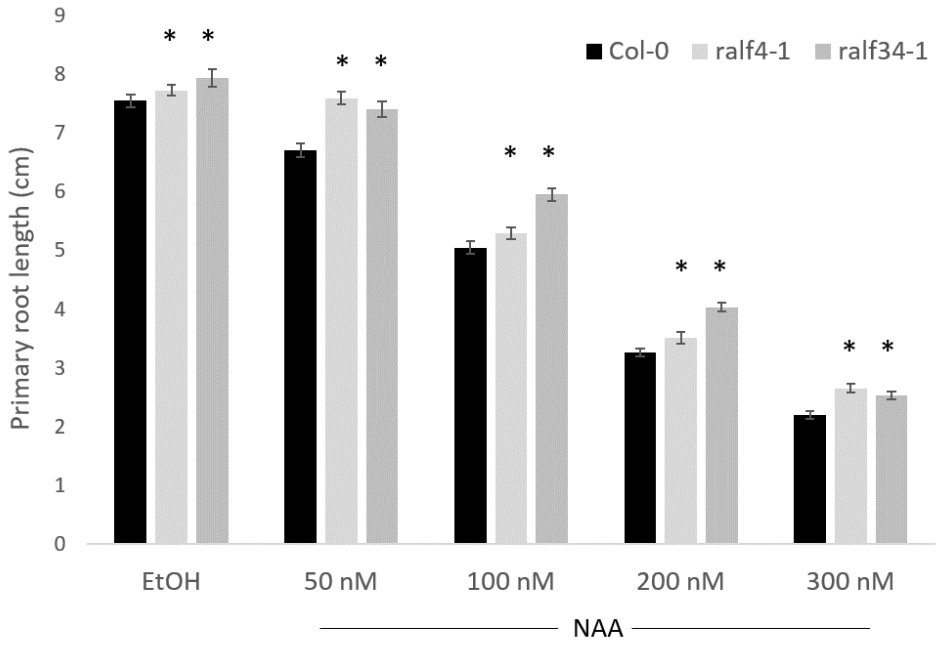


Figure 8. Primary root lengths (cm) of seedlings 10 days post germination in absence (EtOH) or presence of various quantities of the plant hormone auxin (NAA) in wild type (Col-0, n= 63, 59, 59, 59, 41), and RALF T-DNA insertion mutants (*ralf4-1*: EtOH – P= 0.0367 (n= 48); 50 nM – P= 0.0003 (n= 40); 100 nm – P= 6.06E-08 (n= 46); 200 nm – P= 2.37E-11 (n= 49); 300 nM – P= 0.0003 (n= 26); and *ralf34-1*: EtOH – P= 0.0477 (n= 48); 50 nM – P= 3.20E-07 (n= 56); 100 nm – P= 0.0270 (n= 41); 200 nm – P= 0.0361 (n= 43); 300 nM – P= 2.39E-05 (n= 24)); Graphs show average of 3 biological replicates ± standard error, asterisks indicate statistical significance ($P < 0.05$).

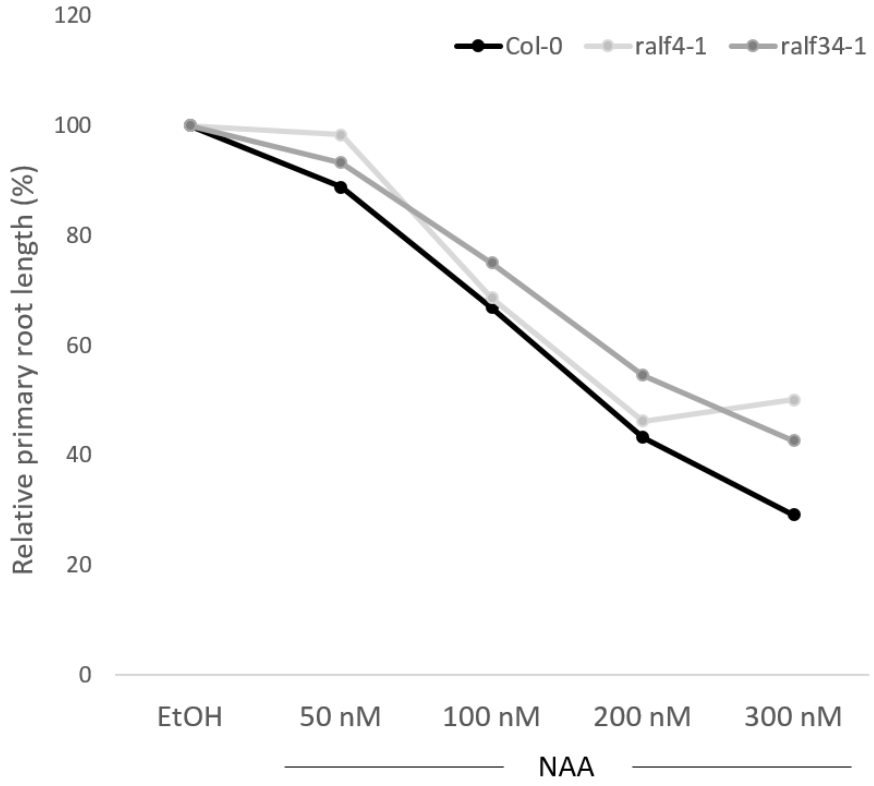


Figure 9. Relative primary root length changes after auxin treatments shown as percentages (%) when compared to original ethanol control, of 10 day post germination wild type (Col-0) and RALF T-DNA insertion mutants (*ralf4-1* and *ralf34-1*) seedlings, average of two biological replicates.

Primary root lengths of 10 day post germination *ralf4-1* and *ralf34*-1 seedlings were longer than wild type at all NAA concentrations compared to wild type control at the same dosages (**Figure 8**). Furthermore, the *ralf* mutants showed a slight insensitivity to increasing levels of exogenously applied auxin, resisting auxins root arrest properties (**Figure 9**). This insensitivity suggests there is no auxin response or a weak secondary response. As hormone crosstalk is prevalent throughout plant development, equally within cell elongation, future investigations should observe the role phytohormones such as the previously

mentioned brassinosteroids, or the plant hormone cytokinin, which regulates the differentiation of cells by antagonising auxin, on these RALF peptides (Dello Ioio et al., 2008; Vert et al., 2008).

CONCLUSION

Hormones have canonically taken centre stage in the control of primary root growth. Among them, cytokinin, auxin, and brassinosteroids are three key players regulating the division and elongation patterns controlling the switch from meristematic region, to elongation zone (Grieneisen et al., 2007; Hacham et al., 2011; Moubayidin et al., 2010). Our work clearly demonstrates that RALF4 and RALF34 act to restrict primary root growth, likely separate from an auxin mediated signalling pathway. Here we provide evidence that *RALF34* is a root meristem, and epidermal layer, highly expressed gene, that can induce a primary root phenotype, which can be amplified by concurrently removing the closely related homologue *RALF4*. Furthermore, *ralf4-1* and *ralf34-1* showed little insensitivity to auxin treatment at multiple concentrations, suggesting a separate role from auxin signalling.

While these RALFs were not specifically associated in affecting the transition zone in these data, there are still potential implications. Previous data (Murphy, 2015; unpublished) has shown *ralf4-1* to upregulate *SHORT HYPOCOTYL2* (*SHY2*), a negative regulator of auxin signalling (Tian et al., 2002). Auxin normally mediates the degradation of the SHY2 protein, however, if the *ralf* mutants are less sensitive to auxin, perhaps there is additional SHY2 mediated

PIN-FORMED (*PIN*) gene regulation, altering auxin responses in the root tip (Blilou et al., 2005). Thus, further investigations should observe the effects of cytokinin, a known auxin agonist, on the *ralf* mutants to observe for changes in root architecture and sensitivity.

MATERIALS AND METHODS

Growth of *Arabidopsis thaliana* seedlings: Seeds were surface sterilised (70% ethanol – 2 minutes, 10% bleach - 15 minutes, $_dH_2O$ – 5 washes) then stratified at 4°C for 2 days. Seeds were then plated on ½ Murashige and Skoog (MS) agar plates (2.154gL^{-1} MS, 0.1gL^{-1} myo-inositol, 0.5gL^{-1} MES, 10gL^{-1} bacteriological agar, pH 5.7 with 1 M KOH) and germinated vertically under constant white light at 21°C.

Phenotyping of T-DNA insertion lines

The following T-DNA insertion mutant lines were used in our investigations: *ralf4-1*/*at1g28270/SALK_016969* and *ralf34-1/at5g67070/SALK_004441*. Primary root phenotyping was photographed and root lengths were measured from the bottom of the hypocotyl to the root tip, using ImageJ software (http://imagej.nih.gov/ij/).

Root and cell length measurements: Primary root measurements were carried out on seedlings grown to 7 days post germination. Primary root length was measured [using ImageJ software (http://imagej.nih.gov/ij/)] from the base of the hypocotyl to the bottom of the root tip. Cortical cell measurements were

carried out on seedlings grown to 6 days post germination. Seedlings were transferred to propidium iodide stain (Sigma) [10 µg/ml] for 2 minutes then imaged on a Nikon confocal laser scanning microscope with a 514 HeNe laser at 40x magnification. Cortical cells were measured by hand using ImageJ. Cortical cells were measured starting from the quiescent centre, moving through the maturing cells, until the root hair initiation zone (the last cortical cell measured under the first visibly forming trichoblast). The length of the meristem is defined here as the length from the quiescent centre cells in the root tip to the first cortical cell ≤ 30 µM; this is roughly equal to the lengths of the root cap. Final cortical cell lengths were measured approximately 2 cm down from the hypocotyl. Measurements presented are means of three biological replicates.

Quantitative RT-PCR: Total RNA was extracted from lines of interest using an RNeasy kit (Qiagen). Using Superscript II (Invitrogen), cDNA was generated from total RNA (200 ng) via oligo(dT) primers as per instructions. Quantitative PCR reactions were performed in 4 technical repeats, using the SYBR Green QPCR Master Mix (Quanta Biosciences), in our modified reaction protocol: Master mix - 7 µL/reaction (forward primer [100 µM] – 0.1 µL/reaction, reverse primer [100 µM] – 0.1 µL/reaction, SYBR(2X) – 6 µL/reaction, $_{d}H_2O$ – 0.8 µL/reaction) and template cDNA [diluted 1/100 $_{d}H_2O$] – 5 µL/reaction. Gene specific quantification was performed with intron spanning primer pairs (where possible) designed using Primer3 software (http://bioinfo.ut.ee/primer3/). Expression was normalised to *EEF1α4* (At5g60390). Primers used: qRALF34_Fp (AGTTACGGAGGAAGACGACG), qRALF34_Rp (CGGGACCTCTAGCTCTGAAG), qRALF4_Fp (CATTGGCTACGATGCTCTCA), qRALF4_Rp

(CATCCACGCCTGTAAGGATT), qEEF1α4_Fp (ctggaggttttgaggctggtat), and qEEF1α4_Rp (ccaagggtgaaagcaagaaga). All qRT-PCR experiments were performed in three biological replicates (unless otherwise specified) and the data presented represent means ± standard deviation.

Auxin dose response quantification: *Arabidopsis* (ecotype Columbia) seeds were surface sterilised, stratified at 4°C for 2 days, then plated on ½ MS media supplemented with 0 (ethanol), 50, 100, 200, or 300 nM 1-Napthaleneacetic acid (NAA) (Sigma) and grown vertically under constant white light at 21°C. Primary root lengths and lateral root densities were quantified 10 days post germination. Measurements are presented as means of two biological replicates.

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Chapter 6 – Unravelling regulation of *RALF34* expression and the RALF34 signalling cascade

Evan MURPHY and Ive DE SMET

SUMMARY

RALF small signalling peptides have been shown to play a role in many processes including, but not limited to, both primary root elongation and lateral root development. Synthetic RALF peptide treatments induce cell length restriction, in leaf and root tissue, and this effect has been shown to be conserved across species (Tomato, Poplar, and *Arabidopsis*). However, the up- and downstream mechanisms involved in these developmental pathways are largely unknown.

In this chapter we largely utilised *in silico* data mining to identify potential up- and downstream proteins involved in *RALF* mediated signal transduction. T-DNA inserts in highlighted putative interacting proteins were phenotyped, and initial observations suggest some of these candidates may be significantly involved in cell elongation. Furthermore, observations on the levels of transcription of 28 cell wall remodelling enzymes in the *ralf34* mutant background gave insights into potential regulatory mechanisms through which *RALF34* mediates downstream lateral root development. However, as cell wall remodelling is a process involved during many developmental paradigms, future efforts will be focused on concentrating specifically within lateral root development.

INTRODUCTION

Secretory peptides are discrete mechanisms through which plants regulate cell to cell communication, sometimes having a systemic effect (hence *peptide hormone* is occasionally used) (i.e. SYSTEMIN, PROPEP1) and sometimes having a localised controlled effect (i.e. CLE40) (Marmiroli and Maestri, 2014). Peptide-ligands bind to a specific, or a set of specific, protein receptor partner(s) in order to proliferate downstream signalling, known as signal transduction. In *Arabidopsis* these are generally receptor-like kinases, a diverse group of proteins generally consisting of an extracellular receptor domain, a transmembrane domain, a juxtamembrane domain, and an intracellular cytoplasmic kinase domain. In *Arabidopsis* there have been over 600 receptor-like kinase (RLK) family members identified, with 235 leucine-rich repeat receptor-like kinases found therein (LRR RLKs) (Shiu and Bleecker, 2001). In plants, receptor-like kinases are predominantly of the serine/threonine variety, with tyrosine kinases (such as BRASSINOSTEROID INSENSITIVE1 (BRI1) being a minority (See Chapter 9 for more on phosphorylation) (Matsubayashi et al., 2006; Oh et al., 2009; Shiu and Bleecker, 2001). Receptor-like kinases have been implicated in many physiological and developmental processes within a plant, such as: hormone perception (such as the aforementioned BRI1, and BRI1-ASSOCIATED RECEPTOR KINASE (BAK1)) (Clouse et al., 1996; Li et al., 2002), defence response (FLAGELLIN SENSING2 (FLS2), SUPRESSOR OF BIR1-1 (SOBIR1)) (Gomez-Gomez and Boller, 2000; Liebrand et al., 2013), abiotic stresses such as drought and nutrient availability (ABA- AND OSMOTIC STRESS-INDUCIBLE RECEPOR-LIKE CYTOSOLIC KINASE1 (ARCK1)) (Tanaka et al., 2012), cell wall

development (CATHARANTHUS ROSEUS RLK1-like (CrRLK1Ls)) (Boisson-Dernier et al., 2011; Cheung and Wu, 2011), shoot apical meristem development and maintenance (through CLAVATA1 (CLV1), and co-receptor proteins CLAVATA2 (CLV2) and CORYNE (CRN)) (Clark et al., 1997; Jeong et al., 1999; Muller et al., 2008), root apical meristem development and maintenance (through ARABIDOPSIS CRINKLY4 (ACR4) and the receptor-like protein CLV2 (CRN)) (Meng and Feldman, 2010; Stahl et al., 2013), and lateral root initiation (through ACR4) (De Smet et al., 2008) (see Chapter 2 for further details on peptides and receptor-like kinases in development).

Recently, the receptor for the RAPID ALKALINIZATION FACTOR1 (RALF1) peptide was identified as one of the CrRLK1-like RLKs, FERONIA (FER) (Haruta et al., 2014). Through the application of synthetic RALF1 peptide upon *Arabidopsis* seedlings, it was shown, utilising mass spectrometry, that 5 phosphopeptides were highly differentially regulated – the receptor-like kinase FER, ARABIDOPSIS PLASMA MEMBRANE H^+-ATPASE ISOFORM2 (AHA2), CALCIUM DEPENDENT PROTEIN KINASE9 (CPK9), PENETRATION3 (PEN3)/ARABIDOPSIS THALIANA ATP-BINDING CASSETTE G36 (ATABCG36), and a novel FER-related RLK ERULUS (ERU) (Haruta et al., 2014). Interestingly, only ERU was decreased upon RALF1 application. To identify if either of the two identified receptor-like kinases were potentially the RALF1 receptor, T-DNA mutants for FER and ERU were screened in presence and absence of RALF1 peptide. It was shown through growth assays and cytoplasmic calcium reporters (aequorin), that the *fer4* knockout was insensitive to RALF1 application while *eru* showed no changes

in sensitivity (nor did 17 other RLKs). This suggested FER was a receptor for RALF1; however, to further illustrate the connection, RALF1 was shown to directly bind to FER through co-immunoprecipitation. Furthermore, the phospho-status of the previously identified AHA2 was unchanged post RALF1 application in the *fer4* knockout mutant. The proton pump has previously been shown to be phosphorylated specifically at Ser^{899} after induction with bacterial flagellin, flg22, causing apoplastic alkalinisation, which upon RALF1 treatment AHA2 was also shown to be phosphorylated at this residue (Gomez-Gomez et al., 1999; Nuhse et al., 2007). However, AHA2 has been shown to be phosphorylated on at least 7 residues, suggesting this protein participates in many different signalling cascades (Haruta et al., 2015; Stecker et al., 2014). However, the model stands that upon RALF1 binding, FER causes direct phosphorylation of AHA2, which downregulates the AHA2 activity, increasing the apoplastic pH, creating an optimal environment for downstream cell wall remodelling enzymes to restructure the growing root (Haruta et al., 2014) (See Chapter 3 – 'Understanding the RALF family – a tale of many species' for a schematic).

Originally, FER was characterised through analysis of the *fer* knockdown and found it was essential for fertilisation and normal pollen tube elongation (Escobar-Restrepo et al., 2007). Later, FER was linked to two other closely related receptor-like kinases, HERCULES (HERK1) and THESEUS1 (THE1), also belonging within the 17 members of the CrRLK1-like family (Guo et al., 2009). Phenotypic analysis of *herk1 the1* double mutants show dwarf phenotypes which are enhanced in the *bri1* background, connecting these RLKs to brassinosteroid signalling. Application of brassinolide (the most biologically

active brassinosteroid) to *Arabidopsis* seedlings upregulated the expression of *HERK1*, *THE1*, and *FER* significantly. Interestingly, like FER, the RALF1 peptide was also linked to brassinosteroids, as the irAtRALF1 knockdown line was shown to be less sensitive to exogenously applied brassinolide than wild type (Bergonci et al., 2014). Oppositely, the closely related RALF23 peptide was less sensitive to exogenously applied brassinolide only when overexpressed (Srivastava et al., 2009). The peptide was confirmed to be cleaved by the Golgi localised plant subtilisin-like serine protease (subtilase) SITE-1 PROTEASE (AtS1P) *in vivo*, by crossing the *s1p-3* knockout mutant with the *AtRALF23*-myc overexpressing line, recovering the bushy overexpression phenotype. These are the first examples of functional proteins being identified in the RALF mediated cell elongation developmental pathway.

In this chapter, we probe several protein pathways, up and downstream, through which RALF peptides may participate. Firstly, to understand the potential upstream mechanisms through which our peptides of interest, RALF4 and RALF34, may be regulated, we began an *in silico* investigation into transcriptional regulatory networks. This highlighted a novel, light-regulated pathway through which RALF peptides might be regulated. We then probed protein-protein interaction networks and cross referenced that to co-expression networks of closely expressed genes to observe for genes that encode proteins involved in cellular processes such as signal transduction. The network results gave insight into various proteins that may be involved in RALF-mediated signalling, including: a leucine rich repeat (LRR) protein, LRR receptor-like kinases, and a protein kinase. Furthermore, we continued the investigation in the

role AtS1P plays in RALF related development. Lastly, to explore downstream effects of RALF signalling, we generated and cross referenced a cell wall remodelling gene database to a lateral root specific gene database (through which *RALF34* was identified), which highlighted numerous cell wall remodelling enzymes of interest. We explored T-DNA mutants in several of these genes to observe lateral and primary root phenotypes.

RESULTS AND DISCUSSION

Upstream and downstream of RALF34: an in silico approach

Utilising CORNET 2.0 for *Arabidopsis*, an *in silico* tool for generating interaction networks between query genes and precompiled expression datasets, we input the gene codes for *RALF4* (*At1G28270*) and *RALF34* (*At5G67070*) to identify potentially interacting proteins with similar developmental functions as the RALFs, and also looked for cellular processes such as signal transduction, to identify potential receptor proteins; furthermore we observed for downstream candidates such as expansins and cell wall remodelling enzymes (De Bodt et al., 2010; De Bodt et al., 2012). We then used *Arabidopsis* eFP browser to correlate our *in silico* candidates, with data mined expression profiles, to see if any of our putative receptor candidates overlap expression with *RALF34*. Lastly, we also began investigating the previously mentioned SITE1-protease for a role in RALF mediated signalling, as peptide processing is very important for ligand-receptor binding (Srivastava et al., 2009).

Regulatory transcription factors

We initially used CORNETs 'TF tool' – which analyses transcription factors and DNA binding proteins – to identify prospective upstream regulatory proteins. Inputting the two *RALF* genes separately generated distinct transcription factor networks with RALF34 showing 19 putative interactions (**Figure 1**) and RALF4 showing 13 (**Figure 2**), and 1 protein interacting with both (*COV1*, *At2G20120*).

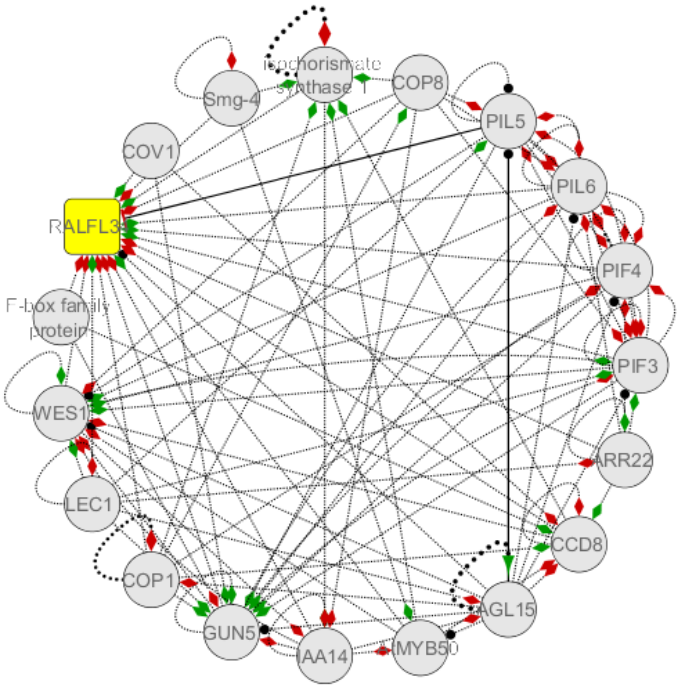


Figure 1. Transcription factor network consisting of 19 proteins, all of which interact with *RALF34* (in yellow); *PHYTOCHROME INTERACTING FACTOR 3-LIKE 5* (*PIL5*) shows a direct (solid black line) repression of *RALF34*, while the others show putative unknown interactions; solid black line – experimentally confirmed, dotted line – experimentally unconfirmed, arrow head – direct activation, T head – direct repression, circle head – direct unknown, green diamond – indirect activation, red diamond – indirect repression.

The *RALF34* network was compromised of numerous light-regulated genes, and transcription factors of various functions outside of root specific processes (GO biological processes) except for, interestingly, SOLITARY ROOT (SLR)/IAA14 (**Figure 1**), which is a gene shown to be inherently important to lateral root development (Fukaki et al., 2002), and we previously showed *RALF34* to be upregulated in the *slr-1* background (see Chapter 4 – RALFs in lateral root development).

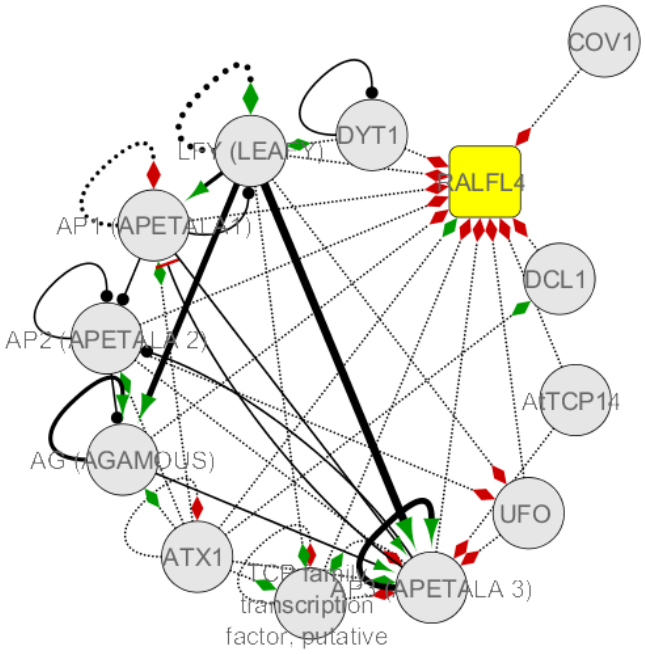


Figure 2. Transcription factor network consisting of 13 proteins, all of which interact with *RALF4* (in yellow), however, no direct interactions indicated; solid black line – experimentally confirmed, dotted line – experimentally unconfirmed, arrow head – direct activation, T head – direct repression, circle head – direct unknown, green diamond – indirect activation, red diamond – indirect repression.

The *RALF4* transcription factor network was less informative for root research however, identifying mostly shoot derived transcription factors; though, the appearance of at least one overlapping protein (COV1) is a positive sign (**Figures 1-2**). Furthermore, in a brief observation to correlate the *in silico* identified light regulated transcription factors (**Figure 1**), such as PIL5, to a light mediated process, we observed that expression of p*RALF34*::n3xRFP was

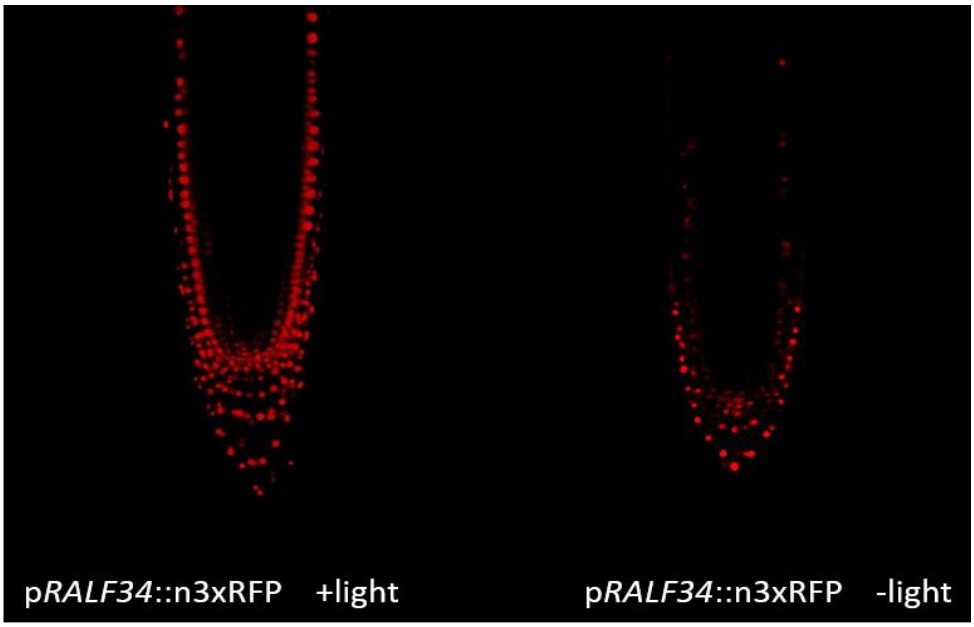


Figure 3. Expression of *pRALF34::n3xRFP* in (left) wild type grown under normal conditions for 4 days; (right) wild type grown in the dark for 4 days and exposed to light for 1 hour (40x magnification).

reduced in dark grown seedlings, potentially linking *RALF34* to some of the *in silico* identified light-regulated genes (**Figure 3**). This suggests these *in silico* networks have the potential to correctly identify interacting signalling partners. An ongoing yeast-1-hybrid screen on the *RALF34* promoter is being explored for further potential upstream transcriptional regulations (which already showed overlap with one of the *in silico* identified genes, *AGL15*).

Protein-protein interactions

To investigate into potential protein-protein interactions, we utilised CORNETs 'PPI tool' – which analyses protein-protein interactions (both experimental and predicted) (**Figure 4**). Interestingly, inputting *RALF4* and *RALF34* separately, *RALF4* generates a protein interaction network with 15 other RALF peptides and no other connections (**Figure 4A**), whereas, *RALF34* generates a protein interaction network of two parts, one half consisting of 14 RALF peptides (which did not include RALF4), the other consisting of 38 proteins of various developmental functions. From this, we selected *At4G02630*, a protein kinase family protein, and *At3G08680*, leucine-rich repeat transmembrane protein kinase as potential candidates for RALF34 mediated signal transduction, as these proteins are putatively involved in receptor protein tyrosine kinase function, and serine/threonine kinase activity according to their GO biological process.

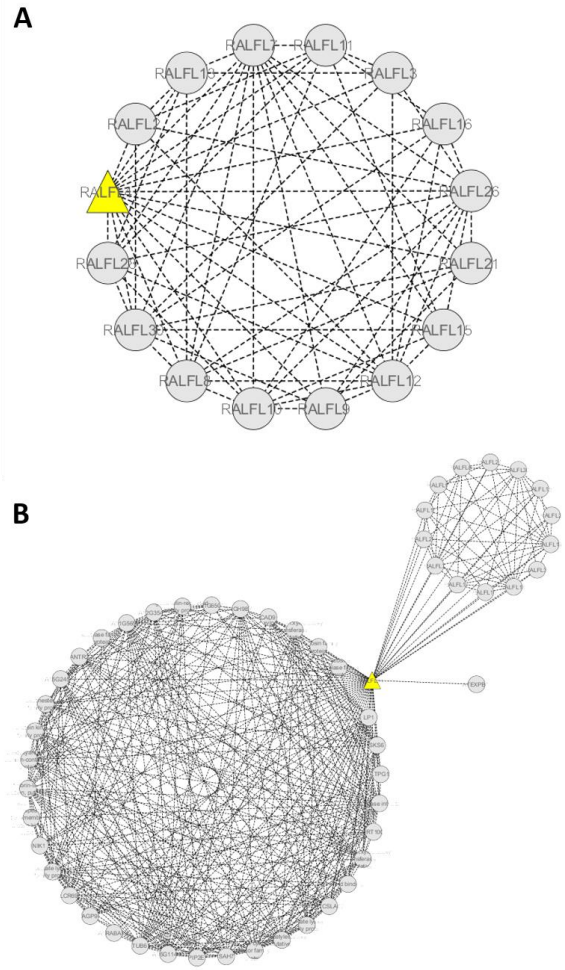


Figure 4. (A) Protein-protein interaction network generated around *RALF4* (yellow) which highlighted 15 interacting partners, all other members of the RALF peptide family; (B) Protein-protein interaction network generated around *RALF34* (yellow) which separated into 2 lobes: right- 14 RALF family peptides, central – *RALF34* connected to an expansin and, left - 38 various proteins, including 2 kinase proteins.

Co-expression analysis

We then utilised CORNETs co-expression tool, with an aim to overlap genes of interest that co-express with *RALF4* and *RALF34* with the protein-protein interaction hits generated previously (**Figure 4**). The *RALF4* co-expression network generated a list of 50 interactions and highlighted some interesting developmental functions such as regulation of cell differentiation, maintenance of organ identity, and pollen tube growth, however, most of these genes related to aerial tissues and thus, most were not relevant to the aim of this study (**Figure 5A**); it also identified an LRR family protein, *At5G25550*, however, a T-DNA insertion line was not available. The *RALF34* input generated a network of 50 genes that was much less specific in the biological processes identified, with the majority being involved in gene expression, metabolic processes, and cellular processes (**Figure 5B**); though, two further LRR proteins were identified, *At3G20820* and *At3G56370*, suggesting again, there may be a signal transduction element to these networks. However, only 3 genes overlapped between the *RALF34* coexpression and protein-protein interaction networks, and 0 genes overlapped between the *RALF4* networks, suggesting we should be cautious when interpreting these data. Applying more stringent filters to the *in silico* databases may help narrow the search field in future research.

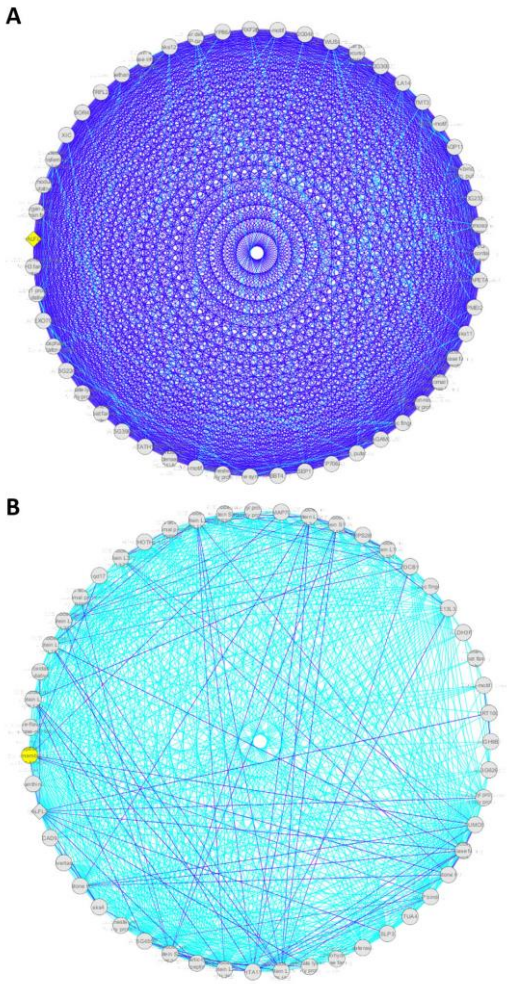


Figure 5. (A) Coexpression network generated from *RALF4* showing top 50 interacting gene; primarily dark blue lines indicate strong correlation; (B) Coexpression network generated from *RALF34* showing top 50 interacting genes, primarily light blue lines indicate weaker coexpression confidence than the *RALF4* network; line colour indicates coexpression correlation coefficient, dark is stronger.

To correlate *At4G02630*, *At3G08680*, *At5G25550*, *At3G20820*, and *At3G56370* with our RALF peptides further, we used *Arabidopsis* eFP browser to highlight potential overlapping expression patterns with our genes of interest (Brady et al., 2007; Winter et al., 2007) (**Figure 6**).

We used *RALF1* as a control for low root expression, *RALF4* showed slightly higher expression in the root tip, and *RALF34* showed significantly higher expression throughout the meristematic region, as well as the pericycle cell layer, which was shown experimentally (see Chapter 4 – RALFs in lateral root development). All of our candidate genes showed increased levels of

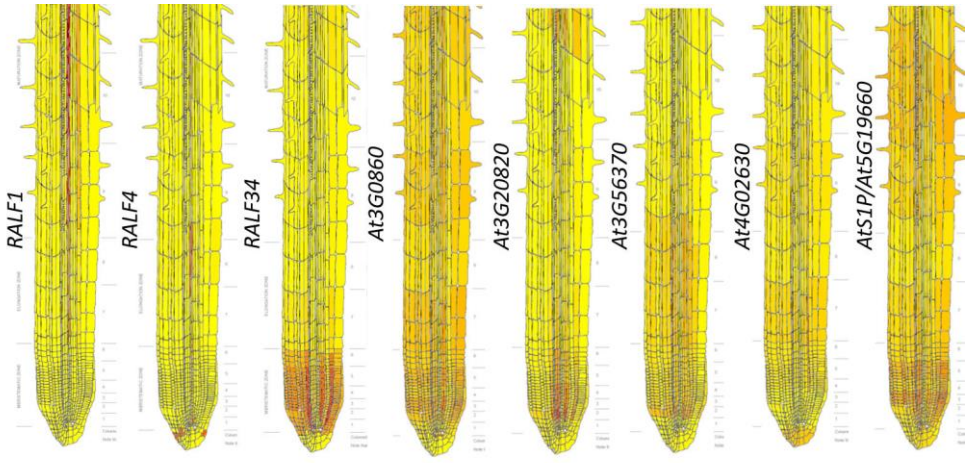


Figure 6. Schematic heat map representation of expression profiles of *RALF1* (far left), *RALF4* (second), and *RALF34* (third), and our novel proteins of interest, *At3G0860*, *At3G20820*, *At3G56370*, *At4G02630*, and *AtS1P/At5G19660*; deeper colour indicates higher potential expression.

expressed in the root tip compared to *RALF1* (highlighting a potential correlation to *RALF34*), furthermore, *At3G56370* has significantly increased expression in young xylem pole pericycle tissue, and *At3G20820* has increased expression in the pericycle and very high expression in the lateral root cap, also overlapping significantly with *RALF34*. Lastly, *AtS1P* has high expression throughout the root, with concentration levels in the meristematic zone, xylem, and xylem pole pericycle tissues. With these expression profile in mind, we obtained T-DNA insertion mutants and began an overall investigation into the role these putative signal transduction proteins play in primary root development.

Loss-of-function mutants for selected candidates increases primary root length and lateral root density

To explore the role of *RALF* peptide-linked signal transduction candidates in primary root growth and lateral root development, we utilised T-DNA insertion lines *at3g20820-1*, *at3g56370-1*, *at3g56370-2*, *at4g02630-1*, *at3g08680-1*, and *ats1p-3* to investigate the effects of downregulating the levels of these genes in *Arabidopsis* plants (**Figure 7-8**).

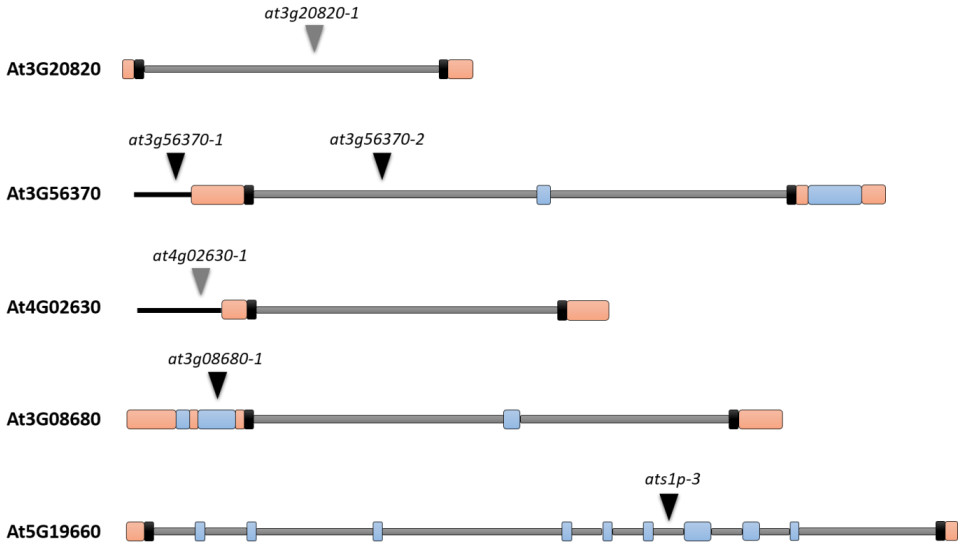


Figure 7. Map of the 5 identified *leucine-rich repeat kinase*, *protein kinase*, and *SITE-1 PROTEASE* (*ATS1P*) genes of interest: At3G20820 (*at3g20820-1*, T-DNA insert in the EXON), At3G56370 (*at3g56370-1*, T-DNA insert in the PROMOTER, *at3g56370-2*, T-DNA insert in the EXON), At4G02630 (*at4g02630-1*, T-DNA insert in the PROMOTER), At3G08680 (*at3g08680-1*, T-DNA insert in the 2nd INTRON), At5G19660 (*ats1p-3*, T-DNA insert in the 7th EXON) ; red box – UTR, black box – start/stop codon, black bar – PROMOTER, gray bar – EXON, blue box – INTRON, gray triangle – unconfirmed insert, black triangle – confirmed insert.

A plant line with a single T-DNA insertion in any one of the receptor/kinase candidates (blue bars) shows a significant increase in root length over wild type of at least 10%, with the LRR protein kinase (*at3g08680-1)* mutant producing a substantial increase of 29% over wild type (**Figure 8A-B**). Oppositely, the *ats1p-3* mutant (yellow bars) caused a significant reduction in primary root length, of around 20% (**Figure 8A-B**). Interestingly, this phenotype is opposite to that previously reported for *s1p-3*, as they observed a small increase in the primary root length, under different growth conditions (Srivastava et al., 2009, supplemental). Our data presents an interesting problem however; if you remove the processing enzyme, the peptide remains in its prepropeptide, inactive form, and thus, the *ats1p-3* would phenocopy the *ralf34* mutant and produce longer roots; however, since we observed significantly shorter roots, it is likely AtS1P is not the processing enzyme of RALF34, or of any of the RALF family members involved in negative regulation of cell elongation. However, since all four of the LRR and kinase proteins produced convincing primary root phenotypes, ranging from 10% to 29% enhanced lengths over wild type, we investigated their lateral root phenotypes (**Figure 8C**).

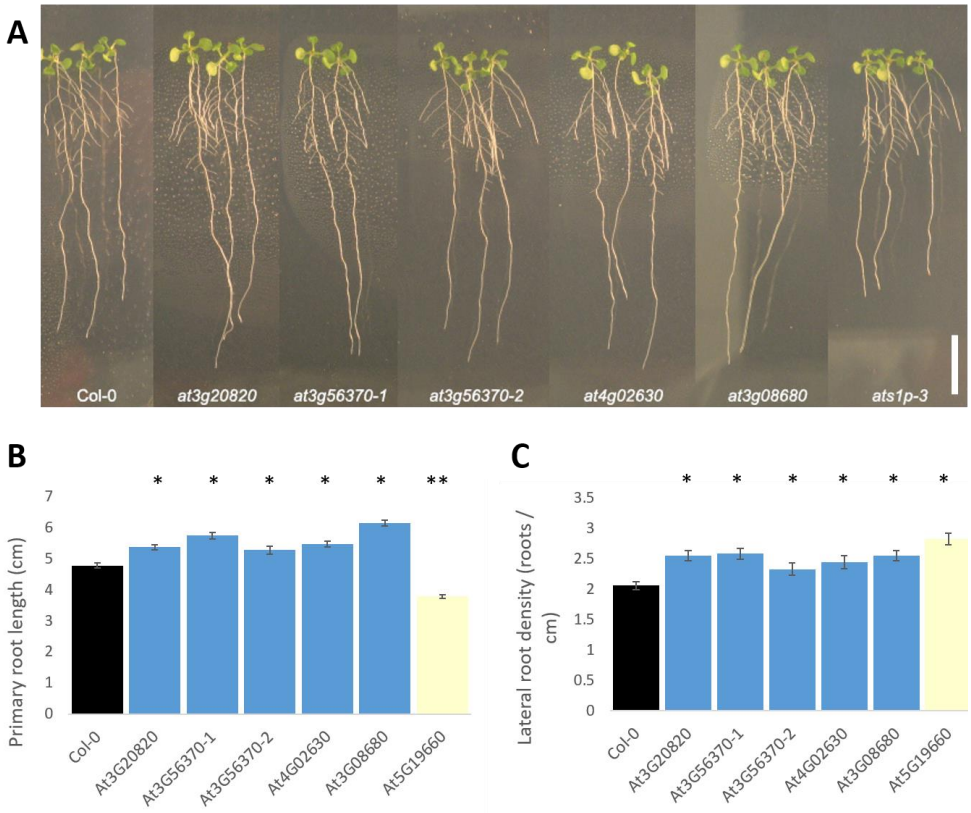


Figure 8. (A) Plate phenotypes of 7 day post germination (dpg) ½ MS grown seedlings of Col-0, *at3g20820*, *at3g56370-1*, *at3g56730-2*, *at4g02630*, *at3g0860*, and *ats1p-3/At5G19660*; (B) Primary root lengths (cm) of 7 dpg seedlings in wild type (Col-0, n= 119), and LRR and protein kinase T-DNA mutants (*At3G20820*, P= 4.59E-07 (n= 79); *At3G56370-1*, P= 1.99E-11 (n= 66); *At3G56370-2*, P= 0.0008 (n= 55); *At4G02630*, P= 1.46E-07 (n= 65); *At3G08680*, P= 9.40E-23 (n= 91); *At5G19660/ats1p-3*, P= 1.13E-20 (n= 95)); (C) Lateral root densities (lateral roots / cm) of 7 dpg seedlings in wild type (Col-0, n= 119), and LRR and protein kinase T-DNA mutants (*At3G20820*, P= 4.84E-06 (n= 79); *At3G56370-1*, P= 8.54E-06 (n= 66); *At3G56370-2*, P= 0.0269 (n= 55); *At4G02630*, P= 0.0020 (n= 65); *At3G08680*, P= 6.53E-06 (n= 91); *At5G19660/ats1p-3*, P= 1.37E-09 (n= 95)); Graphs show average of 3 biological replicates ± standard error, asterisks indicate statistical significance ($P < 0.05$) (*higher, **lower than control).

Lateral root densities were all increased significantly in the *lrr* and *kinase* mutants, and interestingly, also in *ats1p-3* (**Figure 8C**), which is a process

RALF34 has been instigated in playing a role during the early formative stages of development (see Chapter 4 – RALFs in lateral root development). Interestingly, some of the phenotypes of these *lrr* and *kinase* mutants are seemingly stronger than the *ralf34-1* or *ralf4-1* phenotype alone, specifically *at3g08680-1* appears to be a strong mutant producing both a longer primary root and more emerged lateral roots (**Figure 8B-C**). These data would suggest that if this protein were to act in a RALF mediated pathway, it might act as a protein regulated by more than one RALF peptide, as the phenotype is very similar to the enhanced phenotype of the *ralf4-1 ralf34-1* double mutant (see Chapter 5 – RALFs role in primary root growth). Protein receptors binding more than one ligand is not uncommon amongst small signalling peptides, as observed in the CLE family of peptides, where the high similarity within the family has shown numerous CLEs to bind to the BARELY ANY MERISTEM (BAM) receptors (Hord et al., 2006; Jun et al., 2010; Shinohara et al., 2012). To further characterise these LRRs and kinases with RALF mediated signalling, we will use 35S::*RALF4/34* overexpression (currently in development) to test for specific sensitivities in each mutant background.

In silico approach to identify potential downstream cell wall remodelling candidates involved in lateral root development

Recent observations suggest the receptor-like kinase FERONIA binds to the RALF1 peptide, phosphorylating the plasma membrane AHA2 protein reducing its activity, and increasing the apoplastic pH, generating an environment optimal for cell wall remodelling enzyme activity (Haruta et al.,

2014). However, the specific downstream enzymatic mechanisms through which the RALFs and FERONIA act are largely unknown. Since *ralf34* has been shown to cause proliferative cell divisions during lateral root development (see Chapter 3 – RALFs in lateral root development), we began an investigation into the downstream mechanisms with a lateral root specific focus.

We therefore collated a database of 770 cell wall remodelling enzymes, proteins, and affiliated genes from three large studies focusing on the cell wall, abscission, and lateral root emergence (Cai and Lashbrook, 2008; Kumpf et al., 2013; Swarup et al., 2008). This novel cell wall database was cross-referenced against a highly specific lateral root initiation gene database (1920 genes), which was generated through fluorescence activated cell sorting (FACS) on pericycle cells undergoing asymmetric cell division (De Smet et al., 2008). Utilising FACS, 16 genes were identified as 'highly lateral root initiation specific', and the receptor-like kinase ACR4, which was shown to play a vital role in promoting formative cell divisions during lateral root organogenesis, was identified (De Smet et al., 2008). Also among the 16 highly differentially regulated lateral root specific genes, was the small signalling peptide *RALF34*, which belongs to the RALF family of peptides shown to play roles in cell elongation (Murphy and De Smet, 2014; Pearce et al., 2001), and *EXPANSIN1* (*EXPA1*), a member of the large EXPANSIN family of cell wall modifying proteins (Shcherban et al., 1995; Wei et al., 2011). This illustrates the efficacy of the FACS technique in identifying genes involved in lateral root development (*ACR4*) and potentially in cell wall remodelling.

Through cross-referencing the 770 cell wall gene database against the 1920 gene FACS database, 28 novel genes (plus *EXPA1*) were identified as being

differentially regulated immediately before, and during the first round of asymmetric cell division of lateral root development, with 1 gene (At4G13210) identified as highly differentially regulated to a similar level as RALF34 and EXPA1 (**Figure 9A**). The novel genes were then correlated as either similar, anti, or divergent, to the expression pattern of the peptide RALF34, to further stratify potential roles of the relevant cell wall proteins (**Figure 9B**).

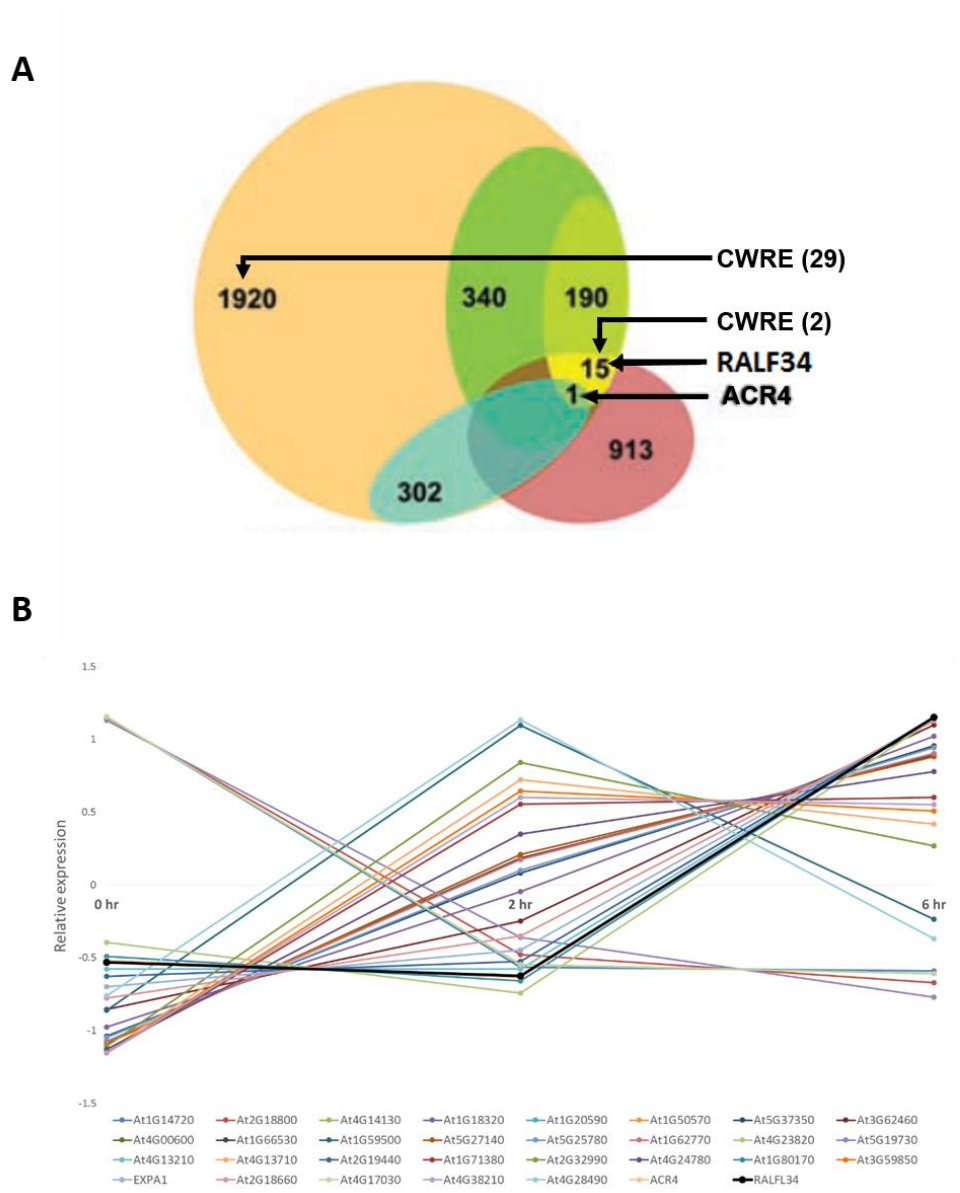


Figure 9. (A) FACS dataset (De Smet et al., 2008) showing 1920 highly differentially regulated genes during lateral root initiation; highlighted are the 29 cell wall remodelling enzyme genes, 2 of which are found within the 15 candidate lateral root development regulator genes; (B) Microarray data showing relative gene expression levels of the 28 identified CWRE genes, and RALF34, over a 0 hr, 2 hr, and 6 hr time point post auxin (10 µM NAA) treatment (De Smet et al., 2008).

CWREs are differentially expressed in ralf34

Quantitative real-time polymerase chain reaction (qPCR) was performed on *ralf34-1* cDNA using all 28 novel cell wall gene specific primers (**Table 1**) to

elucidate potential transcriptional regulation in this lateral root phenotype specific mutant (**Figure 10**).

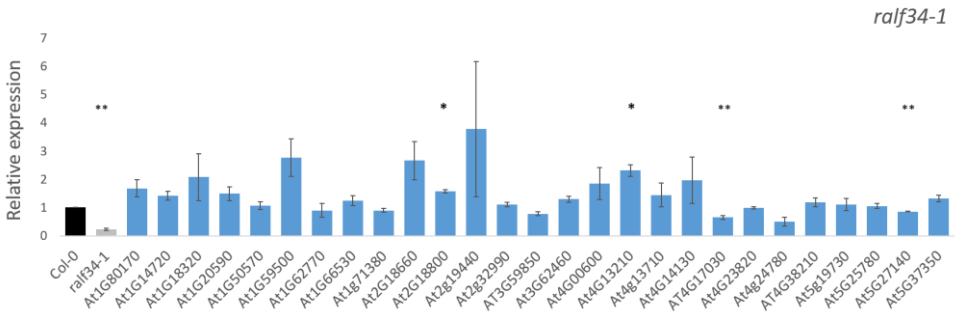


Figure 10. Relative gene expression levels of 28 cell wall remodelling genes quantified through quantitative real-time polymerase chain reaction (qPCR) in the *ralf34-1* background; Graphs show average of 3 biological replicates ± standard error, asterisks indicate statistical significance ($P < 0.05$) (*higher, **lower than control).

It's clear that several cell wall related genes are up- and downregulated in the *ralf34-1* background, suggesting their downstream role in cell wall modification, potentially during RALF-mediated lateral root development (see Chapter 4 – RALFs in lateral root development) and/or cell wall elongation (see Chapter 5 – RALFs in primary root development).

Loss-of-function in cell wall remodelling enzyme mutants decreases primary root length and lateral root density

To explore the role of *RALF* peptide-linked cell wall enzyme candidates in primary root growth and lateral root development, we utilised T-DNA insertion lines that were available in all 28 genes. Here we present data in *at3g62460-1*, *at1g66530-1*, *at1g50570-1*, *at5g37350-1*, and *at1g14720*, to investigate the effects of downregulating the levels of these genes in *Arabidopsis*

plants (**Figure 11**). A plant line with a single T-DNA insertion in any one of the cell wall enzyme candidates, shows a significant decrease in root length over wild type of at least 10% (**Figure 11A**). Lateral root densities were increased significantly in *at3g62460-1*, *at1g50570-1*, *at5g37350-1*, and *at1g14720-1* (**Figure 11B**), which is a process *RALF34* has been identified as playing a role during the early formative stages of development (see Chapter 4 – RALFs in lateral root development). Interestingly, these were not genes specifically highly up- or downregulated in the *ralf34* mutant; as such, these phenotypes may not be related to downstream RALF34 signalling, or, as cell wall enzymes generally belong to large families of proteins, there is an inherent redundancy. Potentially, using a more specific transcriptomic technique, such as synchronising lateral root development through NPA-to-NAA hormone transfers, would give a better overview of the genetic information in the *ralf34* mutant background. On-going phenotypic analysis is being performed on those genes which were highly differentially regulated, such as *At4g13210* (up) and *At4g17030* (down).

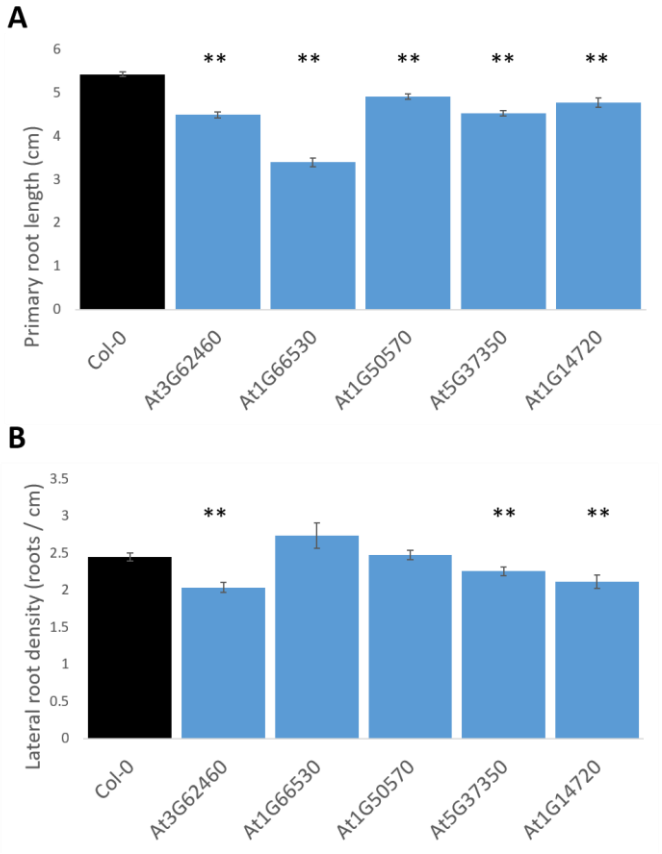


Figure 11. (A) Primary root lengths (cm) of 7 days post germination seedlings in wild type (Col-0, n= 150), and cell wall remodelling T-DNA insertion mutants (*At3G62460*, P= 5.23E-22 (n= 112); *At1G66530*, P= 3.01E-32 (n= 61); *At1G50570*, P= 2.06E-09 (n= 103); *At5G37350*, P= 4.29E-22 (n= 108); *At1G14720*, P= 6.18E-07 (n= 52)); (B) Lateral root densities (roots / cm) of 7 days post germination seedlings in wild type (Col-0, n= 150), and cell wall remodelling T-DNA insertion mutants (*At3G62460*, P= 1.46E-06 (n= 112); *At1G66530*, P= 0.1171 (n= 61); *At1G50570*, P= 0.7453 (n= 103); *At5G37350*, P= 0.0150 (n= 108); *At1G14720*, P= 0.0021 (n= 52)); Graphs show average of 3 biological replicates ± standard error, asterisks indicate statistical significance (P < 0.05) (*higher, **lower than control).

CONCLUSIONS

Secreted peptides such as RALF34 have been found significant for ever more cellular processes, such as the aforementioned lateral root development and cell elongation. Thus, identifying the ligand-receptor pairs is important for understanding the nature of the signalling pathways. Our work used an *in silico* approach to identify potential interacting partners with overlapping phenotypes.

Previously, *ralf34-1* has been shown to produce longer primary roots than wild type (See Chapter 5 – RALFs role in primary root growth). This phenotype is enhanced when *ralf34-1* is crossed with the closely related *ralf4-1*, generating even longer primary roots, which many of our LRR and kinase proteins that coexpressed with *RALF34* mimicked. Further analysis using 35S::*RALF34* overexpression lines need to be used to see if the putative receptor phenotypes are insensitive to additional peptide, or if the *ralf* mutant phenotype can be recovered. Lastly, we observed whether *ralf34* mutant phenotypes were potentially due to the increased level of cell wall remodelling gene expression. Further analysis into specific cell wall enzyme T-DNA lines may reveal *ralf34* opposite phenotypes.

MATERIALS AND METHODS

Growth of *Arabidopsis thaliana* seedlings: Seeds were surface sterilised (70% ethanol – 2 minutes, 10% bleach - 15 minutes, $_dH_2O$ – 5 washes) then stratified at 4°C for 2 days. Seeds were then plated on ½ Murashige and Skoog (MS) agar plates (2.154gL^{-1} MS, 0.1gL^{-1} myo-inositol, 0.5gL^{-1} MES, 10gL^{-1} bacteriological agar, pH 5.7 with 1 M KOH) and germinated vertically under constant white light at 21°C. For dark grown *pRALF34::n3xRFP*, seeds were plated as above, exposed to light for 6 hours, then germinated, and grown for a further 4 days post germination in total darkness (control seedlings were grown under normal light), then imaged on a Nikon confocal microcope.

Phenotyping of T-DNA insertion lines: The following T-DNA insertion mutant lines were used in our investigations: *at3g20820-1/SALK_119747*, *at3g56370-1/SALK_079802*, *at3g56370-2/SALK_038787*, *at4g02630-1/SALK_042434*, *at3g08680-1/SALK_106115*, *ats1p-3/at5g19660/SALK_020530*, *at3g62460-1/SALK_62460*, *at1g66530-1/SALK_074366C*, *at1g50570-1/ SALK_089278C*, *at5g37350-1/SALK_110287*, and *at1g14720-1/SALK_058173C*. Phenotyping was analysed under a Leica stereo dissection microscope at varying magnifications to observe and count the emerged lateral roots. Lateral roots were counted, marked, and then photographed. Root lengths were measured from the bottom of the hypocotyl to the root tip, using ImageJ software (http://imagej.nih.gov/ij/).

CORNET: Co-expression network: *RALF* co-expression databases were generated using Correlation coefficient >0.8 and P-value < 0.05, top 50 genes, correlation of query genes with neighbours and between neighbours, in at least

(1) selected databases, all databases selected. Protein-protein interaction network: RALF protein databases were generated using the CORNET PPI tool, selecting interactions of query protein(s) with neighbours and interactions between neighbours, in all selected databases, all databases selected for both experimental and predicted interactions. Transcription factor network: RALF transcription factor database was generated using the CORNET TF tool selecting interactions of query protein(s) with neighbours and interactions between neighbours, in all selected databases, all databases selected for both confirmed and unconfirmed interactions.

Quantitative RT-PCR: Total RNA was extracted from lines of interest using an RNeasy kit (Qiagen). Using Superscript II (Invitrogen), cDNA was generated from total RNA (200 ng) via oligo(dT) primers as per instructions. Quantitative PCR reactions were performed in 4 technical repeats, using the SYBR Green QPCR Master Mix (Quanta Biosciences), in our modified reaction protocol: Master mix - 7 µL/reaction (forward primer [100 µM] – 0.1 µL/reaction, reverse primer [100 µM] – 0.1 µL/reaction, SYBR(2X) – 6 µL/reaction, $_{d}H_2O$ – 0.8 µL/reaction) and template cDNA [diluted 1/100 $_{d}H_2O$] – 5 µL/reaction. Gene specific quantification was performed with intron spanning primer pairs (where possible) designed using Primer3 software (http://bioinfo.ut.ee/primer3/). Expression was normalised to *EEF1α4* (At5g60390). All qRT-PCR experiments were performed in three biological replicates (unless otherwise specified) and the data presented represent means ± standard deviation. Primers used:

Table 1. Primers used for gene expression analysis in downstream *ralf34* assay.

qRALF34_Fp	AGTTACGGAGGAAGACGACG
qRALF34_Rp	CGGGACCTCTAGCTCTGAAG
qAT1G59500_Fp	TGAGACGAGTCACAGCAGAG
qAT1G59500_Rp	CGTGCCGGGATTAGACAAAG
qAT1G66530_Fp	CAACTAACCCCTTGCTGCTC
qAT1G66530_Rp	CGAAATCAGCCGAAACGAGT
qAT5G37350_Fp	CAAGTTGCGCGAGTGTTACT
qAT5G37350_Rp	CTCACTGAGATCCCCATGCA
qAT4G14130_Fp	TCTCGTCATGTGTTGCTCCT
qAT4G14130_Rp	AAACTTGTCGCCGGAAACTC
qAT2G18800_Fp	GGGACCATTCGTAGCCTCTT
qAT2G18800_Rp	ATCCCTCTCTGCGAAAACCA
qAT1G14720_Fp	GTGTGTGCTTCCATTACCGT
qAT1G14720_Rp	CTGCAGGAGTTGTTATCGCC
qAT3G62460_Fp	CTTCGAAACCTCACCAGCAC
qAT3G62460_Rp	CCGGAGCTGTAAACCTTTGC
qAT4G00600_Fp	AAATGTCTCCAACCAACCGC
qAT4G00600_Rp	GATAAAACCGGGTGCAGTCC
qAT1G18320_Fp	ACTCGGGTTGATGATGGGTT
qAT1G18320_Rp	CAGCAGAGAAAACCAAACCCA
qAT2G18660_Fp	ACCGGCATCAGTATTAGCGA
qAT2G18660_Rp	GCTGGTAGTCGGAGTCAAGA
qAT1G80170_Fp	TCAGGCACCAAGATTCTGGT
qAT1G80170_Rp	CCACGTTGGCAATTTTGGTG
qAT4G13210_Fp	CGTGGTGTCAACGTGCATAT

qAT4G13210_Rp	ATACCATCACCATCCGCCAT
qAT1G50570_Fp	TTCTCTGATTGGGTTGGGCT
qAT1G50570_Rp	GAAGCAGTTGTGAAACCGGT
qAT1G20590_Fp	CACAAGGCAAAGCTCGATCA
qAT1G20590_Rp	ATGGCCAACACCTTGCAATT
qEEF4_Fp	ctggaggttttgaggctggtat
qEEF4_Rp	ccaagggtgaaagcaagaaga

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Chapter 7 – Conclusions and Future Perspectives

Our planet is growing rapidly in population and with that comes a demand for resources. In this study we used the model plant *Arabidopsis thaliana* as the roots are easily visualised, the genome has been sequenced, and there are many tools widely available to work with, making it an excellent choice to study novel, and unknown characterised proteins and their effects on root development.

In **Chapter 4** we demonstrated in *Arabidopsis* that the small signalling peptide RALF34 plays a significant role during the early formative divisions of lateral root development. Specifically, through the use of the *ralf34-1* and *ralf34-2* mutant alleles, we observed abnormal lateral root shape, extra cellular divisions, as well as a higher density of stage 1 primordia. We then showed that the early marker gene for lateral root founder cells, *GATA23*, was specifically altered in the *ralf34-1* mutant line. Interestingly, the *ralf34-1* mutant (which knocks out *GATA23* expression) does not show the same phenotype as the GATA23-RNAi knockdown lines – ie. *ralf34-1* has more and GATA23-RNAi show fewer lateral roots. This may indicate a potential feedback mechanism, or separate pathway involved in these two signalling modules.

To investigate the root specific nature of RALF34, we generated fluorescent transcriptional reporters. This showed an interesting expression pattern of *pRALF34::n3xRFP* that strongly expresses specifically in developing lateral root primordia before the first asymmetric cell division; a pattern strongly

overlapping with the p*GATA23*::GFP fluorescent line. Interestingly, *RALF34* expression extends beyond the developing lateral root primordia, potentially suggesting RALF34 plays a role in lateral root spacing/organisation within the root. As of yet, there is no published fluorescent line showing an expression pattern similar to p*RALF34*. Utilising T-DNA insert mutant lines we observed a marked decrease in lateral root initiation and further stage progression during lateral root development in the *rafl34-1* and *ralf34-2* mutants when accompanied by the removal of the shoot derived sources of auxin / additional shoot derived signals. Further investigation needs to be performed into the role RALF34 has during the early events of lateral root initiation. What controls *RALF34* flanking expression? To investigate this, we are using *in silico* analyses and a yeast-1-hybrid study is attempting to examine transcription factor-DNA interactions (see Chapter 6). Other small signalling peptides (CLE7/26) have been shown to express specifically within, or specifically excluded from, developing lateral root primordia (Czyzewicz et al., 2015), also potentially playing roles in spacing or lateral root initiation. However, RALF34 appears to be the first small signalling peptide identified to play a role in potentially organising pre-branch sites (potentially through GATA23). How does RALF34 control *GATA23* expression? We have attempted to identify potential receptor proteins (see Chapter 6), which may be involved in the downstream signalling directly affecting *GATA23* regulation. However, it is also possible that *GATA23* is downstream to a key transition stage that is not reached in the *ralf34* mutant. RALF34 was previously shown to be regulated by CYCD3;1, a key cell cycle regulatory protein, suggesting RALF34 may be regulated by or regulate – the cell cycle – during lateral root development (de Jager et al., 2009).

Another pertinent question is why is shoot derived auxin necessary for *RALF34* mediated lateral root initiation/progression, or is it another shoot derived molecule? Utilising a gain of function approach would further characterise this process. RALF34 potentially affects the auxima maxima required to initiate lateral root division, hence, removing RALF34 via *ralf34-1*, means cells don't reach the auxin maxima required when the shoot derived auxin is removed. Furthermore, other plant hormones such as gibberellin and brassinosteroids may be acropetally transported down through the shoot, regulating RALF34 mediated lateral root development. An alternative to the previous assay would be to overexpress *RALF34*, and we might potentially see the opposite and observe proliferative divisions post-shoot excision. Furthermore, after severing the shoot tissues, we can apply exogenous auxin (or other hormones) to see if it is specifically shoot derived auxin or another shoot derived signal (photosynthate/peptide) that is required to drive lateral root development. Lastly, *ralf34* produce more lateral roots, a trait that could potentially be highly advantageous in crops; since *RALF34* is present in rice, wheat, oilseed rape, maize, etc it would be valuable to look at root architecture in *ralf34* mutants (if available), or even exogenous RALF34p peptide treatments, as was done for CLE26 (Czyzewicz et al., 2015).

Chapter 5 focused on the mechanism of cell elongation in the primary root. Previously, RALF1 and RALF23 peptides had been shown to have an effect on primary root growth in *Arabidopsis* (Bergonci et al., 2014; Srivastava et al., 2009). Here, we showed that *RALF34* was a highly expressed root gene, with strong expression in the root apical meristem. We observed *ralf34* and the

closely related *ralf4* mutants produce longer primary roots, and that this phenotype was enhanced in the *ralf4 ralf34* double mutant. These observations were correlated to cell size, where the average mature cortical cell length was also longer than wild type, which also was further enhanced in the *ralf4 ralf34* double mutant. Crop potential of longer roots is clearly advantageous, showing once more, the translatability of this work. Lastly, we observed a minor partial resistance to exogenously applied auxin in the *ralf4* and *ralf34* mutants, opposing auxin induced growth arrest. Here, it will be important to understand the role of auxin mediated signalling and how this acts through these RALFs – or potentially, if auxin is not the main protagonist on RALF34 signallling, how do the other (brassinosteroids, GA, cytokinin…) effect RALF mediated cell elongation. Future research should focus on downstream components in these RALF pathways that mediate cell elongation, such as cell wall remodelling enzymes (see Chapter 6). Auxin has long been associated with an 'acid growth theory', whereby auxin stimulates cell wall loosening through lowered pH, and RALF1 has already been implicated in associating with a hydrogen pump, AHA2, so there is potential that RALF4 and RALF34 may act through similar mechanism (Haruta et al., 2014; Rayle and Cleland, 1992). Do RALF4/RALF34 act on AHA2 in a similar manner to RALF1? Identifying a receptor (Chapter 6), or observing the phosphostatus of AHA2 in the *ralf4*, or *ralf34* mutant backgrounds would provide the answers to this question.

Chapter 6 looked at the potential up- and downstream proteins involved in RALF mediated signalling. Through *in silico* data mining, four LRR and kinase proteins were identified which all had *RALF34*-like expression profiles

in the root tip. Utilising T-DNA knockout lines, it was shown these mutants produced significantly longer primary roots and higher lateral root densities than wild type. Furthermore, the AtS1P processing enzyme, previously shown to cleave RALF23 (Srivastava et al., 2009), was analysed for root specific phenotypes in the *s1p-3* mutant, which showed a significantly shorter primary root and higher lateral root density than wild type. Further investigation needs to be performed to correlate these receptor proteins to RALF34 mediated signalling. Observation of physical interaction of ligand to receptor (ie. photoaffinity labelling) is technically challenging and time consuming, but necessary. In addition, application of synthetic peptide or crossing the 35S::*RALF34* overexpression line into the receptor candidate mutant backgrounds and observing for sensitivities would be a much quicker screen. The intriguingly strong opposite phenotype from *s1p-3* suggests this cleavage enzyme does not regulate RALF proteins involved in negative regulation of cell elongation, however, this may have revealed the potential existence of RALFs that function in positive regulation of cell elongation. Further study should observe the primary lengths of *ralf23* and the other *ralf* family members to observe their roles in primary root length to indeed see if there exists a divergent role within this family of peptides. In the second half of this chapter we examined the downstream effects of the *ralf34* mutant on 28 cell wall remodelling enzymes. This showed a wide array of up- and downregulation on several genes. We examined five T-DNA mutant lines for primary root and lateral root phenotypes. All five showed decreased primary root length, however only three showed slight significant decreases in lateral root density. The transcriptional changes we observed in the *ralf34* background were not

substantial, suggesting these candidates may not be involved in our processes of interest, or, our technique was not specific enough. Application of more specific techniques, like timing of lateral root initiation through use of NPA and NAA hormones, or inducing primary root growth using brassinolide might give greater insight into the downstream effects of RALF signalling. If we applied RALF34 peptide would we observe more radical changes to cell wall gene expression? Perhaps using a gain of function approach would also give insight into the enzymatic changes downstream to RALF34. Furthermore, are these changes like to potential Ca^{2+} signalling in RALF34? In Chapter 3 we showed the *RALF34* expression pattern was potentially linked to mechanical induction and stretch response. Calcium signalling has been linked to cell wall remodelling and root elongation (Conn et al., 2011; Han et al., 2015), therefore, it's clear Calcium mediated signalling should also be investigated in *RALF34*.

In the **appendix** we present a published article which focuses on identifying interacting partners with the receptor-like protein kinase ACR4, which was identified in the same assay as RALF34 (De Smet et al., 2008). My work focused on optimising a phosphatase assay, to identify whether phosphatase activity levels where altered in the *acr4* mutant background, and whether these levels were similar to *pp2a* mutant levels. Interestingly, the levels of phosphatase activity were reduced in the *acr4* background, however, were not significant when compared to wild type. The paper goes on to identify PP2A-C3 as an interacting partner with ACR4, showing direct protein-protein interaction, with PP2A-C3 dephosphorylating ACR4 and ACR4 oppositely, phosphorylating PP2A-C3. I further worked on 12 *pp2a* T-DNA insertion mutants, attempting to quantify and ascertain root specific phenotypes with a

similarity to *acr4* (unpublished). This work will help to characterise important factors involved in lateral root development signal transduction. The recent evidence that PP2A directly interacts with ACR4 shows the root specific nature of this enzyme, however, it will require significant research to uncover further protein interactions involved during lateral root development and primary root growth.

Taken together, in this thesis we have used a multidisciplinary approach to uncover the signalling cascades revolving around key genes involved in primary and lateral root growth (**Figure 1**).

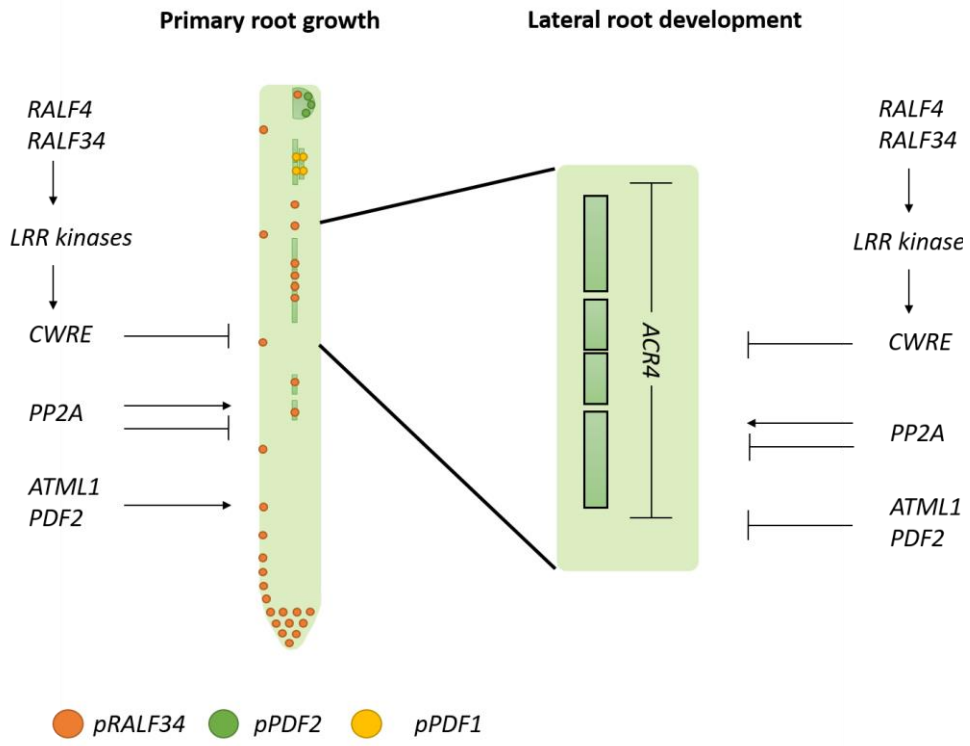


Figure 1. Schematic overview of the genes we investigated and the roles they play in primary root growth and lateral root development. Highlighted are the expression profiles (orange, *RALF34*, green, *PDF2*, and yellow, *PDF1*) showing their specificity to these root processes. Illustrated on the **left** is the putative RALF4/34 cascade through which primary root/ cell elongation is mediated, as well as the roles PP2A (varied) and ATML1 and PDF2 played. Illustrated on the **right** is the putative RALF4/34 cascade through which lateral root initiation is mediated, as well as the roles PP2A (varied) and ATML1 and PDF2 played (potentially through ACR4).

Using computational *in silico* data mining (**Chapter 6**), I identified several factors, including transcription factors, of which *RALF34* expression is downstream. I validated one of these interactions (**Chapter 3**), showing the important lateral root gene *SLR*, is a negative regulator of *RALF34*. I correlated

the *in silico* generated *RALF34* expression patterns (**Chapter 6**) to *in vivo* expression (**Chapter 3**) showing strong primary root and lateral root expression. I then investigated the expression pattern and phenotype of the *AtS1P* cleavage enzyme, shown to cleave RALF23, which strongly overlaps with *RALF34*, as cleavage is important for peptide activity (**Chapter 6**). The primary root length phenotype of *s1p* did not match that of *ralf34* suggesting there may be several cleavage enzymes, perhaps highly specific to each RALF, as this phenotype was significantly opposite to that of *ralf34* (**Chapter 5**). I then identified several LRR and kinase proteins through *in silico* data mining that fit the receptor and cell signalling criteria, and had putative overlapping expression profiles with *RALF34* (**Chapter 6**). Furthermore, we identified 28 cell wall remodelling genes downstream to *RALF34* that are putatively correlated to the lateral root initiation process (**Chapter 6**). Utilising T-DNA insertion mutants I performed phenotypic analysis on the above candidates with the view to elucidate roles in both primary- and lateral root development. The *RALF4* and *RALF34* genes were shown to play a role in cell elongation (**Chapter 5**) as well as lateral root development (**Chapter 3**), and several of the putative receptor genes, and cell wall enzymes were also correlated to these processes (**Chapter 6**). *RALF34* was identified in the same assay as *ACR4*, and has a similar lateral root phenotype (**Chapter 3**). Thus, I presented our paper on ACR4 which aimed to identify downstream targets of the receptor-like kinase. The paper showed ACR4 to be regulated by the PP2A holoenzyme, a phosphatase protein involved in almost all plant developmental processes. Further work (not shown here) aimed to investigate the many subunits of the PP2A family and their involvement in lateral root development. This highlighted the importance for this multifaceted

enzyme to be further investigated, linking it to specific primary and lateral root derived mechanisms, and in time, potentially linking it to *RALF34* mediated signalling.

These chapters have uncovered novel roles for many genes and proteins, largely involved in lateral root development, and primary root elongation. Clearly, there are many exciting avenues that await further investigation. Hopefully this work has contributed positively to the development of root architecture research and will be used as a foundation for future research to be built upon.

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Appendix – Reciprocal regulation between ACR4 and PP2A-3 regulates formative cell divisions in the *Arabidopsis* root

Submitted as a research article to *PNAS*

SUMMARY

The receptor-like kinase ARABIDOPSIS CRINKLY 4 (ACR4) is a mechanism inherent to controlling formative cell divisions in the *Arabidopsis* root. Phenotypic analysis of the *acr4* mutant shows a proliferation of cell divisions during the early stages of lateral root development. Interestingly, *acr4* also shows a strongly disorganised root apical meristem, a phenotype shared with mutations in the PP2A phosphatase holoenzyme. In this paper we identified a catalytic subunit of the PP2A holoenzyme, PROTEIN PHOSPHATASE 2A-3 (PP2A-3), as a novel regulator of formative cell divisions, and an interacting protein with ACR4 at the phosphorylation level. PP2A-3 was shown to dephosphorylate ACR4 potentially acting as a molecular switch, playing an integral role in directing the subcellular localisation of the ACR4 protein.

Author contribution: I contributed primarily to the work performed on **Figure 3D**, associating ACR4 phosphatase activity levels to those of PP2A. Furthermore, I helped with *in silico* analysis and general writing aspects of the article.

BIOLOGICAL SCIENCES - PLANT BIOLOGY

Reciprocal regulation between ACR4 and PP2A-3 regulates formative cell divisions in the *Arabidopsis* root

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ABSTRACT

In plants, the generation of new cell types and tissues depends on coordinated and oriented formative cell divisions**.** The plasma membrane-localized receptor kinase ARABIDOPSIS CRINKLY 4 (ACR4) is part of a mechanism controlling formative cell divisions in the *Arabidopsis* root. Despite its important role in plant development, very little is known about the molecular mechanism ACR4 is affiliated to and its network of interactions. Here, we used various complementary proteomic approaches to identify ACR4-interacting protein candidates that are likely novel regulators of formative cell divisions and that could pave the way to unraveling the molecular basis behind ACR4-mediated signaling. We identified PROTEIN PHOSPHATASE 2A-3 (PP2A-3), a catalytic subunit of PP2A holoenzymes, as a novel regulator of formative cell divisions and as the first described substrate of ACR4. Our results furthermore expose a tight post-translational regulation in the associated biochemical network through reciprocal regulation between ACR4 and PP2A-3 at the phosphorylation level.

SIGNIFICANCE STATEMENT

Plant growth and development are mediated through a wide range of proteins, including receptor kinases and phosphatases. The plasma membrane-localized receptor kinase ARABIDOPSIS CRINKLY 4 (ACR4) is part of a mechanism controlling formative cell divisions in the *Arabidopsis* root. However, the regulation of ACR4 signaling and how it impacts cell divisions remains completely unknown.

We have discovered that ACR4 phosphorylates the PP2A-3 catalytic subunit of the PP2A phosphatase holoenzyme, and that PP2A dephosphorylates ACR4. This furthermore exposed a tightly regulated point in the associated biochemical network that regulates formative cell divisions in plant roots. Furthermore, since a PP2A phosphatase holoenzyme including the PP2A-3 catalytic subunit has – amongst many other processes – also been implicated in cell division plane orientation, this further connects ACR4 to regulation of cell division.

KEYWORDS

Arabidopsis thaliana | formative cell division | phosphorylation | ACR4 | PP2A-3

INTRODUCTION

Plants rely on coordinated formative cell division for the formation of new cell types and tissues (1). For example, in the *Arabidopsis* root, the development of columella cells and regularly positioned lateral roots require a tight coordination of formative cell division and differentiation (1-3). In the primary root apical meristem, columella stem cells − upon formative cell division − give rise to new stem cells and daughter cells that will differentiate into columella cells (4) (**Fig. S1 *A***). Several proteins that play a role in this process have been identified (5-12), and small regulatory networks have been proposed (6, 10, 13). During lateral root initiation, pericycle cells at the xylem pole undergo a limited number of anticlinal formative cell divisions, giving rise to a core of small daughter cells flanked by larger ones. Subsequent periclinal formative cell division of the small central cells will start forming the dome-shaped lateral root primordium (7) (**Fig. S1 *B***). Also here, several factors that control these formative divisions, often overlapping with those in the primary root tip, have been identified (3, 7, 11, 14, 15). However, our knowledge on the mechanisms and signaling networks mediating formative cell divisions is sparse and is largely derived from transcriptional data (16).

Reversible protein phosphorylation represents a major mechanism regulating cell signaling (17, 18), and several kinases have been shown to play a role in (lateral) root development (7, 8, 11). For example, the plasma membrane-localized receptorlike kinase ARABIDOPSIS CRINKLY 4 (ACR4) is present in the pericycle during early stages of lateral root development, marks the plasma membrane in the primary and developing lateral root tips (**Fig. S2**),

and is part of a mechanism controlling formative cell divisions in the *Arabidopsis* root (3, 7, 19). ACR4 possesses an extracellular ligand binding domain, a transmembrane helix, and an intracellular domain that contains the juxtamembrane and the C-terminal subdomains, which flank the core kinase domain with serine/threonine kinase activity (**Fig. 1 *A***). The intracellular juxtamembrane domain is a likely recruitment site for interacting proteins and essential to facilitate downstream signaling (20, 21). Although ACR4 was the first receptor kinase to be assigned a role in root development (7), our knowledge about the ACR4 signaling pathway in the root remains limited (19, 22).

In addition to post-translational modifications such as phosphorylation, developmental programs and cellular functions largely rely on interactions between proteins, forming complex networks to control biological processes (23, 24). While membrane proteins play a crucial role in many biological processes, knowledge of the *in planta* membrane interactome is limited (24). Notwithstanding the recent progress with respect to global analyses of membrane protein interactions, so far ACR4 was not represented in a membrane-linked *Arabidopsis* interactome (25). Therefore, the objective of this study was to use ACR4-centered, protein-focused systems biology approaches to gain insight into the ACR4 signaling cascade and to identify new potential regulators of formative cell division.

RESULTS AND DISCUSSION

Mapping putative ACR4 interactions. To identify novel regulators of formative cell division in *Arabidopsis*, we combined *in silico*, tandem affinity purification (TAP), yeast two-hybrid (Y2H), and phage display approaches to define potential ACR4interacting proteins. Given the technical difficulties associated with plasma membrane proteins, we focused on intracellular ACR4 domains for our *in vitro* and *in vivo* studies (**Fig. 1 *A***).

We first interrogated available protein-protein interaction (PPI) databases for experimental and predicted interactions by applying the PPI tool within CORNET 2.0 (26, 27) to ACR4 (AT3G59420). This resulted in a network with 85 nodes which mainly lacked experimental validation (**Fig. S3 and Table S1**). Second, we applied a TAP approach to *Arabidopsis* cell suspension cultures (28) expressing the N- or Cterminally tagged ACR4 intracellular kinase domain (**Fig. 1 *A* and Fig. S4**). This analysis resulted in 4 putative ACR4-interacting proteins, of which 2 occurred in at least 2 biological replicates and in both assays with N- and C-terminally tagged ACR4 intracellular kinase domain, and were not present in any *in house* background list, namely 4-HYDROXY-TETRAHYDRODIPICOLINATE REDUCTASE 1 (HTPA REDUCTASE 1/DAPB1, AT2G44040) and 4-HYDROXY-TETRAHYDRODIPICOLINATE REDUCTASE 2 (HTPA REDUCTASE 2/DAPB2, AT3G59890) (**Table S1**). Based on the TAP data alone, the two other candidates, PROTEIN PHOSPHATASE 2A-3 (PP2A-3, AT2G42500) and PROTEIN PHOSPHATASE 2A-4 (PP2A-4, AT3G58500), could not be conclusively identified as *bona fide* ACR4-interacting proteins as they were only detected in

one technical repeat. Third, we applied a conventional high throughput Y2H assay to screen for potential interactions with the ACR4 intracellular domain (**Fig. 1 *A***).

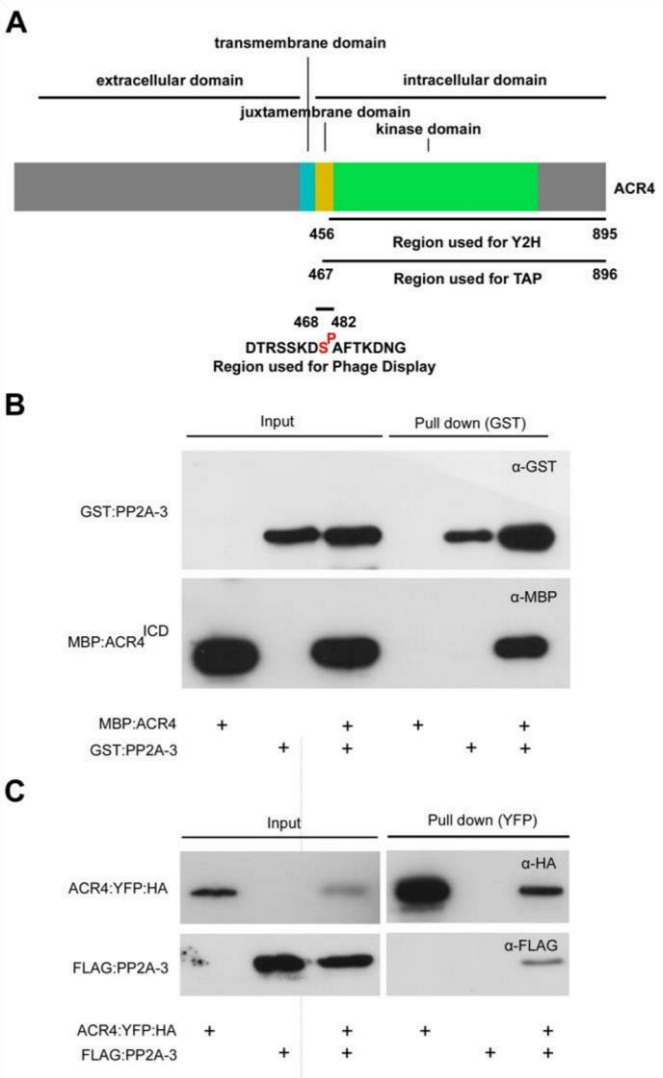


Figure 1. ACR4 interacts with PP2A-3. (A) Schematic representation of ACR4 with key domains and regions used for protein-protein interaction studies indicated. **(B)** *In vitro* GST pull-down experiment using GST:PP2A-3 and MBP:ACR4^{ICD} according to indicated combinations (+). PP2A-3 and ACR4^{ICD} were detected by Western blotting with anti-GST and anti-MBP antibodies, respectively. **(C)** *In planta* YFP pull down experiment using ACR4:YFP:HA and FLAG:PP2A-3 transiently co-expressed in tobacco leaves by *Agrobacterium* infiltration according to indicated combinations (+). PP2A-3 and ACR4 were detected by Western blotting with anti-FLAG and anti-HA antibodies, respectively.

This revealed 4 potential ACR4-interacting proteins with high confidence (**Table S1**). Finally, we screened a synthetic 15-mer peptide encompassing the Ser^{475} phosphorylation site within the intracellular juxtamembrane domain of ACR4 (**Fig. 1 *A***) against a 21amino acid phage-peptide library (29, 30). When the resulting consensus binding motifs for the phosphorylated peptide were queried against the *Arabidopsis* protein database using the sequence pattern search function in Scansite 2.0 (31), over 4000 potential ACR4-interacting proteins were identified (**Table S1**). Taken together, our complementary approaches identified several putative ACR4-interacting proteins, but when we searched for overlap between the different approaches this was limited to absent (**Table S1**). This could mean that different approaches yielded different subsets of putative ACR4-interacting proteins or that we picked a large number of likely false-positives.

***In silico* quality assessment of putative ACR4 interactions.** To increase the confidence in the potential ACR4-interacting proteins listed in **Table S1** and to select candidates for functional analyses, we performed in depth *in silico* quality assessment. Correlated gene expression is an indicator of co-functionality of genes in common pathways and processes (32), and interacting proteins are often significantly coexpressed. First, we used CORNET 2.0 (26, 27) to globally explore co-expression of *ACR4* and genes encoding potential ACR4-interacting proteins. Pairwise correlations showed some co-expression (Pearson correlation coefficient > 0.55) between ACR4 and some CORNET (2/85) and phage display hits (44/4402) (**Table S1**). Second, to further support potential PPIs in the root tip and during lateral root initiation, we used available cell- and tissue-specific

transcript profiling datasets (7, 33). Visualization of root tip *in silico* expression patterns for TAP (3/4) and Y2H candidates (2/4) through the BAR Arabidopsis eFP Browser (34), revealed distinct expression patterns that, at least partially, overlapped with the *ACR4* expression domain (**Fig. S5**). In addition, some of the CORNET (8/85) and PHAGE DISPLAY candidates (360/4402) were also transcriptionally differentially regulated in a transcriptome study of pericycle cells undergoing lateral root initiation (7) (**Table S1**). Based on the above observations, we generated a priority list for CORNET, TAP, Y2H and PHAGE DISPLAY hits, narrowing down the number of candidates from 4495 to 525 (**Table S1**).

To globally assess interactions between ACR4 and potential interacting proteins, we used the PPI tool within CORNET 2.0 (26, 27). Indeed, several of the CORNET, Y2H, TAP and prioritized PHAGE DISPLAY hits are connected with ACR4 and with each other in predicted and experimentally validated protein-protein interaction networks focusing on pairwise interactions (**Table S1**). To gain insight in the molecular functions represented in this protein-protein interaction network, we determined which Gene Ontology (GO) categories are statistically overrepresented in this network. Several overrepresented GO categories with respect to biological process (5%) and molecular function (5%) relate to phosphorylation (**Table S1**), which is in agreement with the fact that ACR4 is a receptor kinase. Taken together, through our *in silico* assessment we increased the confidence in a subset of potential ACR4-interacting proteins (**Table S1**).

ACR4 interacts with PP2A-3. Taking the results of **Table S1** into account allowed us to impose additional criteria to select candidates for functional analyses. Among the candidates selected by at least two approaches and with a high score in the priority list (**Table S1**), sequences that match isoforms of catalytic PP2A C subunits were retrieved. These were PP2A-3 (AT2G42500) and PP2A-4 (AT3G58500), which form a subclade in the family of 5 *Arabidopsis* PP2A C subunits (35). In general, the PP2A heterotrimeric holoenzyme, which is a major, highly conserved eukaryotic serine/threonine phosphatase, consists of a catalytic C subunit, a type A scaffolding/regulatory subunit, and a type B regulatory subunit (17, 36). In *Arabidopsis*, PP2A phosphatases have been implicated in various hormone-regulated, cellular and developmental processes, including spatial control of cell division and columella organization, and in innate immunity but little is known about their dynamic and highly regulated function (36-39). In the context of our focus on formative cell division, we selected PP2A-3 (and PP2A-4) for subsequent in depth functional characterization.

We first confirmed the interaction between the intracellular ACR4 domain and PP2A-3 using a protein overlay assay. This showed that PP2A-3 can interact with both the naïve (endogenously phosphorylated at a limited number of residues in *E. coli* through an unknown mechanism) and fully *in vitro* autophosphorylated ACR4 intracellular domain (29), but not with the negative controls POLYPYRIMIDINETRACT-BINDING PROTEIN (PTB) and BOVINE SERUM ALBUMINE (BSA) (**Fig. S6**). In addition, gel-filtration analysis further confirmed the interaction between the ACR4 intracellular domain ($ACR4^{ICD}$) and PP2A-3 (**Fig. S7**). The elution profile showed the

presence of ACR4^{ICD} and PP2A-3, as a higher molecular weight complex, in comparison with the elution position of ACR4^{ICD} alone. Subsequently, the purified recombinant MBP:ACR4^{ICD} was effectively pulled down with GST:PP2A-3 *in vitro* (**Fig. 1 B**). Moreover, in *Nicotiana benthamiana* transient expression assays, ACR4:YFP:HA was able to co-immunoprecipitate FLAG:PP2A-3 *in planta* (**Fig. 1 *C***). Taken together, these data strongly indicate that ACR4 and PP2A-3 interact with each other.

ACR4* and *PP2A-3* are co-expressed.** As mentioned above, *Arabidopsis* eFP Browser data (34) suggested that *PP2A-3* is weakly expressed in columella stem cells and in the root apical meristem (**Fig. S5**). To confirm this, we analyzed seedlings expressing a *pPP2A-3::n3xGFP* fusion and assessed expression in the root tip. In 5 independent transformants we indeed observed *pPP2A-3::n3xGFP* expression in the root tip, but *PP2A-3* was more broadly expressed than *ACR4* (7, 19, 40) (**Fig. 2 *A* and Fig. S2**). In addition, we observed *PP2A-3* expression during early lateral root initiation (**Fig. 2 *B), which also overlapped with *ACR4* expression at this stage (7) (**Fig. S2**). Taken together, these results show that *ACR4* and *PP2A-3* are expressed in overlapping domains, further supporting that they can physically interact.

PP2A-3 is involved in columella stem cell differentiation. To test genetically if PP2A-3 plays a role in ACR4-mediated lateral root initiation and/or stem cell regulation, we analyzed primary root length, lateral root density and columella stem cell differentiation in a previously characterized *pp2a-3* mutant (36). The

primary root of *pp2a-3* is slightly longer, while the emerged lateral root density is not significantly affected compared to wild type (**Fig. 2 *C-E***). With respect to columella stem cell differentiation, we observed slightly less differentiation in *pp2a-3* compared to wild type (**Fig. 2 *F***). These limited effects could be due to redundancy with the closely related PP2A-4. Indeed, a double *pp2a-3 pp2a-4* mutant displayed even less columella differentiation and severely disrupted cell organization in the root tip compared to wild type (**Fig. 2 *F* and *G***), which is similar to *acr4* (7, 19). However, given the dramatic effect on primary root growth (**Fig. 2 *C-D***), lateral root densities or pericycle division patterns could not be unambiguously analyzed in the *pp2a3 pp2a-4* background.

Short root phenotypes associated with disrupted cell division and/or cellular patterning in the root tip are often associated with a loss of quiescent center cell identity (41, 42). To assess if the quiescent center was absent in the disrupted root tip of *pp2a3 pp2a-4,* we analyzed the expression of the quiescent center marker *QC184* (9). Surprisingly, notwithstanding the dramatic impact on the regular cellular pattern in the root tip, *QC184* expression was not abolished, and even appeared to expand into the columella (**Fig. 2 *H***). The latter might suggest that the stemness gradient in *pp2a-3 pp2a-4* is perturbed. To further explore and confirm this, we applied cantharidin – a potent and selective PP2A inhibitor (43) – to the *QC184* and *pWOX5::GUS* markers (9), which supported that quiescent center identity is not lost when interfering with PP2A activity (**Fig. 2 *H***). In addition, *WOX5* expression is similarly altered in the *clavata3/embryo surrounding region 40* (*cle40*) mutant, and ACR4 is a target of CLE40 signaling (19, 22).

In summary, our observations indicate a role for PP2A-3 (and likely PP2A-4) in cellular patterning during lateral and primary root development that – to some extent – overlaps with ACR4 function.

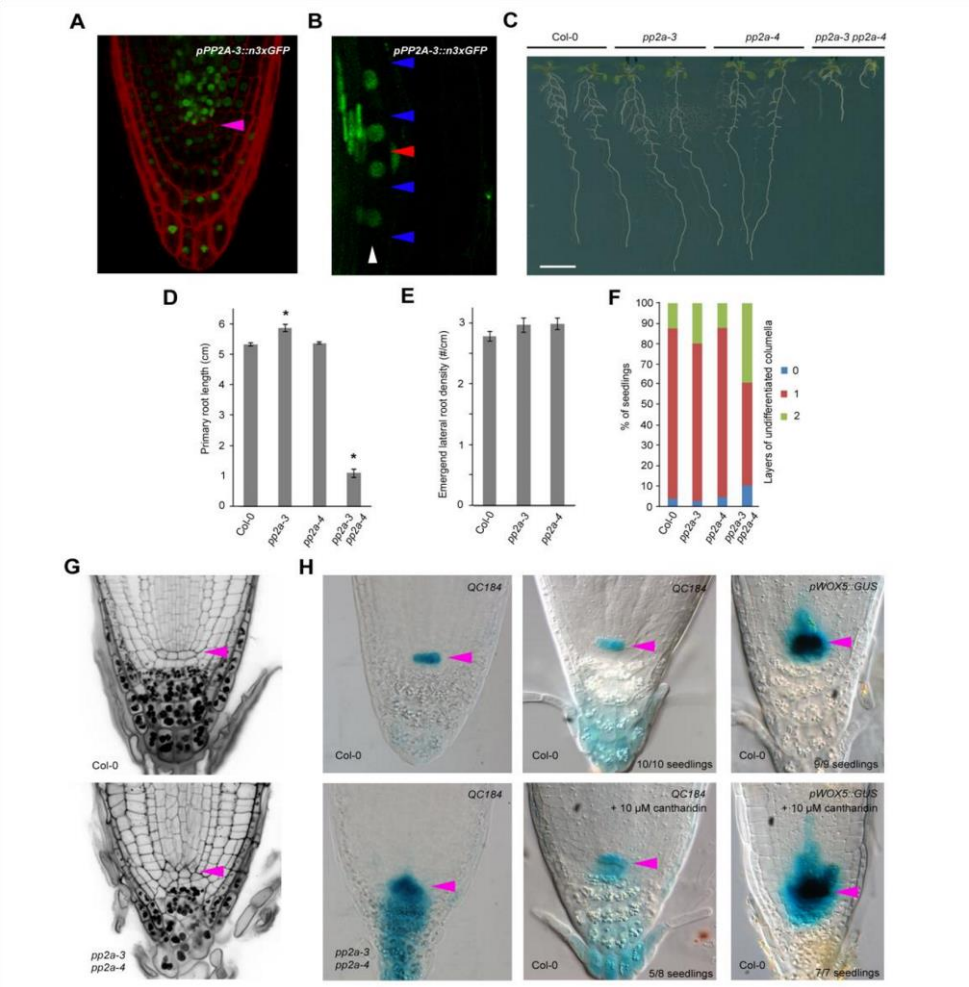


Figure 2. PP2A-3 mediates stem cell divisions. (A-B) Representative images of *pPP2A-3::n3xGFP* in the primary root tip (A) and during lateral root initiation (B) of 4 day old seedlings. pink arrowhead, quiescent center; red arrowhead, boundary between position of two adjacent pericycle cells before asymmetric cell division; blue arrowhead, subsequent asymmetric cell divisions; white arrowhead, pericycle. **(C-E)** Primary and lateral root phenotype of *pp2a-3*, *pp2a-4*, and *pp2a-3 pp2a-4* seedlings compared to the wild-type control (Col-0) at 9 days after germination (C). Scale bar, 1 cm. Quantification of primary root length (D) and emerged lateral root density (E). The bar graphs indicate the average ± standard error and n = 19 for *pp2a-3*, 18 for *pp2a-4*, 18 for *pp2a-3 pp2-4* and 20 for Col-0. Statistical significance (Student's *t*test) compared to Col-0 is indicated: *, p-value < 0.01. **(F-G)** Quantification of columella stem cell daughter cell differentiation (n = 176 for *pp2a-3*, 402 for Col-0, 214 for *pp2a-4* and 286 for *pp2a-3 pp2a-4)* (F) and irregular cellular pattern in *pp2a-3 pp2a-4* (G). Pink arrowhead, quiescent center. **(H)** Expression of *QC184* and *pWOX5::GUS* in 5-day-old *pp2a-3 pp2a-4* seedlings or in 5-day-old seedlings grown on 10 µM cantharidin. Representative pictures with number of seedlings with similar expression pattern indicated. Pink arrowhead, quiescent center.

ACR4 phosphorylates PP2A-3. PP2A activity and function in eukaryotic cells is regulated via post-translational modification of PP2A subunits (44). For example, PP2A complex assembly depends on the phosphorylation status of the catalytic subunit and phosphorylation of protein phosphatases has been shown to inactivate the enzyme (44, 45), but this has not been demonstrated in plants. To evaluate if ACR4 affects the phosphorylation status of PP2A-3, we compared the phospho-proteomes of Col-0 and *acr4* seedlings. However, although we could detect a peptide (NH_3GAGYTFGQDISEQFNHTNNLK-COOH) for PP2A-3 (or PP2A-4) in all samples, we did not observe any (differential) phosphorylation. Since PP2A holoenzymes act in various pathways (39, 46-48) and since ACR4 only acts in a few cells, it is likely that subtle differences mediated by ACR4 could be masked. Therefore, we explored if PP2A-3 could be phosphorylated by ACR4 *in vitro*. Indeed, *in vitro* kinase assays demonstrated that purified recombinant SUMO:ACR4^{ICD}, but not a mutant inactive version of ACR4^{ICD} with K540A and D641A amino acid exchanges (mACR4^{ICD}), could phosphorylate purified recombinant PP2A-3 (PP2A-3::6xHIS) under two conditions (see ***SI Materials and Methods***) (**Fig. 3 *A***). In the first condition autophosphorylated SUMO:ACR4^{ICD} was directly incubated with PP2A-3 and in the second condition the two proteins were first incubated together before initiating the kinase reaction with ATP (**Fig. 3 *A*, lane 4 versus lane 5**). In both cases, the autoradiogram clearly indicated phosphorylation of PP2A-3, but there appeared to be a reduction in the intensity of the ACR4^{ICD} under the second condition. This could be attributed either to masking of available phosphorylation sites in ACR4^{ICD} through complex formation with PP2A-3 or to dephosphorylation by a potentially active PP2A-3 phosphatase enzyme when

in complex with $ACR4^{ICD}$. Subsequently, we identified the ACR4-dependent PP2A-3 phosphosites from the *in vitro* kinase assay using high resolution mass spectrometry analyses. This revealed a total of 9 – so far unknown – phosphorylated residues of which five are at Ser, three are at Thr and one is at Tyr (**Fig. 3 *B* and Table S2-3**). Mapping these sites on a three-dimensional homology model of *Arabidopsis* PP2A-3, based on the structure of the catalytic chain within the trimeric human PP2A enzyme, showed that these residues were predominantly solvent exposed even in the trimer structure and, therefore, quite likely accessible for phosphorylation by ACR4 (**Fig. 3 *C* and Table S3**). Taken together, our results pinpoint PP2A-3 as a substrate for ACR4 kinase activity.

Phosphorylation of the tail of the PP2A catalytic subunit plays an important role in regulating the assembly – and thus activity – of PP2A holoenzymes (44). We therefore explored PP2A activity in cellular extracts prepared from *acr4* seedlings using a PP2A phosphatase assay system (39, 49, 50). In our hands, this revealed a decrease in PP2A activity of about 19% in *acr4* compared to wild type, which was similar to that associated with the PP2A regulatory subunit mutant *rcn1* (22%) and the catalytic subunit mutant *pp2a-3* (16%) (**Fig. 3 *D***). Although the results did not achieve statistical significance (p-value for *acr4* = 0.083), likely because the ACR4 impact on PP2A activity is diluted, they are suggestive of ACR4 being required for some of the cellular PP2A activity.

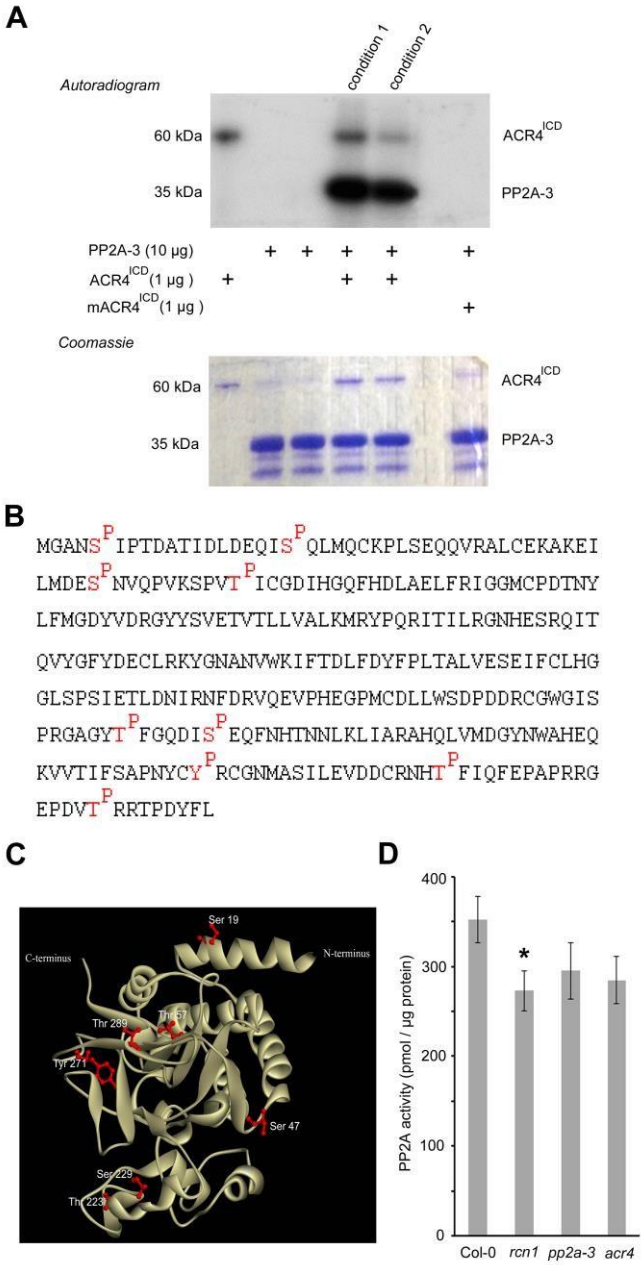


Figure 3. PP2A-3 is phosphorylated by ACR4 kinase. (A) Autoradiogram for coincubated PP2A-3 and ACR4 kinase (ACR4^{ICD}) as indicated (+) (upper panel). From left to right, 1 µg of autophosphorylated ACR4^{ICD} (lane 1), 10 µg of PP2A-3 (lane 2), 10 µg of PP2A-3 with [γ-^{32}P]ATP and cold ATP (lane 3), 1 µg ACR4^{ICD} was first autophosphorylated with [γ-^{32}P]ATP and kinase assay performed with 10 µg PP2A-3 (condition 1) (lane 4), 1 µg of ACR4^{ICD} was pre-incubated with 10 µg of PP2A-3 in kinase buffer for 1 hour at room temperature and the reaction subsequently initiated with 25 µM ATP and 2µCi of [γ-^{32}P]ATP (condition 2) (lane 5), blank (lane 6), and 1 µg of inactive ACR4^{ICD} (mACR4^{ICD}) with 10 µg of PP2A-3 (lane 7). Corresponding Coomassie blue-stained gel is shown as a loading control (lower panel). **(B)** Summary of ACR4-mediated phosphosites as determined by mass spectrometry of *in vitro* phosphorylated PP2A-3 and mapped on PP2A-3 protein sequence from TAIR. **(C)** Homology model of PP2A-3 showing 7 of the 9 phosphorylated residues (red ball and stick). **(D)** Bar diagram for PP2A activity detected in whole seedlings protein extracts of 12 day old seedlings as average of three biological repeats (with 3 technical repeats each) ± standard error. Statistical significance (Student's *t*-test) compared to Col-0 is indicated: *, p-value < 0.05.

PP2A-3 dephosphorylates ACR4. With respect to receptor kinases, PP2A has been shown to modulate the phosphostatus of BRI1 and the co-receptor BAK1 (39, 51, 52), so we also evaluated if PP2A-3 dephosphorylated ACR4. Unfortunately, commercially available purified *Arabidopsis* PP2A is not available, but PP2A is conserved in eukaryotes (44, 53). Therefore, to determine whether ACR4 could be a substrate of PP2A, we used – similar to Wu and colleagues (51) – a purified mammalian PP2A, consisting of the catalytic subunit PP2Ac and the scaffold subunit PP2Aa, to dephosphorylate the $ACR4^{ICD}$ that had been phosphorylated *in vitro*. This demonstrated PP2A-mediated dephosphorylation of the phosphorylated $ACR4^{ICD}$ (**Fig. 4 *A***), and pinpoints ACR4 as a substrate for PP2A phosphatase activity.

To assess the biological importance of altering the PP2A-mediated phosphorylation status of ACR4, we analyzed ACR4:GFP in the root of the *pp2a-3 pp2a-4* double mutant. This revealed a weak GFP signal in *pp2a-3 pp2a-4*, with hardly any membrane localization, as compared to the control (**Fig. 4 *B***). To explore this further, we investigated ACR4:GFP in the presence of the PP2A inhibitor cantharidin. This revealed a reduced membrane association of ACR4:GFP upon inhibiting PP2A activity within 5 hours, while the membrane localization of FORMIN HOMOLOG 6 (FH6), shown through the routinely used membrane marker FH6:GFP (54), was largely unaffected (**Fig. 4 *C-D* and Fig. S8**). We furthermore confirmed that the effect on ACR4:GFP is not due to cantharidin affecting *ACR4* expression levels (**Fig. S9**). Overall, these results suggest that membrane localization of ACR4 is dependent on PP2A-3 (and potentially PP2A-4).

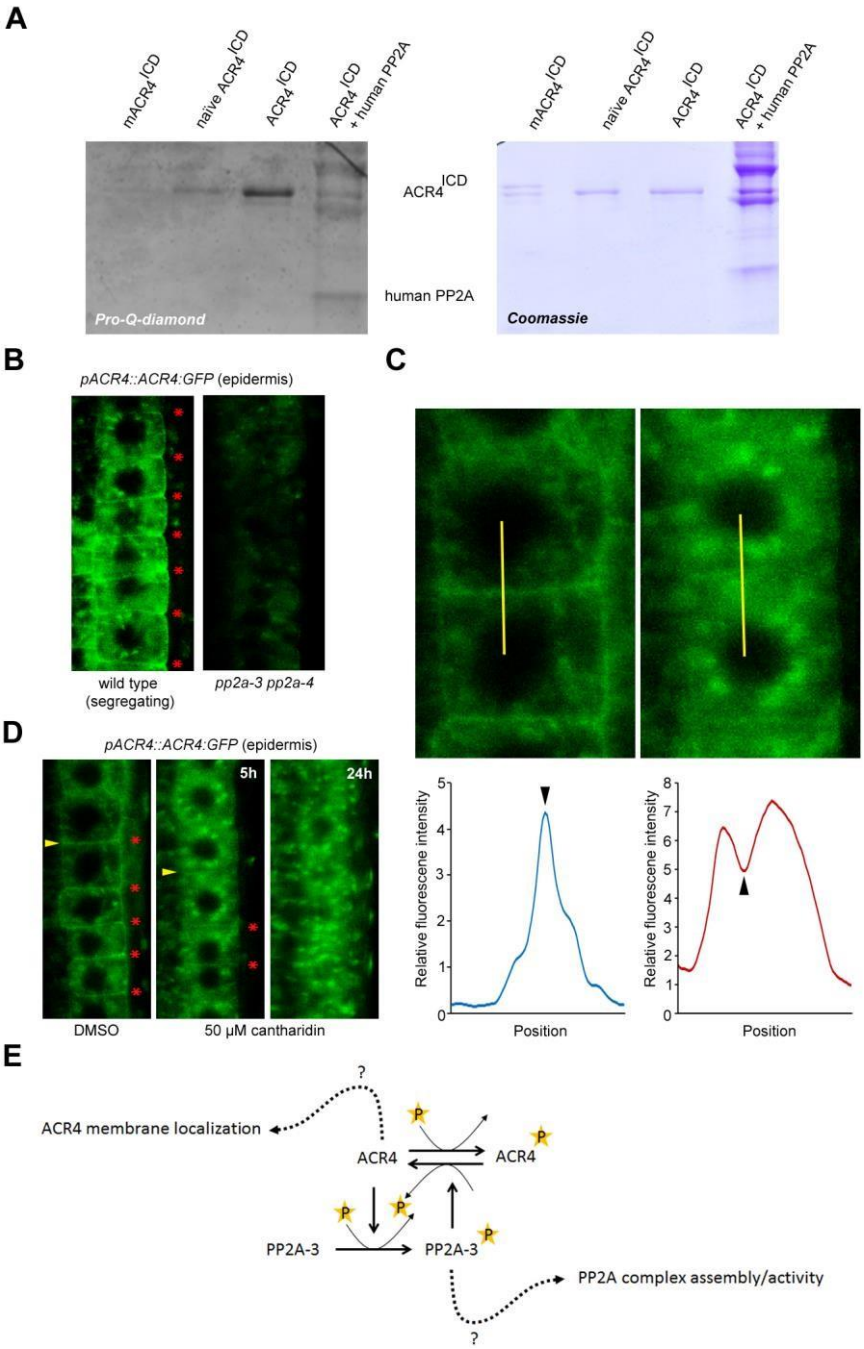


Figure 4. PP2A-3 dephosphorylates ACR4. (A) Pro-Q-diamond stained gel showing the phosphorylation status of $ACR4^{ICD}$ co-incubated without or with PP2A-3 as indicated (left panel). Corresponding Coomassie blue stained gel (right panel) is shown as a loading control. **(B-D)** Localization of ACR4:GFP in *pp2a-3 pp2a-4* background (B) and following treatment with DMSO or cantharidin (C-D) and. Red asterisk, plasma membrane with ACR4 localization; arrowhead, plasma membrane analysed in (C). (C) Quantification of GFP signal across the yellow line as smooth average graph. Arrowhead, position of plasma membrane. **(E)** Tentative model for the interactions between ACR4 and PP2A-3.

CONCLUSIONS

Interactions between membrane-associated proteins and soluble proteins are essential for signal transduction and for regulating plant growth and development. However, knowledge of the membrane protein interactome is limited in multicellular organisms, and only recently a mating-based split ubiquitin system (mbSUS) provided some insight in the *Arabidopsis* membrane-based interactome (25, 55). Unfortunately, the receptor kinase ACR4 was not included in this large scale analysis. Here, we used various approaches to identify potential ACR4-interacting proteins, and to correlate these putative interactions with formative cell divisions and root development. Based on all our analyses, we generated a prioritized list of potential ACR4-interacting proteins that are possibly involved in formative cell division and cell-to-cell communication.

Starting from these ACR4-interacting candidates, we identified PP2A-3 as the first described ACR4 substrate and showed that PP2A-3 plays an important role in the control of columella stem cell divisions and/or differentiation. The PP2A heterotrimeric holoenzyme consists of a catalytic C subunit, a type A scaffolding/regulatory subunit, and a type B regulatory subunit (17). Since ROOTS CURL IN NAPHTHYLPHTHALAMIC ACID1 (RCN1) was shown to regulate cell divisions and differentiation in the root tip (38), this is a likely PP2A type A subunit to build the PP2A holoenzyme involved in these processes, but the relevant regulatory B subunit remains to be identified. In this context, PP2A-3 or PP2A-4 are part of a TONNEAU1 (TON1)/TON1-RECRUITMENT MOTIF (TRM) family/PP2A (TTP) complex required for preprophase band formation and spatial control of cell division, and in this TTP

complex, FASS is the regulatory B'' subunit (36). Taken together, this possibly might explain how ACR4 can affect formative cell divisions, namely through regulating PP2A-mediated control of the orientation of cell divisions. Previously, it was shown that PP2A complexes associates with membranes in growing seedlings and that PP2A may interact with plasma membrane components (38, 46). Similar to the PP2A effect on BRI1 where dephosphorylated BRI1 is internalized (51, 52) and on BAK1 (39), we showed that PP2A can dephosphorylate ACR4. In this context, we also showed that PP2A activity affects the membrane localization of ACR4. On the basis of the available data, we propose a tentative model whereby on the one hand ACR4 phosphorylates the PP2A-3 catalytic subunit of the PP2A holoenzyme, possible facilitating complex assembly, and on the other hand, PP2A dephosphorylates ACR4, regulating its membrane localization and possibly activity (**Fig. 4 *E***). The balance between these two likely affects formative cell divisions and cell differentiation in the root.

MATERIALS AND METHODS

Detailed information on materials and methods used in this study is provided in ***SI Materials and Methods***.

Plant materials, constructs and growth conditions. T-DNA lines were previously described: *pp2a-3*, *pp2a-4* and *pp2a-3pp2a-4* (1), *acr4-2* (2), *rcn1-6* (3). The FLAG:PP2A-3 construct was previsously published (4). For phenotyping and expression studies, seeds were surface sterilized and stratified at 4 ℃ in the dark for 2 days. Seeds were germinated on vertically positioned

Petri dishes in a growth chamber at 21°C under continuous light (100 µmol m^{-2} s^{-1} photosynthetically active radiation).

Protein extraction and Phosphatase Assay. *Arabidopsis* lines for *acr4-2*, *rcn-1-6*, *pp2a-3*, and Col-0 were gas-sterilised (3mL 100% HCL + 97mL thin bleach solution) for 4 hours then stratified for 2 days at 4°C. Seeds were sown on ½ Murashige-Skoog medium (including myoinositol and MES) and 1% bacteriological agar. Approximately 5g of seedlings, grown until 12 days post germination, were ground whole in liquid nitrogen. Fresh phosphatase storage buffer [50mM Tris-HCl pH 7.5, 0.05% (v/v) Triton X-100, 100uM EDTA, 500uM PMSF, 0.05% (v/v) β-mercaptoethanol, 10% (v/v) glycerol, 0.5% (v/v) protease inhibitor cocktail (Sigma)] was added (½ volume/weight) directly to the frozen tissues and ground to a fine paste, then filtered through miracloth (in a cold room) to remove cell debris. The resultant cell lysate was centrifuged for 10 min at 4°C at 13,000 *g*. Endogenous phosphate was removed using the supplied Sephadex G-25 columns (Promega) according to the manufacturer's instructions. Protein levels were quantified using the Bradford assay. PP2A phosphatase activity was measured using a molybdate dye-based phosphatase assay kit according to the manufacturer's instructions. The reactions were incubated at 37°C for 30 mins before being stopped by the addition of the molybdate dye and additive mixture. The glass bottomed 96-well plate was read on a Tecan Infinite M200 Pro spectrophotometer at 30°C, utilizing 25 flashes at 600 nm, with 4 reads per well (within a well-border of 1,700µm). The phosphate released by samples was determined by comparing the protein samples to the standard curves using Magellan v7.0 software. The experiment was performed in three independent biological repeats.

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 (56).

Histochemical GUS assays. Details for GUS assays in ***SI Materials and Methods***. For microscopic analysis, samples were cleared with 90% lactic acid or as described in ref. (57). Samples were analyzed by differential interference contrast microscopy (Olympus BX53) and a stereomicroscope (Leica MZ16).

Biochemical assays. Details for biochemical assays in ***SI Materials and Methods***.

Mass spectrometry. Details for mass spectrometry in ***SI Materials and Methods***.

FOOTNOTES

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As I sit here alone in the cold, grey office, illuminated at night like a building wanting to be forgotten, with hazy emissions of dust-filled light falling on to aged, worn journals of research long made inconsequential, I think to myself. That is, I reflect on what thinking used to be. I remember… Or I believe I remember the feeling of thought… an idea manifesting into a corporeal substance, thick with inspiration, smooth with ingenuity. Or, was it thin and rough, like a volatile splinter snatching at the innermost constituents of the mind, searching, clinging for purpose. I suppose it doesn't really matter. I can't feel either anymore. Fuzz. Fluff. Warm, stale, patronising confusion obstructs the thoughts that would normally pervade the intrinsic synaptic functions connecting the what from the who am I. Who am I? I am who I've always been, which is who I am always to be. Thoughts don't change that. Is a thought part of you? Are you, what you think and are you thinking what you are to become? Are we as easily transitory as a thought? One moment solid in rigidity, so carefully fashioned with repose and conviction, yet the next, fluid and malleable, soon vaporous and ethereal, then, gone, evaporated, dissolved, forgotten. As I sit here alone in the cold, dark office, I find solace in knowing that I am, and will ever be, more than just a thought.