

**Factors Regulating Cortex Cell File
Proliferation under Low Phosphorus Stress
in *Arabidopsis thaliana* Roots**

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Abstract

Radial patterning of root hair bearing cells (trichoblasts) in *Arabidopsis thaliana* (arabidopsis) follows the type 3 root hair patterning mechanism whereby the radial positioning of trichoblasts is coordinated according to the position of underlying cortex cells (Pemberton *et al.* 2001). Epidermal cells which are positioned over the cleft between two underlying cortex cell files adopt trichoblast identity. Low phosphate stress in arabidopsis roots has been show to result in an increase in the number of cortex cell files from 8 to 12-16, which in turn results in an increase in the number of trichoblast position cells (Zhang *et al.* 2003, Cederholm and Benfey, 2015). However, little is known about what mechanisms control this proliferation in cortex tissue. Zhang *et al.* (2003) demonstrate that *eir1* (an allele of *pinformed2*, *pin2*) mutants are deficient in this response to low P. This finding suggests that *PIN2* (an efflux facilitator of directional auxin transport) is required to orchestrate proliferation of cortex cell files under low P. This implies that directional auxin transport and, ultimately, auxin response are required to enable proliferative divisions in cortex tissues under low P. First of all, a deeper anatomical understanding of the cortex cell file number response to low P was developed. That showed that the divisions which lead to the increase in cortex cell file number occur in the first cortex cell next to the quiescent centre. It was also found that the anatomical changes only significantly affect the number of cortex cell and trichoblast cell files, suggesting that it is a cortex specific response to increase radial root hair density. It was

further discovered that the root is sensitive to up to 400 μ M P and responds with increased cortex cell file number within 24 hours. I also showed that this response is independent of iron concentration.

It was next hypothesised that the role of directional auxin transport implied that other *PIN* and *AUX/LAX* genes may be required as well as activating *AUXIN RESPONSE FACTORS (ARFs)*. These experiments revealed that no other *PINs* or *ARFs* play a crucial role in this response. However it was found that PIN2:GFP protein changes its subcellular localisation in response to low P, suggesting that changes in auxin flux direction is required. Based on this, it was hypothesised that *PIN2* complementation in the cortex in *pin2* would rescue the phenotype. This was indeed the case, but also for *PIN2* under the *AUX1* promoter, suggesting an additional role for *PIN2* in the epidermis and root cap during the low P response. Mathematical modelling suggested that auxin flux out of the cortex, mediated by *PIN2*, would be required for the response. However, results testing this *in vivo* with the DII:VENUS auxin reporter were inconclusive. It was suspected that ground tissues patterning regulators, *BIRD* genes, may also play a role in tissue patterning under low P. *jkd (jackdaw)* and *mgp (magpie)* mutants did not show any strong phenotype on low P, but expression analysis using reporter lines and reverse transcription qPCR did suggest changes in regulation. It was then hypothesised that *BIRD* genes and *PIN2* may interact, by crossing *pin2* and *jkd* I found that the two interact to a small degree but it is not evident that they interact directly within the same pathway. It was concluded that this response to

low P was controlled by a number of regulatory networks which definitely involves *PIN2* mediated auxin transport with a contribution from *BIRD* genes.

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1. General Introduction

1.1. Arabidopsis root anatomy

One of the first detailed descriptions of cellular anatomy in the arabidopsis (*Arabidopsis thaliana*) root was that of Dolan *et al.*, (1993). This classical model of root anatomy describes the radial organisation of cell types within the arabidopsis root. Starting in the middle and working outwards, the radial organisation consists of a central vascular cylinder (vascular bundle or stele). The vascular cylinder consists of a bundle of procambium (indeterminate vascular precursor cells capable of differentiating in to vascular cells), wherein two xylem poles and two phloem poles form on perpendicular axes (Figure 1.1a). Within the stele (in the meristem/elongation zone), protoxylem and protophloem cells are found at the edge, adjacent to the surrounding pericycle. These are positioned such that each protophloem cell is opposite the other, and each protoxylem cell is opposite the other so that they form two perpendicular axes of this early-forming vascular tissue. Outside of the stele is the pericycle- a ring of ~12 indeterminate cell files whose fate is to form lateral roots later on in the development of the root.

Around the pericycle is a ring of endodermal cell files, whose suberised cell walls protect the vascular tissues from the free flow of water and solutes, acting as a barrier to apoplastic flow of water and solutes (Grebe, 2011). The endodermis, along with the cortex, forms the ground tissue.

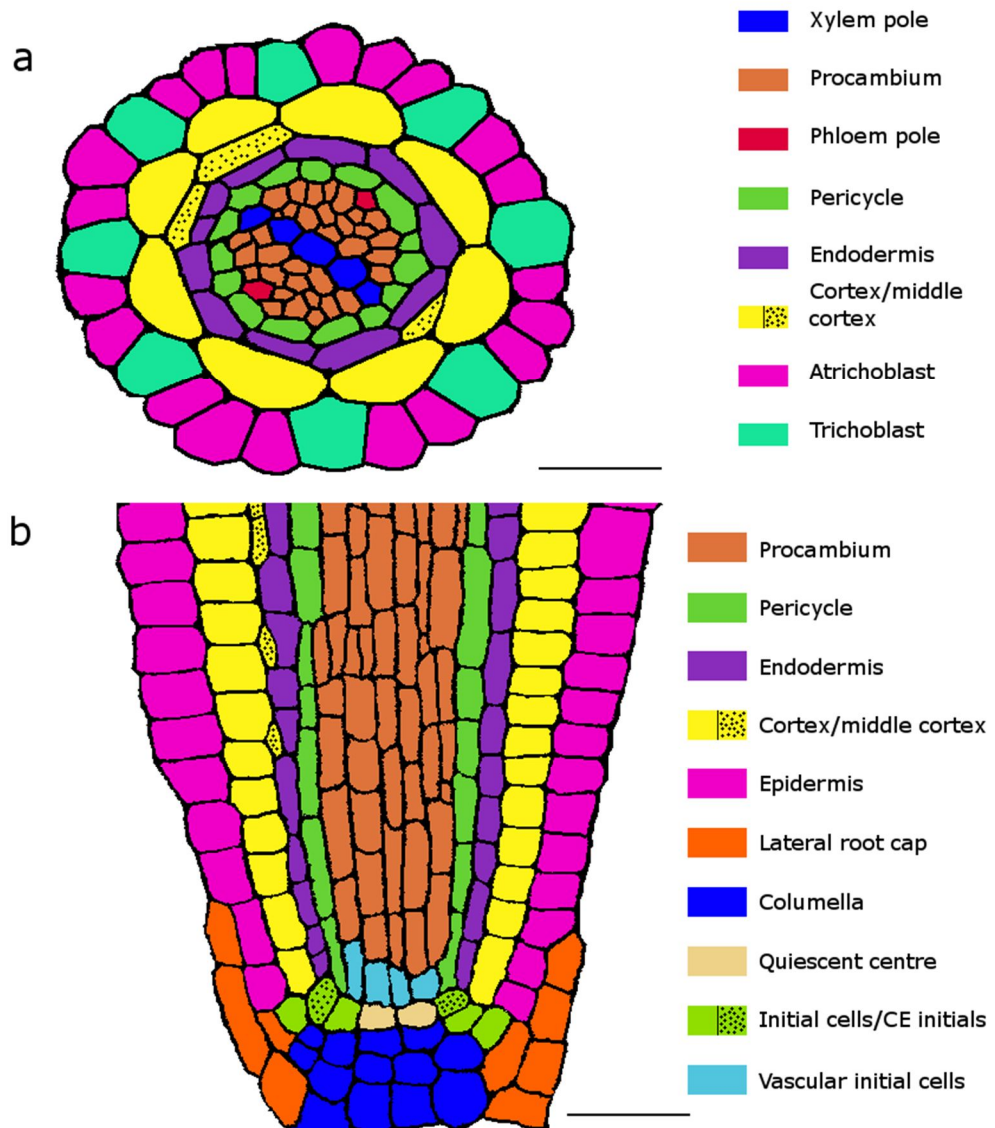


Figure 1.1 Schematic diagrams of radial and longitudinal organisation of tissues in the *Arabidopsis* root. Concentric rings of tissues radiate out from the central vascular cylinder in (a); trichoblast positions in epidermal cells at the cleft between two cortex cells can be seen (turquoise). Layers of middle cortex can be seen beginning to form at the xylem poles (dotted yellow). Organisation of tissues originating from the quiescent centre can be seen in (b); quiescent cells (beige) give rise to initial cells (light green) which divide to form new tissues. Scale bar= 50µm, both panels.

The cortex layer forms a ring of larger volume cells which have several roles amongst plants including regulating the hydraulic conductivity of roots (Fan, et

al. 2007; Rieger & Litvin, 1999) and the formation of aerenchyma by apoptosis (Drew, *et al.* 2000). Finally, surrounding the cortex is the epidermis. However, the epidermis consists of two sub-types of cells; eight epidermal cells take on a hair cell (trichoblast) identity according to their position in relation to the underlying cortex tissue. Each epidermal cell which forms in the cleft between two underlying cortex cell files will take on hair cell identity. Trichoblasts, or root hair cells, go on to produce long, unicellular root hairs. Atrichoblasts do not form any such structure.

A simple marker for this cell type specification is the higher relative cytoplasmic density of trichoblast cells compared with atrichoblast cells which can be visualised by toluidine blue staining (Dolan *et al.*, 1993).

All these tissues in the root originate from the quiescent centre (QC) which is a set of (usually four) indeterminate stem cells at the centre of the meristem (figure 1.1b). Continuous division of these cells gives rise to initial cells in the shootward and rootward vector; shootward initial cells form the stele, endodermis, cortex and epidermis and rootward divisions form the columella and lateral root cap (LRC). Following the divisions of initial cells to form the new tissues, cell specification begins. This is where tissue identities are determined. The specification of trichoblast and non trichoblast cells occurs early after the formation of the epidermis.

Arabidopsis is a member of the Brassicaceae, the Brassica family. This family incorporates a number of plants of importance to mankind including mustards (*Sinapsis sp.*), cabbages and other cole vegetables (*Brassica oleracea*), *B. rapa* (e.g. pak choi) and oilseeds including the British staple vegetable oil-bearing

rapeseed (*Brassica napus*). These species all share a conserved root hair patterning mechanism whereby the identities of root hair-bearing epidermal cell files (trichoblast files) are conferred by their relative position in relation to the underlying tissue. Each epidermal cell file which lies in the cleft between two cortex cells will become a trichoblast file. The variation of root hair patterning amongst plants was studied in some detail by Pemberton, *et al.* (2001); this study examined the root anatomy of 77 species of eudicots from 43 families. The root hair patterning found in Brassicaceae was called 'type 3', i.e. where root hairs occur between the cleft of two underlying cortex cells. They defined two other root hair patterning mechanisms- type 1 is where all epidermal cells, regardless of anatomical context, may become trichoblasts. Type 2 is where any epidermal cell may undergo an asymmetric cell division with the smaller daughter taking on hair identity.

Type 3 root hair patterning is conserved in the Brassica family along with only several others in the order Brassicales (Capparaceae, Limnanthaceae, and Resedaceae), six families in the Caryophyllales, and one species in the Boraginaceae. Thus this patterning is somewhat unusual in the broader context of dicots (type 1 being the most common). Nevertheless, the major brassicas of importance to mankind exhibit type 3 root hair patterning.

1.2 Arabidopsis root architecture

Arabidopsis displays a root architecture typical of dicotyledonous plants whereby one primary root grows from the seed at germination and then elongates, eventually yielding lateral roots from the pericycle layer of stem cells. Unlike many monocotyledonous plants (e.g. rice; Kitomi, *et al.* 2011; Itoh *et al.*, 2005), arabidopsis does not depend upon crown roots for its root system. It is capable of producing adventitious roots from the hypocotyl or other non-root tissues under certain circumstances (Rasmussen *et al.*, 2017), but the majority of the root tissue in an arabidopsis plant under normal conditions is derived from the primary root. This root eventually forms a thick tap root through extensive proliferation of ground tissue and development of procambium in to mature vascular tissues (Baum, *et al.* 2002).

Lateral roots which emerge from the primary root are of great interest in the fields of resource capture and their roles in these processes are well-studied. Changes to lateral root density, vertical emergence angle and radial emergence angle have been investigated in relation to N interactions, P interactions (Svistoonoff *et al.*, 2007; Williamson, *et al.* 2001), water availability and local water availability variation (i.e. hydro-patterning) respectively (Robbins & Dinneny, 2015).

1.3 Auxin control of root growth and development

One prominent factor regulating tissue patterning and growth at the root tip is auxin, a phytohormone whose most abundantly occurring form is indole-3-acetic acid (IAA). Auxin functions as a mobile signal which moves through tissues from cell to cell. In roots auxin flows through the tissue in a reverse-fountain like fashion (or polar auxin transport; PAT), flowing down from the shoot through the vascular cylinder to the root tip, and then back towards the shoot through the root cap and epidermis (Grieneisen *et al.* 2007; figure 1.2b). This constant flow through tissues allows for local accumulation of auxin in subsets of cells, and gradients of auxin concentration to form across tissues. Sites of local auxin accumulation are known as auxin maxima or auxin response maxima. By concentrating in to a subset of cells, auxin can elicit localised responses; for example, lateral root initiation is preceded by an auxin maximum which primes pericycle cells for periclinal division, giving rise to the lateral root meristem (De Smet *et al.*, 2007, Peret *et al.*, 2009). Similarly, quiescence is maintained at the centre of the root meristem by the accumulation of auxin in the cells of the quiescent centre (figure 1.2a). This accumulation of auxin induces the downstream expression of genes which maintain growth-driving divisions and indeterminate cell identity (i.e. stem cell identity). In the root tip this is regulated by the activation of *PLETHORA* (*PLT*) genes by auxin.

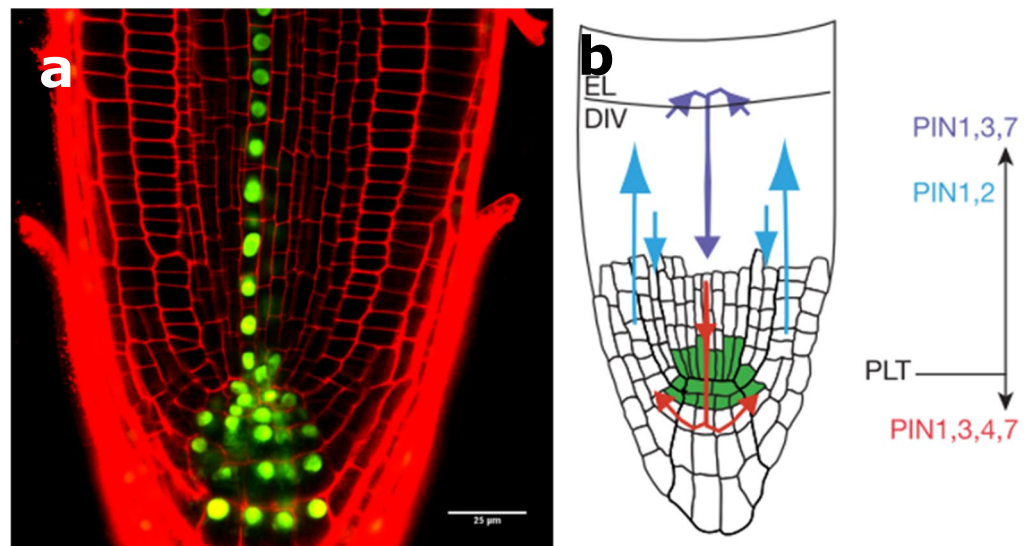


Figure 1.2 Auxin response maxima and reversed-fountain flow of auxin through the root tip

A confocal image of a root tip from an *Arabidopsis thaliana* (Columbia) expressing GFP under the control of the auxin responsive DR5v2 promoter (a). This reporter shows the auxin maximum which is found at the root tip, highest in the QC, columella, vascular initials and the developing xylem pole (as reported by Liao et al., (2015)). Red signal represents propidium iodide fluorescence in the cell walls, green signal represents green fluorescent protein. Scale bar= 25μM. Schematic diagram of polar auxin transport (reversed-fountain) through the root tip (b); arrow colours represent flux as contributed to by PIN proteins denoted in the same colour. DIV: division zone, EL: elongation zone, PLT: PLETHORA (taken from Blilou et al., (2005)).

This auxin maximum at the root tip activates the expression of *PLT* genes which promote the quiescent state of the QC. A lessening concentration gradient towards the shoot then enables the differentiation of cells into new tissues (Mähönen et al., 2014). This auxin maximum is not only required for maintaining root growth and development but is also crucial to the initial establishment of the root meristem in the embryo via the activating AUXIN RESPONSE FACTOR5 (ARF5; Schlereth et al., 2010). Auxin maxima in the

embryonic root also promote the first divisions which lay down the ground tissue, initiating expression of members of the *SHORT-ROOT (SHR)* pathway, also via ARF5 (Möller et al., 2017). Thus the polar flux (i.e. polar auxin transport) of auxin through the root, and the generation of local maxima are crucial to the patterning and growth of the root. In addition to the regulation of development as mentioned above, tropisms (i.e. growth towards/away from a certain environmental cue) are also largely controlled via auxin signalling. Root gravitropism is facilitated by a low to high auxin differential gradient between the downward and upward sides of the root, respectively. This difference in auxin distribution is largely regulated by auxin transporters such as PIN-FORMED2 (PIN2; Rahman et al., 2010a; Sukumar, *et al.* 2009). In this instance, the higher concentration of auxin on the downward side of the root acts to inhibit cell expansion. Meanwhile, the lower concentration on the upper side of the root facilitates cell expansion. This difference in the rate of growth across the root thus results in downward growth upon gravitational stimulation (Band et al., 2012). The process of PAT is facilitated by the subcellular localisation of auxin transport proteins which are often localised in a “polar” fashion, i.e. on one particular side of the cell in order to direct auxin flux in a particular direction (Figure 1.2b).

1.4 Auxin signalling

Auxin is synthesised primarily from either tryptophan or indole (via tryptophan independent pathways) and is highly dynamic in terms of its metabolism in to modified and/or conjugated forms (Korasick *et al.*, 2013). Auxin metabolism is

required as a mechanism of storage and inactivation/activation which allows a rapid response without the need for *de novo* synthesis, as well as its catabolism (Korasick *et al.*, 2013). Release of free IAA from modified auxin has been shown to be important in growth and developmental processes; for example the derivation of IAA from IBA (indole-3-butyric acid) has been shown to be involved in cell expansion and seedling development (Strader *et al.*, 2010, Strader *et al.*, 2011).

Auxin is involved in numerous plant growth and developmental processes including tropisms, embryogenesis, organogenesis and cell expansion. Auxin signals through TIR1-mediated proteolysis of AUX/IAA proteins, which results in de-repression of ARF (AUXIN RESPONSE FACTOR) proteins. ARFs are then active in modulating the expression of a multitude of genes which coordinate the auxin response. Auxin functions in this way by binding TIR1 in a hydrophobic pocket which induces a conformational change enclosing the auxin molecule. This change in conformation enables interaction between AUX/IAA proteins and TIR1 in the SCF ubiquitination complex; resulting in AUX/IAA proteins being targeted for degradation (Tan *et al.*, 2007, Guilfoyle and Hagen, 2007). TIR1 is the most well studied auxin receptor; however it acts redundantly along with several family members, AFB1-5 (Gagne *et al.*, 2002).

A number of ARF proteins have been identified which act downstream of this signalling mechanism. Arabidopsis contains 23 ARF encoding genes and these can be split broadly in to two groups, activating ARFs and repressor ARFs. These two groups of ARFs activate auxin response gene expression or repress it, respectively, both by binding to auxin responsive elements (AREs) in the

promoters of downstream genes. Five ARFs have been shown to act as activators in arabidopsis, these are ARF5, 6, 7 8, and 19. Possibly the most of these is *ARF5* which was first identified from its allele *monopteros*. These plants lack provascular cells and tissue polarity patterning in the embryo which leads to insufficient connectivity of the vasculature and failed organ growth (Hardtke & Berleth, 1998; Krogan, *et al.* 2014). The roles of ARF6 and ARF8 in roots are not well studied, but it is known that ARF8 is induced under N replete conditions (Gifford, *et al.* 2008), and ARF6 and ARF8 are involved in inflorescence development (Nagpal, 2005). ARF7 and ARF19 collectively are crucial to lateral root development because they are required to perceive the initial priming of pericycle cells by auxin (Okushima, *et al.* 2007).

Not all ARFs fall neatly in to either being a repressor or an activator. Some ARFs lack the binding domains which mediate the interaction between ARFs and AUX/IAAs, such as ARF3/ETTIN (ETT). ARF3 functions by interacting with another transcription factor INDEHISCENT (IND), whereby it is able to modulate the expression of other genes. However, in the presence of IAA this association is interrupted leading to cessation of its concerted activity with IND (Simonini *et al.*, 2016).

1.5 Auxin transport

In order to facilitate the generation of the reversed-fountain flow of auxin, or polar auxin transport, through the root, the flux of auxin between cells needs to be regulated in a directional manner. The flow of auxin and its regulation are

controlled primarily by the activity of polar localised plasma membrane anchored transport proteins.

Auxin transport relies on a basic principle of influx and efflux at the cellular level; several transporter types are required for this mode of flux. At apoplastic pH ($\sim$ pH5.5), approximately 15% of auxin exists as protonated IAAH (Delbarre, *et al.*, 2016) which can freely traverse the membrane in its lipophilic form. Whereas majority of extracellular auxin in the apoplast (i.e. IAA⁻), requires active import in to cells. This process is facilitated by auxin uptake proteins such as AUX1 (Marchant *et al.*, 2002). The entirety of auxin at intracellular pH ($\sim$ pH7.0) requires active transport to traverse the membrane (Petrášek *et al.*, 2007; Rubery and Sheldrake, 1974). Accordingly, not only are auxin transporters required for the regulated distribution of auxin but are also required to enable the movement of lipophobic auxin between cells. Auxin influx and efflux are catalysed by AUX/LAX proteins and PIN and ABCB proteins, respectively.

1.5.1 AUX/LAX proteins

AUX/LAX proteins are part of an auxin transporter family whose members are expressed specifically in different tissues. *aux1*, the first AUX transporter mutant to be identified, exhibits an agravitropic phenotype which can be rescued by application of NAA (1-naphthaleneacetic acid). NAA is an auxin analogue which can freely permeate the plasma membrane membranes and acts to promote auxin signalling. This suggested that AUX1 is required for auxin

import (Marchant et al., 1999). Since the identification of AUX1, several related proteins have been studied: this small family of four genes is comprised of *AUX1* (*AUXIN1*) and the *AUX1-LIKE* genes *LAX1*, *LAX2*, and *LAX3* (Péret et al., 2012). These genes encode membrane proteins with multiple membrane spanning domains and form a subclass of the amino acid permease super family. This family of proteins catalyses transport of molecules via substrate:proton symport activity, utilising the chemical potential of intra-/extracellular proton gradients to drive translocation (Young et al., 1999). Specificity for auxin by AUX1 has been demonstrated *in vitro* using heterologous expression, strengthening its role as an auxin specific importer (Carrier et al., 2008, Yang et al., 2006). Mutation of individual or multiple *AUX/LAX* genes results in auxin-related phenotypes including disruption of lateral root emergence and phyllotactic patterning (Marchant et al., 2002, Bainbridge et al., 2008, Swarup et al., 2008). *AUX/LAX* family members have determined roles in plant development such as in embryonic root cell organisation (Ugartechea-Chirino et al., 2010), and members are mostly non-redundant in these roles. For example, while *aux1* mutants display an agravitropic phenotype, *lax1* and *lax2* mutants do not appear to be defective in any aspect of primary root growth (Péret et al., 2012). Péret et al. (2012) showed that the localisation of *AUX/LAX* family members is generally non-redundant (figure 1.3a and b) and that expression of *LAX3* and *LAX1* is induced by auxin but *AUX1* and *LAX2* are not. This means that while the proteins share much amino acid sequence homology and biochemical function, their genomic regulatory regions show more diversity.

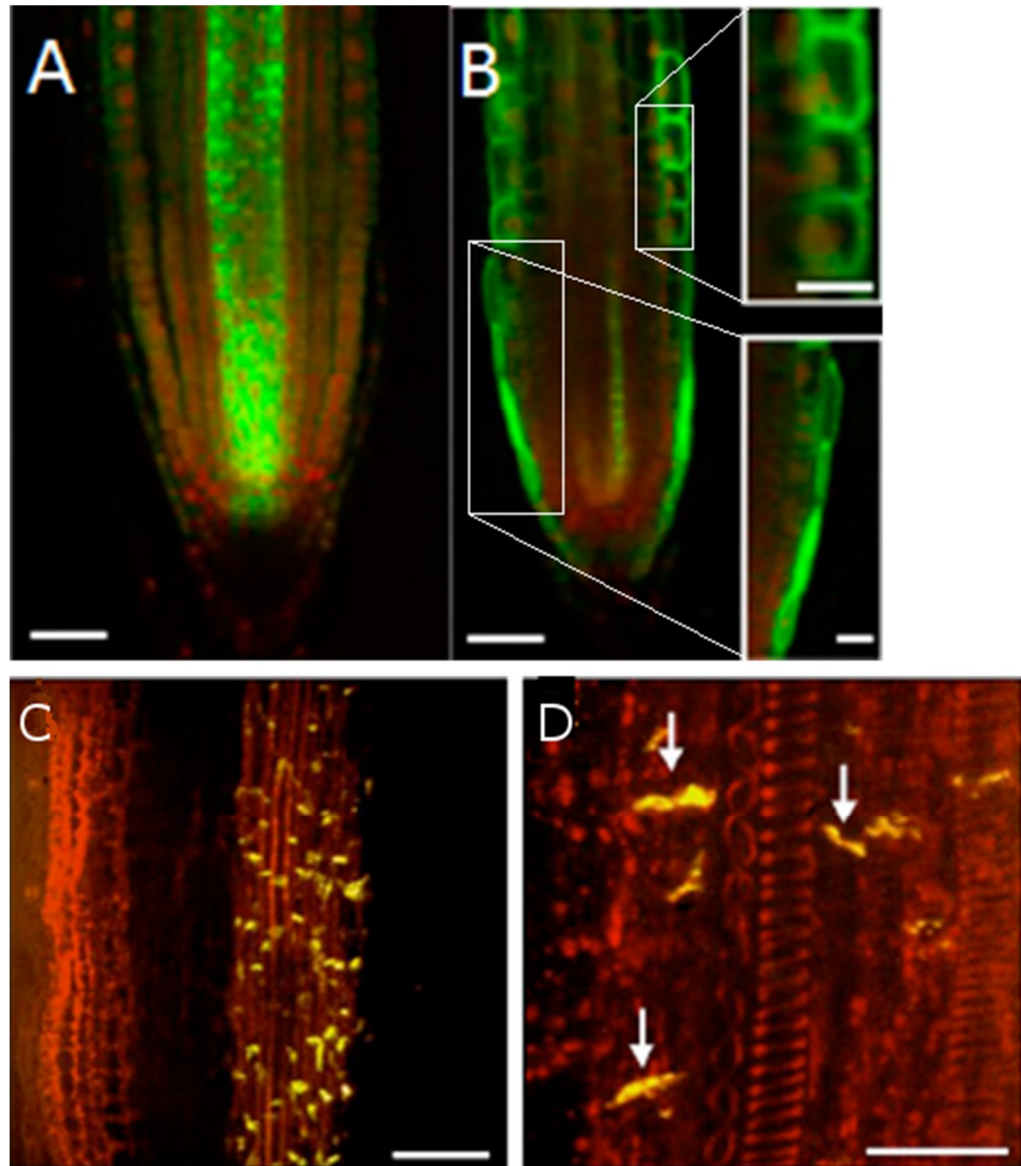


Figure 1.3 Images showing immune-localisation of putative auxin transporters in the Arabidopsis root tip. Non-redundant localisation of LAX2 (a) and AUX1 (b) can be seen, reflecting their different roles in regulating polar auxin transport and auxin homeostasis in the root tip. Polar localisation of PIN1 protein to the apical plasma membrane in the vascular bundle can be seen (c). Closer view of the image in (c) showing localisation of PIN1 protein to one face of the plasma membrane (d). Scale bars= 25μm. Taken from Peret et al., (2012) and Gaelweiler et al., (1998) respectively (a and b, c and d).

1.5.2 PIN proteins

PIN (PIN-FORMED) proteins exhibit polar sub-cellular localisation at the plasma membrane, whereby a particular PIN protein may localise to the upper (shootward) or lower (rootward) side of a cell (figure 1.3c and d). This polar localisation facilitates vectorial efflux of auxin which enables the directionality of auxin flux to be coordinated through variation of localisation by different PINs (Wisniewska et al., 2006; figure 1.2b). PINs constitute a plant specific family whose members are expressed in defined tissue domains and contribute to specific developmental processes (Paponov et al., 2005). PIN1 was first identified in experiments exploring mutants which mimicked treatment with auxin transport inhibitor NPA (naphthylphthalamic acid; Galweiler et al., 1998). Petrasek et al. (2006) *et al.* used heterologous expression systems to quantify the PIN1, PIN2, PIN3, PIN4, PIN6 and PIN7-mediated radiolabelled NAA efflux. There are eight members in the PIN family (PIN1-8), which exhibit various localisation patterns and play a multitude of roles in growth and development. For example PIN2 is involved in mediating asymmetric auxin distribution within the root tip during gravitropic response (Rahman et al., 2010a). PIN1 has multiple developmental roles, with *pin1* mutants exhibiting phenotypes of disturbed shoot apical and inflorescence meristem organisation/phyllotaxis and organ separation in embryos (Vernoux et al., 2000). While the majority of the family members are localised to the plasma membrane and function in intercellular flux, it has been found that PIN5 and PIN8 are localised to

endoplasmic reticulum. Dal Bosco et al. (2012) and Mravec et al. (2009) show how ER-localisation of PIN5 and PIN8 are required for intracellular auxin homeostasis.

Plasma membrane localised PINs which are expressed in the root meristem are: PIN2, PIN3, PIN3, PIN7, and PIN1. These PINs are localised in such a way that they facilitate the polar rootward transport of auxin through the stele/ground tissue (PIN1, PIN2) and its reflux from the root tip (PIN3, PIN4, PIN7) shootward through the epidermis (PIN2) (Blilou et al., 2005; figure 1.2b).

1.5.3 ABC transporters

Some ABC (ATP-binding cassette) transporters, previously also referred to as PGP (p-glycoprotein) or MDR (multidrug resistance-like) transporters have been found to have roles in auxin transport. Four transporters, ABCB1, ABCB4, ABCB19 and ABCB21 have all been implicated in auxin transport and auxin related developmental processes in arabidopsis (Geisler, Aryal, di Donato, & Hao, 2017). Unlike PIN proteins, these ABC transporters do not appear to adopt a polar subcellular localisation in the vast majority of studies, suggesting they are involved in non-polar auxin efflux from plant cells. ABC transporters also differ from PIN and AUX/LAX transporters in that they require energy derived from ATP binding in order to drive transport (Szentpétery et al., 2004).

1.6 Ground tissue patterning and the SHORTROOT/SCARECROW/BIRD pathway

SHORTROOT (SHR) is a transcription factor which is expressed in the stele (Helariutta et al., 2000). However, SHR protein lacks a nuclear localisation signal and so is dependent upon interactions with other proteins which incorporate a nuclear localisation signal (Hirano et al., 2017). SHR protein accumulates in stele cells and then moves outwards in to endodermis where it activates SCARECROW (SCR) expression. SCR then acts to limit further SHR movement by interacting with it and promoting nuclear localisation, thus specifying endodermal cell fate (Cui et al., 2007; figure 1.4). Indeed, *shr* mutants lack an endodermis cell layer but retain a mutant cell layer with cortex identity (Helariutta et al., 2000). The SHR/SCR complex associates with another group of proteins, the INDETERMINATE DOMAIN (IDD) or BIRD proteins (Long et al., 2015; Welch et al., 2007). This is a diverse family of genes which is composed of a number of transcription factors involved in a number of developmental/morphological processes. Coelho (2017) review the phylogeny of this family and their roles in plant development including the specification of bundle sheath cells crucial to carbon concentration in C₄ monocots. In *Arabidopsis* roots the activity of a number of IDD proteins have been characterised as having a role in ground tissue patterning in the meristem. These are *JACKDAW* (*JKD*), *MAGPIE* (*MPG*), *BALDIBIS* (*BIB*), *NUTCRACKER* (*NUC*), *BLUEJAY* (*BLJ*), and *IMPERIAL EAGLE* (*IME*) (Long et al., 2015; Moreno-Risueno et al., 2015). The activity of BIRD genes is also involved in interactions with plant

hormones including auxin, GA, and ABA; Yoshida et al., (2014) show that IDD protein interact with DELLA proteins to orchestrate gene expression downstream of GA signalling, promoting expression of SCARECROW-LIKE3 (*SCL3*).

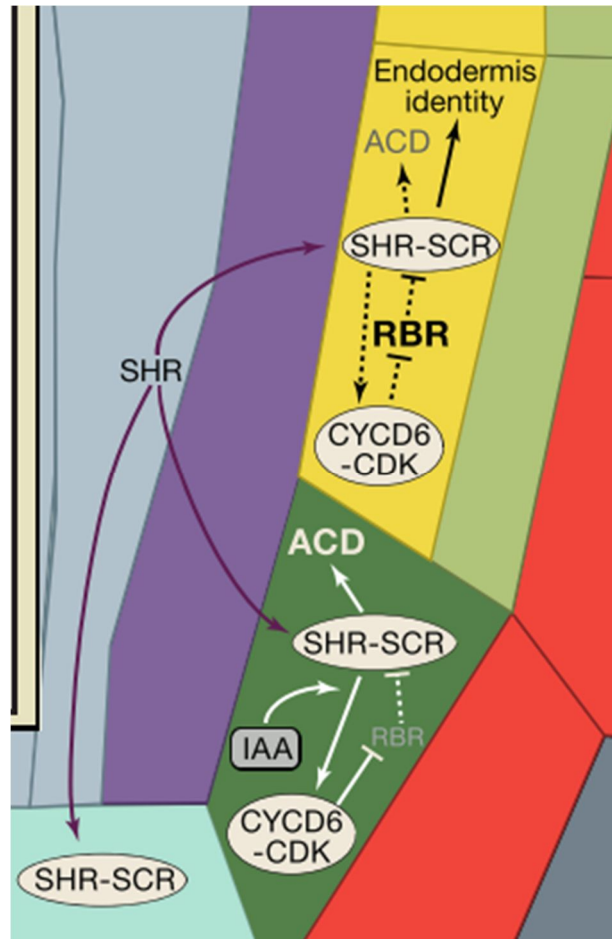


Figure 1.4 SHR moves from stele to endodermis to interact with SCR in ground tissue patterning

Schematic diagram showing the movement of SHR from the stele (light blue) and its role in activating downstream targets involved in controlling ground tissue patterning and specification in the cortex/endodermis initial (dark green), endodermis (yellow), and cortex (light green). SHR moves from the stele to activate and associate with SCR in the endodermis. The SHR/SCR complex activates CYCD6, suppressing RBR which then enables the division to specify cortex and endodermis. IAA: indole acetic acid, SHR: SHORT-ROOT, SCR: SCARECROW, CYCD6: CYCLIN D6, CDK: CYCLIN DEPENDENT KINASE, RBR: RETINOBLASTOMA RELATED. Taken from Wilson et al., (2013)

This makes sense in the context of previous findings since Paquette & Benfey, (2005) found that the integration of GA signalling and SCR activity are involved in the maturation of ground tissue. Cui et al., (2013) found that some IDD proteins directly regulate the expression of some auxin related genes such as *YUCCA5* and *PIN1* and that their mutant alleles exhibited altered auxin response in above ground tissues and root tips. Overall these findings suggest IDD/BIRD genes are integral to anatomical patterning and development, and that part of this is via the modulation of hormone signalling pathways.

Out of the BIRD genes, one particularly interesting candidate in the context of phosphorus deprivation is JACKDAW. Welch et al., (2007) investigated the role which JACKDAW plays in regulating SHORTROOT action and showed how its mutant phenotype exhibits an increase in cortex cell file number in roots. In addition to its extra cortex files, the *jkd* mutant also develops an extra layer of endodermis, indicative of its role in restricting SHR movement. This phenotype was enhanced in the *jkd/scr* double mutant which exhibited a greater number of cortex files but lacked an endodermis layer. Long et al., (2015) later probed the function of BIRD genes and their interactions with SHR and SCR in more detail and came to similar findings. Another BIRD gene, MGP has been shown previously to suppress the *jkd* mutant phenotype (Welch et al., 2007); Long et al., (2015) investigated this further and in conjunction with NUC. They found that the *jkd-mgp-nuc* triple mutant resembled the *scr* mutant in its phenotype in that it displayed defects in division of ground tissue layers. These undivided cell files, however, exhibited endodermal identity which was indicated by the presence of a Casparian strip (Long et al., (2015).

1.7 Ground tissue maturation

The post-germination pattern of primary root anatomy is maintained for around a week, however after this time the root begins to mature and ground tissue maturation begins to occur. The first change which is seen is the emergence of middle cortex tissue. Middle cortex forms from periclinal divisions of the endodermis, yielding a new layer of cortex tissue between the endodermis and the existing cortex (Baum, 2002; dotted yellow in figures 1a and 1b). Paquette & Benfey (2005) show how ground tissue maturation occurs as periclinal division of endodermal cells in the meristem zone, and that this process is dependent upon *SCR* activity. ABA and GA signalling has also been shown to play a role in regulating the decision to form middle cortex (Lee et al., 2016). Interestingly Baum et al., (2002) note that the first middle cortex divisions occur in endodermal cells adjacent to the xylem axis, which can be seen in figure 1.1a.

1.8 Epidermal cell patterning

As mentioned above, *arabidopsis* exhibits an epidermal patterning mechanism whereby root hair-bearing cell files in the epidermis are specified according to their position in relation to the underlying cortex, known as type 3 root-hair patterning (Pemberton, *et al.* 2001). This process is regulated by the leucine-

rich receptor kinase-like protein, SCRAMBLED (SCM), which resides on the anticlinal walls of epidermal cells facing the adjacent walls of underlying cortex cells. A cortex cell file-cleft specific signal, which is not yet identified, interacts with SCM in order to convey positional information (Kwak & Schiefelbein, 2008). Within the epidermis, a negative feedback mechanism functions to specify positions of root hair cells in the lateral dimension (i.e. between epidermal cells). This mechanism has come to light through the discovery of mutants such as *werewolf* (*wer*) (Ryu et al., 2005), *transparent testa glabra* (*ttg*) (Bernhardt, 2003), and *glabra2* (*gl2*) (Masucci et al., 1996) which exhibit a hairy root phenotype with cells at non-hair positions producing root hairs. Mutants of the antagonistic factors of the latter genes such as *caprice* (*cpc*) (Wada, et al. 1997) and *tryptichon* (*try*) (Schellmann et al., 2002) have a hairless root phenotype. This led to the finding that an antagonistic relationship works between these factors to result in a steady state whereby some cells take on trichoblast identity and some take on atrichoblast identity (Lee & Schiefelbein, 2002; Song et al., 2011). In essence, this mechanism functions whereby the WER/TTG/GL3/EGL3 complex promotes GL2 which blocks trichoblast identity. However, this complex also activates CPC/TRY/ETC which moves to neighbouring cells and inhibits WER activity. WER negatively regulates CPC/TRY/ETC protein complex, which negatively feeds back on this loop. This results in a situation where when WER activity overcomes the activity of CPC/TRY/ETC from the neighbouring cells, it will adopt trichoblast identity. Strong WER activity in trichoblast cells then promotes CPC/TRY/ETC movement to neighbouring cells which strongly blocks trichoblast identity causing them to

adopt atrichoblast identity (figure 1.5). Kwak & Schiefelbein, (2007) showed that by itself this mechanism does result in a statistically significant likelihood for epidermal cells to pattern themselves in an alternating hair-non-hair manner. However, this mechanism by itself ignores the position of the epidermal cells in the context of underlying cortex cells. They found that SCM is required for the formation of contiguous files of trichoblasts and non-trichoblasts where trichoblast files sit in the cleft between two cortex cells. They also showed that SCM does this by inhibiting *WER* expression in cells in the trichoblast position, thus biasing the balance of the feedback mechanism to define trichoblast identity in this position.

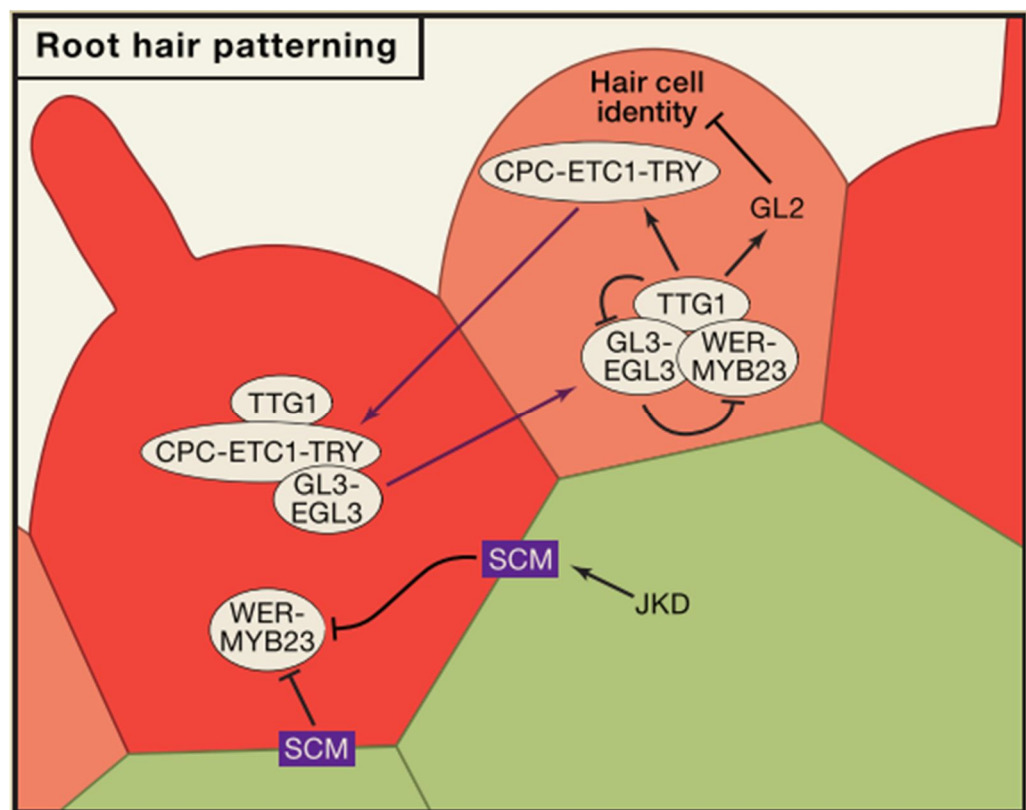


Figure 1.5 SCM provides a positional cue for determining trichoblast cell fate.

Schematic diagram showing the interactions between factors involved in specifying endodermal cell fates. SCM suppresses WER which acts to repress hair cell fate via intercellular movement and association with a complex to promote GL2. CPC: CAPRICE, ETC1: ENHANCER OF TRYPTICHON1, TRY: TRYPTICHON, TTG1: TESTA GLABRA1, GL2: GLABRA2, GL3: GLABRA3, EGL3: ENHANCER OF GLABRA3, WER: WEREWOLF, SCM: SCRAMBLED, JKD: JACKDAW. Taken from Wilson et al., (2013).

1.9 Nutrient requirements of plants

Plants require an array of inorganic mineral nutrients for basic metabolic, structural and biochemical process which are required for life and to thrive in a competitive environment. These nutrients must be mined from the surrounding soil in which the plants are rooted and can only be reached from within the limits of the root system (or mycorrhizal system, the network of hyphae of symbiotic fungi associated with roots; Laplaze, *et al.*, 2015). These nutrients are available in varying quantities depending on the quality and type of soil; in addition this may also be affected by the chemical properties of the minerals and the soil itself.

In general, plants require some elements in higher quantities (macro-nutrients) and others in lower quantities. Macro nutrients include nitrogen (N), potassium (K), sulphur (S), magnesium (Mg), calcium (Ca), and phosphorus (P)- elements which are all required for larger scale processes including amino acid/nucleic acid biosynthesis, signalling, osmotic homeostasis, and energy cycling.

Nitrogen composes 1-5% of plant dry matter (Jämtgård *et al.*, 2010), acquired mostly in the form of nitrate or ammonium, amino acids present in the soil can also be utilised by plants (Jämtgård, *et al.* 2010). Plants which are capable of forming symbiotic relationships with nitrogen-fixing bacteria are able to harness atmospheric N in a more direct manner, primarily via rhizobial symbioses. Legumes (Fabaceae) are the most well studied example of this, and house nitrogen fixing bacteria, known as rhizobia, in nodular root structures which are initiated by signalling between the plant and bacteria (reviewed in

Laplaze, *et al.* 2015). N is essential for the manufacture of nucleic acids, amino acids, chlorophyll, phytohormones and other molecules crucial to plant function.

K⁺ is the most abundant cation in plant cells, and is found in concentrations around 100mM in non-deficient cells. Rapid fluxes in cell K⁺ concentration lead to a sharp change in osmotic potential; in specialised cells this causes deformation of the cell, an important process in stomatal closure, which occurs via K⁺ channels such as KAT1 in arabidopsis (Corratgé-Faillie *et al.*, 2017) and KZM2 in maize (*Zea mays*; Gao *et al.* 2017).

Micro nutrients are required in lower concentrations. This array of nutrients is variable and accounts for numerous different functions within plants. Micro-nutrients include zinc, boron, manganese, copper and even some specific minerals in very trace amounts such as selenium and arsenic.

1.10 Plant phosphorus requirement

Phosphorus is a crucial macronutrient for plants, playing a critical role in many cellular signalling and energy cycling processes as well as forming part of important biological molecules such as phospholipids and the phospho-sugar backbone of deoxyribo- and ribonucleotides. Applied phosphates are relied on heavily as a fertiliser input in agricultural systems to maintain optimal plant health, providing 50-60% of required phosphorus; estimates of global application rates are around 15 million tonnes per year (Smil, 2000). UK application of phosphorus to arable land and use in animal feed is 86,000t per year (Cooper & Carliell-Marquet, 2013). However, the addition of phosphorus

fertilisers to soil has an adverse effect on surrounding soils and waterways as a result of run-off from agricultural land. Phosphorus run-off represents the primary cause of eutrophication with 2-3t per million people per day entering UK watercourses (Baker et al. 2015; Kato *et al.* 2007).

Additionally, phosphorus is primarily sourced from rock phosphate which is mined from finite sources. In 2010, 176 million tonnes of rock phosphate were extracted from natural reserves- the demand for rock phosphate is expected to reach its peak by 2030 (Baker et al., 2015; Cordell, Drangert, & White, 2009) and resources could be depleted in 50-200 years (Baker et al. 2015; Rosmarin *et al.* 2011).

Phosphorus concentration in replete plant cells is generally at low millimolar concentrations, suggesting that this is the physiologically optimal phosphorus concentration (Baker et al., 2015). However, the phosphorus concentrations in soil are usually at the low micromolar level and exist in a variety of forms including those which cannot be acquired by plants. This is because at low pH, phosphorus precipitates in the presence of oxides and hydroxides of iron and with calcium at higher pH (Lin et al. 2014; Raghothama, 1999). Intensive plant growth also has a strong negative impact on the concentration of usable phosphorus in soils, meaning that low concentrations can become a serious problem for growth on agricultural land (Baker et al., 2015; Guo, *et al.* 2000). Thus plants must be able to detect and respond to environmental phosphorus concentrations in order to take up and assimilate it effectively.

1.11 Sensing of phosphorus by plants

Sensing of nutrient availability is a crucial step in the adaptations of the plant to this. The sensing mechanisms of some nutrients are relatively well understood, for example Gifford, *et al.* (2008) show that nitrate responsive *ARF8* induction is sensitive to glutamine/glutamate metabolism which is at an early stage in the nitrogen assimilation process. Blocking glutamine synthesis blocked *ARF8* induction altogether, suggesting that this response results from N sensing in the form of downstream metabolites rather than inorganic nitrate. However, the exact mechanisms which plants use to detect P have not been fully elucidated. Despite the exact pathways not being clear, one nitrate transporter, now dubbed a nitrate transceptor, NRT1.1 has been identified as being able to sense N (Li *et al.*, 2017). Despite this, it is known that plants are able to detect the concentrations of phosphorus present at local and systemic scales, i.e. in their immediate soil environment and internal, physiological concentrations. This information can then be relayed through signalling mechanisms to orchestrate the biochemical, physiological and anatomical/architectural adaptations required to enhance its uptake and availability to them. Different phosphorus use/uptake efficiency responses are mediated according to whether the deficiency is detected on a local or systemic scale (Chiou & Lin, 2011). This makes sense as it is important for a plant to respond differentially to potentially short term starvation and more long term depletion of phosphorus before it starts changing metabolism and remobilising

resources. Some factors have previously been shown to act as long distance signals in phosphate stress; for example microRNA399 is expressed under low P and moves through phloem from shoot to root to degrade transcripts of PHO2. PHO2 is an E3 ubiquitin ligase which acts by degrading PHR1 (which promotes phosphate deficiency responses) (José Manuel Franco-Zorrilla et al., 2007). Thibaud et al. (2010) investigated the transcriptomic responses of plants to local and systemic Pi starvation and how they differ. The researchers used a split root assay in which half of a root system was exposed to low Pi (0Pi) and the other to high Pi (500 μ M Pi). The authors then categorised genes as responding to local stress (different in low compared to high sides of the root system) or systemic stress (different in low or high Pi compared with roots grown completely in low or high Pi). Using this method they could distinguish between root tissues responding to Pi concentrations in the growth medium and those responding to signals originating from different parts of the plant. They found 301 genes repressed and 240 genes induced by low Pi and that most of these changes occurred in response to local starvation (73% and 83% respectively). They found that many of the differences in gene expression between systemic and local responses represented different facets of dealing with Pi deficiency. For example, they saw that genes involved in the synthesis of phospholipids were downregulated systemically; concomitant with previous reports of membrane remodelling under low Pi (Härtel, *et al.* 2000; Nakamura, 2013). Conversely, locally regulated genes included those which are associated with root/cell growth and cell wall modelling responses such as ethylene response factors and expansins. This is not surprising considering the ethylene

sensitive root hair responses which occur under Pi starvation (Zhang, *et al.*, 2003).

1.12 Growth and developmental responses to low phosphorus

Numerous studies have focussed on the effects which the availability of different nutrients have on root growth, architecture and anatomy. Under experimental conditions of controlled mineral deprivation, some nutrients appear to have distinctive effects on roots. For example nitrogen deficiency induces increased lateral root elongation compared with N replete conditions (López-Bucio, *et al.* 2003), whereas P deficiency leads to dramatic shortening of the primary root, increased lateral root density and longer, denser root hairs (López-Bucio *et al.*, 2002).

Phosphorus is available to plants in the form of phosphate salts in the soil whose low solubility can be a limiting factor to their uptake by plant roots (Raghothama, 1999; Richardson *et al.* 2009). Under phosphate limiting conditions plants make various adaptations in order to enhance their phosphorus uptake and use efficiency. These include altered expression of phosphate transporters, remobilisation of reserves from older to younger tissues (Remy *et al.*, 2012), secretion of organic acids (H. Liao *et al.*, 2006) and changes to their root growth, architecture and anatomy. These include changes to gross root architecture but also alterations in radial anatomy and cell fate patterning within the root and on the epidermis.

Some plant responses to low phosphorus are well documented; in particular it is known that root hair length/density and lateral root density increase, which occurs along with a reduction in primary root growth rate (Bates & Lynch, 1996; Ma, *et al.* 2003). Increasing root hair density and elongation has been shown to enhance phosphorus uptake and provide a competitive advantage in phosphorus limiting conditions (Bates & Lynch, 2000a, 2000b, 2001).

With regard to increased root hair density, there are three main mechanisms by which this can occur. These are: an increase in the number of trichoblast cell files in the epidermis, a decrease in the length of trichoblasts in the longitudinal section, and an increase in the number of trichoblast position cells forming root hairs. Another mechanism by which root hair density can be increased is by the formation of ectopic root hairs; i.e. the formation of root hairs from cells that would otherwise be atrichoblast cells. However, the latter mechanism has not so far been seen in wild type *Arabidopsis* in limiting or non-limiting phosphorus conditions (Ma *et al.*, 2001; Zhang *et al.*, 2003).

It has also been identified that low phosphorus affects the radial patterning of primary root tissues and this alteration in radial patterning may be an adaptation to enhance radial root hair density by increasing the number of H position epidermal cells. Zhang *et al.* (2003) show that under low phosphorus conditions, the number of cortex cell files in the *Arabidopsis* primary root increases from 8 to around 16. They propose that this is a mechanism to increase the number of epidermal cells which occur in the H position and by doing so increase radial density of trichoblasts. Indeed, they demonstrate that the average number of hair position epidermal cells is increased from 8 to 16.4

in low phosphorus grown plants and that the theoretical and observed root hair densities are approximately tripled. However, the molecular mechanisms which govern this anatomical change are not understood. Zhang et al., (2003) showed that the auxin transporter, PIN2 is required for this process but how this protein might be responsible for orchestrating the change and what other factors are involved will require more work to be uncovered.

1.13 Molecular mechanisms regulating anatomical responses to low P

Only a handful of studies have investigated the regulatory mechanisms underpinning this adaptive response. Cederholm & Benfey (2015) studied the role which root growth factors (RGFs) play in the regulation of cortex proliferation. Cederholm & Benfey, (2015) demonstrated distinct roles for RGF1 and RGF2 in regulating root responses to low phosphorus in arabidopsis. Using high-resolution transcript profiling, the authors identified a suite of genes whose expression is changed under phosphorus stress and focussed on the role of the RGFs. They show that RGF1 and RGF2 transcripts are increasingly induced in the cortex with increasing time post transfer from high to low P (24-48 hours), indicating that they only start to become active in this cell type in response to low Pi (0Pi). In mutant studies they found that RGF2, along with another signalling peptide, RGF3, are required for regulating root growth under low Pi which is severely reduced under low Pi in the *rgf1rgf2rgf3* triple mutant. This phenotype could not be rescued by exogenous application of recombinant RGF1 peptide. Together these suggest separate roles for RGF1 and RGF2 in the

low phosphorus response. Indeed they argue that *rgf1* mutants exhibit hypersensitivity to low Pi with regard to the number of cortex cells, which is then returned to wild-type levels upon supplementation with 10nM RGF1 peptide. However, they define 'hypersensitive' as a ratio of cortex cells compared to wildtype of 1.2 under low P. This most likely equates to approximately 10-11 radial cortex files which is normal or even slightly less for wild type low P response. In fact, they show that *rgf2* mutants exhibit a decrease in the number of cortex cell files under low phosphorus which might hint at a role for this peptide. Either way, the expression patterns and responses of these mutants for these peptides certainly seem to suggest a role for them in regulating cortex divisions under low phosphorus conditions.

Although there is limited published work on cortex cell patterning in Pi starvation response there is some insight in to some mechanisms which may be involved in radial patterning of ground tissue in arabidopsis. One group of proteins which seems particularly important in this area of radial patterning is SHOTROOT (SHR) and its movement limiting regulators SCARECROW (SCR) and the BIRD proteins JACKDAW (JKD), MAGPIE (MGP), NUTCRACKER (NUC), and BALDIBIS (BIB). Even though no work has focussed on the potential role of BIRD genes in regulating radial anatomy adaptations to low P, Ticconi et al., (2009) showed that there are interactions between SCR, SHR and a P₅-Type ATPase PDR2 during phosphorus deprivation (0Pi). They demonstrate how PDR2 is required for maintaining meristem activity in phosphorus starved roots. PDR2 appears to be required to maintain SHR and SCR activity after 2 days post-

transfer to low P media, with mutants exhibiting SHR and SCR in extra cell layers when compared with high P (0.5 x MS) grown plants. However, this experiment may need to be approached with some caution since most other studies look at low P response later than this (i.e. 9 days or more on low P or 5 days or more post transfer) (Cederholm & Benfey, 2015; Zhong Ma et al., 2003; Ma, *et al.* 2001; Zhang et al., 2003). Thus it could be that *pdr2* plants are showing an accelerated response or sensitivity to P deprivation, which therefore might cast doubt on the role of PDR2 in stabilising SHR and SCR under P deprivation. Additionally this further raises the question of what SHR and SCR activity might look like in WT Columbia after a longer period of P deprivation. However, even though the effect of PDR2 in this respect is questionable, its role in phosphate availability perception and response is certainly interesting and may represent a negative regulator in low P response.

1.14 Other transcriptional regulators of low P response

As well as signalling peptides and transcription factors directly related to meristem activity and differentiation, there is evidence that WRKY transcription factors are also involved in activating adaptations to low phosphorus in arabidopsis (Devaiah, *et al.* 2007; Devaiah & Raghothama, 2007; Wang et al., 2014). These studies investigate the role of WRKY75 and WRKY45 which are expressed differentially under low phosphorus stress and regulate the expression of a number of phosphorus acquisition related genes. Wang et al., (2014) showed that the transcription factor WRKY45 was upregulated during

low phosphorus stress and is active in the plants' adaption to P starvation. They found that WRKY45 upregulation was associated with an upregulation of PHOSPHATE TRANSPORTER1-1 (PHT1-1), a plasma membrane Pi transporter which is active in Pi uptake and that WRKY45 binds to motifs in this transporter's promoter, implying direct regulation. They demonstrated that WRKY45 indirectly mediates Pi uptake by measuring Pi uptake in WRKY45 over-expression and knock-out lines, finding that Pi over- and under-accumulated in these lines respectively. Similarly to WRKY45, Devaiah et al. (2007) demonstrate how WRKY75 is induced by low Pi stress and is responsible for activating the expression of phosphate transporters and several other phosphate responsive factors. They report that *WRKY75* RNAi lines show hypersensitivity to low Pi, and have an altered root architecture compared with wild type plants.

A major transcription factor identified as having an influential role on low phosphorus responses is the MYB-like transcription factor PHR1 which appears to mediate a great number of low Pi induced responses. PHR1 protein interacts with the PHR1-binding site motif (GNATATNC) which is upstream of a number of low Pi associated genes such as *PHOSPHATE TRANSPORTER1* (*PHT1*) and *PHO1*, which encodes a membrane protein involved in loading Pi in to root xylem (Chiou & Lin, 2011; Rubio et al., 2001). PHR1 has also been shown to regulate and interact with two other transcriptional regulators SPX1 and SPX2 according to Pi status. This mechanism is crucial to the low Pi response since *PHR1* expression levels do not seem to be affected by Pi status and thus its activity must be mediated post-transcriptionally by a Pi dependent interaction

(Isabel et al., 2014). The importance of post-translational regulation of PHR1 is further highlighted by the activity of SIZ1, a SUMO E3 ligase which has been shown to mediate the degradation of PHR1. Under P replete conditions, this acts to block the P-stress responses induced by PHR1. *siz1* mutants display a constitutive low P phenotype including a shortened primary root and increased lateral root density (Miura et al., 2005).

1.15 Hormonal regulation of low P response

As previously mentioned, PIN2 has already been shown to be important for the radial patterning responses to low phosphorus in arabidopsis. Clearly this suggests that auxin transport and response in the root is critical to signalling low phosphorus stress and initiating changes in cell division and tissue patterning in the root meristem. One of the most notable responses to low phosphorus is an increase in root hair density and length, and it has been previously suggested that ethylene could play a role in regulating position dependent trichoblast differentiation (Michael, 2001; Tanimoto, *et al.* 1995). Zhang et al. (2003) investigated the roles which auxin and ethylene have on root hair patterning under low phosphorus in arabidopsis. They studied the responses of ethylene and auxin signalling mutants to low (1uM) and high (1000um) phosphorus conditions. This study demonstrated how manipulating ethylene signalling had a large effect on root hair density by treating plants with 1-amino-cyclopropane-1-carboxylate (ACC; an immediate ethylene precursor) and ethylene inhibitors aminoethoxyvinylglycine (AVG), 1-methylcyclopropene (MCP) and silver thiosulphate (STS). The study separated the roles of auxin and

ethylene in the cortical and epidermal responses respectively; it was shown that ethylene signalling components are required for increases in root hair density and length under low phosphorus. However, this increase in root hair density was primarily due to a decrease in trichoblast length and an increase in the number of trichoblasts forming hairs (24% in high P (1000uM) to 90% in low P (1uM; Ma et al. 2001), but had no influence on cortex cell number. This was replicated on high P media by ACC treatment which also promoted root hair length, density and ectopic root hair formation which was inhibited by AVG on low P media.

Ma et al., (2001) revealed a complex cross talk between low phosphorus and ethylene signalling in root hair traits; the researchers found that most ethylene mutants had lower root hair density and shorter root hairs at low and high P. The ratio of root hair density and length at low P to high P was found to be lower in all ethylene mutants and *eir1* (a mutant allele of *PIN2*) when compared with Columbia. However, interestingly total root hair length was higher in *ein6* than Columbia; this could suggest a complex signalling mechanism, although the figure is from only six observations and the mean Columbia value is within the error of the *ein6* values. This suggests that ethylene may be important for root hair initiation but is more important for increasing density and elongation under low P than in non-limiting conditions. Interestingly, *eir1* had the strongest effect on the reduction in root hair density and length out of all the mutants studied. This either suggests that auxin flux in the cortex and epidermis directly influences root hair traits at the epidermal scale or that it is mediated by auxin-ethylene cross-talk wherein auxin flux is crucial. Indeed interactions

between auxin and ethylene have been shown to be important for root hair elongation (Pitts, *et al.*1998; Strader *et al.* 2010). While the above work demonstrated that ethylene signal transduction and auxin transport are important for root hair traits under low P, they showed that ethylene signalling is not required for increasing cortex cell number and H-position epidermal cells. The only mutant which exhibited a decrease in the cortex proliferation response to low P was *eir1*, suggesting that auxin is important for this response. Lucas *et al.*, (2011) demonstrated that as part of the function of SHR in maintaining root indeterminacy and the stem cell niche, it appears to have a regulatory influence on several of the PIN proteins including PIN2. The researchers tracked the localisation of PIN1, PIN2, PIN3, PIN4 and PIN7 over time in wild type and *shr* mutants. They found that the expression of all of these PINs was drastically reduced up to, and undetectable (by immunolocalisation) by 10 days of age. PIN2 also showed a change in localisation in the cortex; switching from an acropetal polarity to basipetal in the elongation zone. This implies that SHR is an important regulator of PINs, and in relation to phosphorus stress, may be an important factor in directing auxin flux via regulation of PIN2 and possibly other PINs.

As well as auxin and ethylene, there is some indication that cytokinin (CK) may also play a role in regulating Pi starvation responses. Several studies have suggested that a downregulation in cytokinin activity may be important in the response to low Pi. Franco-Zorrilla *et al.* (2005) show that CK signal reception genes are downregulated under low phosphorus and that some low Pi responses were enhanced in CK signalling mutants. Franco-Zorrilla *et al.* (2002)

similarly showed that mutations in CRE1 cytokinin receptor suppressed cytokinin mediated down-regulation of Pi starvation responses. Additionally, (Martin et al., 2000) showed that exogenous application of CK to phosphorus starved plants repressed the expression of genes related to phosphorus starvation response. These findings indicate that CK is likely to have a negative role in the responses to Pi starvation in arabidopsis.

1.16 Aims and objectives

In the context of the current literature, there are many gaps in our knowledge regarding the increased cortex cell file number response to low P in arabidopsis roots. Following the hypothesis that the cortex response to low P is important, this study aims to answer three main questions:

1. How does the increase in cortex cell files in arabidopsis roots in response to low P manifest spatially, temporally, and in relation to P concentration?
2. What regulatory factors are involved in/required for this response?
3. How do these factors function and interact to orchestrate this response?

Chapter 2 Materials and methods

2.1 Plant growth conditions

Plants used for all high and low P experiments were grown under 12 hours light, 12 hours dark conditions at 21°C in a growth chamber.

All seeds were sterilised with 5% sodium hypochlorite (VWR) for 5 minutes before being rinsed with 70% ethanol with 0.01% Triton X-100 (Sigma-Aldrich) once, then several times with sterile water. Seeds were then sown on agar plates, sealed with micropore tape (3M) and cold treated overnight to promote even germination.

Plants grown for propagation and selection were grown under 24 hour light conditions at 21°C in a growth chamber, or on Levington M3 compost in glasshouse conditions.

2.2 Plant growth media

Plates used for low P experiments were made using the following recipe for generating media with different concentrations of phosphate:

Nutrient	Low Pi high Fe	High Pi high Fe	Low Pi Low Fe	High Pi Low Fe
KNO ₃	3mM	3mM	3mM	3mM
Ca(NO ₃)H ₂ O	2mM	2mM	2mM	2mM
MgSO ₄ 7H ₂ O	0.5mM	0.5mM	0.5mM	0.5mM
(NH ₄) ₂ SO ₄	425µM	0	425µM	0
NH ₄ H ₂ PO ₄	50µM	1mM	50µM	1mM
H ₃ BO ₄	25µM	25µM	25µM	25µM
ZnNa EDTA	2µM	2µM	2µM	2µM
(NH ₄) ₆ Mo ₇ O ₂₄ 4H ₂ O	1µM	1µM	1µM	1µM
KCl	50µM	50µM	50µM	50µM
MnSO ₄ H ₂ O	2µM	2µM	2µM	2µM
CuSO ₄ 4H ₂ O	1µM	1µM	1µM	1µM
Fe-EDTA	50µM	50µM	5µM	5µM

0.5g/L of 2-(*N*-morpholino)ethanesulfonic acid (MES; Sigma Aldrich) was added and the pH of the media was adjusted to 5.7 using potassium hydroxide (KOH; Sigma Aldrich). Media was then added to bottles containing 8g agar/L and autoclaved before being used to pour plates.

All plates which were made for propagation of plants for seed production, genotyping, crossing or selection of transformants were made using Murashige and Skoog nutrient salts at half strength (2.2g/L). 0.5g/L MES was added and the media was adjusted to pH 5.7 using KOH and gelled using bacto agar at a strength of 1%. This media was then autoclaved and used to pour plates.

2.3 Pseudo-Schiff propidium iodide staining

Roots for PS-PI staining were harvested from the plate and fixed immediately in a 50% methanol 10% acetic acid aqueous solution and kept at 4°C overnight. Roots were then rinsed in water before being incubated at room temperature in 1% periodic acid for 40 minutes. Tissue was rinsed 2-3 times and then left in pseudo-Schiff PI solution for 1-2 hours (0.015N hydrochloric acid (HCl; VWR), 100mM sodium metabisulphite (ThermoFisher scientific); 50ul/ml propidium iodide (Sigma-Aldrich) solution [1mg/ml] freshly added each time). Roots were again rinsed 2-3 times with water before being mounted on slides with 40% glycerol (VWR) 30% chloral hydrate (Sigma-Aldrich) and left in the dark over night to clear.

2.4 Microscopy and image processing

Stained cleared roots were imaged using a Leica SP5 confocal microscope. A 488nm laser was used to excite PI fluorescence for imaging, PI fluorescence was collected using either a photomultiplier tube (PMT) or hybrid detector at between 600-800nm.

To image green fluorescent protein (GFP) and yellow fluorescent protein (YFP)/VENUS fluorescence, tissue was stained for 2-3 minutes using 5-10µl propidium iodide (PI)/ml in deionised H₂O before being rinsed in deionised H₂O. Roots were then mounted in deionised H₂O on glass slides under glass coverslips. Images were generated using a Leica SP5 or SP8 microscope. For GFP, 488nm laser emission was used for excitation and PMT or hybrid detectors collecting fluorescence emission between 500-550nm. For YFP/VENUS, 514nm laser emission was used for excitation and fluorescence emission was collected at 520-550nm.

For the generation of optical sections from pS-PI stained/cleared roots, Z-stacks of roots were generated using LAS-X Leica software then dissected in orthogonal view using ImageJ image analysis software. Cell counts were performed using a cell counter function in the same software.

Light-sheet microscopy was performed using a Zeiss lightsheet Z.1 and initial image processing performed using Zeiss ZEN software prior to final image analysis. All imaging utilised a 514nm laser used to excite YFP and propidium iodide. Root tips were excised from plants and kept upright whilst being stained in 5-10 μ l propidium iodide (PI)/ml in deionised H₂O and mounted in to a glass capillary using low melting temperature agarose (Sigma-Aldrich) mixed with fluorescent beads from the PS-Speck Microscope Point Source Kit (ThermoFisher Scientific). Roots were then imaged using the Multiview function in ZEN software.

Imagej was used to assemble multiview stacks in to a final stack using fluorescent beads as points of interest for registration in the multiview processing work-flow in imagej.

Fluorescence was measured using imageJ image analysis software based on pixel density which was then corrected for by calculating the intensity per unit area of a measured space. This technique makes up for the effect of concentration of fluorophores in a smaller space (i.e. smaller or larger nuclei, or the human error of selecting smaller or larger measurement areas) potentially indicating a weaker/stronger signal.

2.5 RNA extraction and cDNA synthesis

2-3mm root tips were excised from 14 day old primary roots and frozen in liquid nitrogen. Tissue was then ground whilst frozen before being further ground following the addition of 1ml Trizol reagent (Invitrogen). 200ul chloroform was added to each sample which was then briefly vortexed and centrifuged at 14000rpm in a microcentrifuge for 15 minutes. The aqueous layer from each sample was then removed and added to 500ul isopropanol and then RNA precipitated overnight at -20°C. Samples were microcentrifuged at 14000rpm in a microcentrifuge for 15 minutes and the resulting pellet was then aspirated. The pellet was washed with 70% ethanol and spun at 14000rpm again for 5 minutes before being aspirated and dried. Each pellet was then resuspended in 20ul deionised, autoclaved (DA) water.

~500ng each sample was then treated with DNase (ThermoFisher Scientific) to remove contaminating genomic DNA according to the manufacturer's instructions. RNA was quantified using a NanoDrop 2000 (ThermoFisher Scientific)

RNA was then used for reverse transcription with Super Script II reverse transcriptase kit (Roche) to produce cDNA. The following reaction was used for this step:

RNA	500ng
Riboblock RNase inhibitor	0.25µl
DNase	0.25µl
Buffer	1µl
DA water	made up to 9.75µl

RNA was incubated at 37°C for 30 minutes, then 1µl 50mM EDTA (ThermoFisher Scientific) was added and it was then incubated for a further 10 minutes at 65°C.

cDNA was then synthesised using RNA from the previous reaction:

RNA	100ng
(10mM) Oligodeoxythymidine (oligodT) 10mM	0.5µl
DA H ₂ O	make up to 6.5 µl

RNA was incubated at 65°C to anneal the oligodT to polyadenosine tails of mRNAs and then chilled on ice. Then the following was added:

Buffer (10x)	2µl
RNase inhibitor	0.25µl
(deoxyribonucleotide triphosphates) dNTPs (10mM)	1µl
Reverse transcriptase	0.25µl

The final reaction mix was then incubated at 50°C for 60 minutes followed by a deactivation step at 85°C for 5 minutes.

2.6 Quantitative-PCR

cDNA was then used to perform qPCR with Sensi-mix Hi-ROX q-PCR master mix (Roche) in a Light cycler 480 II qPCR machine (Roche) using the following reaction mix:

Per reaction:

2x HiRox SenSimix buffer	5µl
forward primer (10µM)	0.25µl
reverse primer (10µM)	0.25µl
AD H ₂ O	2.5µl

cDNA as synthesised above was dilute 20x and then 2µl added to each well: this large volume was added to dampen the effects of pipetting error.

2.7 Molecular cloning

PCR

All PCRs for cloning fragments from genomic DNA or copy DNA were performed as modifications of the following protocol:

Initial melting: 94°C 5min
Melting: 94°C 30sec
Annealing (xx) 30sec
Extension 72°C (30sec/500bp) x35
Final extension 72°C 7min

The annealing temperature was modified according to the predicted melting temperature of the primer-template dimer being used; the annealing temperature was set at 1-2°C above the predicted melting temperature in order to ensure high annealing fidelity.

The following reagents were used to perform each reaction:

5x PCR buffer (New England Biolabs)	10µl
forward primer (10µM)	1µl
reverse primer (10µM)	1µl
dNTPs (10mM)	1µl
Template DNA (<10ng)	1µl
Demineralised, autoclaved water	35.5µl
Phusion high fidelity DNA polymerase (New England Biolabs)	0.5µl

PIN2, *MAGPIE*, *BALDIBIS*, and *NUTCRACKER* sequences were all amplified from cDNA produced from RNA extracted from *Arabidopsis thaliana* Columbia root tips as described above. PCRs were performed using primers PIN2_GG_F1, PIN2_GG_R1, BIB_GG_1F, BIB_GG_1R, BIB_GG_2F, BIB_GG_2R, MGP_GG_1F, MGP_GG_1R, MGP_GG_2F, MGP_GG_2R, NUC_GG_1F, NUC_GG_1R, NUC_GG_2F, and NUC_GG_2R respectively. *axr3-1* cDNA was amplified using the following primers: axr3_1F, axr3_1R, axr3_2F, axr3_2R. All

of the previous amplifications, apart from PIN2, required two sets of primers in order to generate two fragments for removing an internal BsaI site from each of these sequences. This was necessary as the Greengate process is dependent upon BsaI restriction at specific end locations.

The *AUX1* promoter was amplified from *Arabidopsis thaliana* Col. 0 genomic DNA using proAUX1_F and proAUX1_R primers. Cloned fragments were then digested with BsaI and ligated in to pGGA001 (for promoters) or pGGC001 (for coding sequences) plasmids as described above. These were then used to construct expression plasmids as described above.

2.8 Gel electrophoresis and purification

Samples were loaded in to 0.7% agarose gels made with 1x Tris borate EDTA buffer (89mM Tris, 89mM boric acid, 2mM EDTA; VWR) with 1ul ethidium bromide (Sigma-Aldrich) 10mg/ml solution per 50ml gel. Samples were loaded with loading buffer up to 1x concentration according to manufacturer's guidelines (New England Biolabs) Band sizes were measured by running each gel with either 1kb DNA ladder or 100bp DNA ladder (New England Biolabs).

Samples were then run on a gel at 80-100v for 45-90 minutes depending on predicted fragment size. Gels were imaged and photographed using ultra-violet illumination.

Fragments of interest were then excised under ultraviolet illumination using a razor blade. Then DNA extracted using a gel DNA extraction kit (ThermoFisher scientific) and eluting in to 50µl deionised, autoclaved water.

2.9 Restriction digestion

Diagnostic restriction digests were performed according to the manufacturer's directions regarding the use of buffers and incubation times/temperatures. Restriction enzymes and appropriate buffers were sourced from either New England Biolabs or Promega. Reactions were performed as the following but with the appropriate modifications according to temperature/buffer:

Buffer (10x)	2.5µl
DNA (100ng)	
Enzyme	0.5µl
Deionised, autoclaved H ₂ O	21µl

This mix was then incubated at 37°C for 1 hour, followed by a treatment at 85°C for 10 minutes to deactivate the enzyme.

The reaction was then run on a gel as described above and the size of resulting fragments measured according to 1kb DNA ladder (New England Biolabs).

2.10 Assembly of Green Gate entry module plasmids

Fragments of cloned regions were amplified as described in section 2.7.

These were then purified from the agarose gel which was used to separate the PCR product and then digested with BsaI as described above along with the required vector plasmid.

The restriction digestion reaction was then treated with a PCR clean up kit (Qiagen) and eluted in 25µl.

To this 3µl 10x T4 DNA ligase buffer (with ATP; New England Biolabs), 1µl deionised, autoclaved H₂O, and 1µl T4 DNA ligase (New England Biolabs) were added. This was then incubated at room temperature for one hour followed by a 10 minute deactivation step at 70°C

2.11 Greengate destination reactions

The following reaction was used the same way for each assembly for the generation of pCo2:MGP:GFP, pCo2:NUC:GFP, pCo2:BIB:GFP, pAUX1:axr3, pCo2:axr3, pAUX1:PIN2, and pCo2:PIN2:

Entry modules (A-F)	1.5µl (should be around 100ng/µl)
Destination vector (pGGZ001)	1µl
CutSmart buffer (New England Biolabs)	1.5µl
10mM ATP (New England Biolabs)	1.5µl
NEB Greengate mix (New England Biolabs)	1µl

The following programme was used in a PCR machine for the reactions:

37°C	2min	x30
16°C	2min	x30
50°C	5min	
80°C	5min	

Greengate constructs were transformed into bacteria using the procedures in section 2.12.

2.12 Bacterial Transformation and selection

Transformation and selection

Bacterial transformation was performed by adding 10µl ligation reaction or <5ng plasmid DNA to competent DH5α *Escherichia coli* cells and chilled on ice for 15 minutes. Cells were then heat shocked at 42°C for 90 seconds before being chilled on ice again for 5 minutes. 1ml LB media was added and then cells were incubated at 37°C for 1 hour.

Following incubation of transferred cells, 50µl of the transformation was spread on to a circular plate containing LB media gelled with 1% agar. The cell suspension was then

centrifuged for 5 minutes at 2500rpm in a bench top centrifuge. Following this, the supernatant was removed and the remaining bacteria spread on to another LB plate. For selection of transformed bacteria, the following antibiotic concentrations were used:

Ampicilin (VWR): 100µg/ml, Spectinomycin (Sigma-Aldrich): 100µg/ml, Tetracycline (VWR): 2µg/ml, Rifampicin (VWR): 50µg/ml, Gentamycin (Sigma-Aldrich): 50µg/ml

Plasmid purification

Following the selection of transformed bacterial colonies, positive colonies were picked and added to 5ml liquid LB with antibiotic. This culture was then incubated in a shaking incubator at 37°C overnight. Cultures were then pelleted by centrifuging at ~3500rpm for 5 minutes in a bench-top centrifuge and then lysed and plasmids purified using the GeneJET plasmid purification kit and protocol (ThermoFisher Scientific)

Agrobacterium transformation

10ng plasmid DNA was added to electro-competent *Agrobacterium tumefaciens* cells which were transformed by electroporation using cold electroporation cuvettes which had been stored on ice for at least 20 minutes prior to transformation. After electroporation, the bacteria were added to 1ml LB and incubated at 28°C for 60 minutes. 1/10 of the incubated suspension was spread on an LB agar plate containing the appropriate antibiotics.

All Agrobacteria which were used carried plasmids harbouring resistance genes for gentamycin, rifampicin, and tetracycline. All of the constructs made were in the pGGZ001 backbone which has a bacterial spectinomycin resistance cassette. Therefore for all work using antibiotic selection of *A. tumefaciens* gentamycin, rifampicin, tetracycline and spectinomycin were used at the concentrations specified above.

The plates were then incubated at 28°C for 36-48 hours until visible colonies had grown.

Colonies were then picked and added to a 5ml selective culture and grown over night in a shaking incubator at 28°C.

2.13 Plant transformation

5ml *Agrobacterium* cultures were used to inoculate 250ml of LB with appropriate antibiotics which was grown in a shaking incubator at 28°C overnight. The next day transformation buffer was freshly prepared using DA water (50g sucrose per litre, 0.74g MgCl₂ per litre). *Agrobacterium* cultures were then centrifuged at 8000g for 10 minutes at 4°C. Supernatant was decanted and the pellet resuspended in 250ml transformation buffer. Just before dipping plants, 50µl SILWET® L-77 was added per 250ml suspended *Agrobacterium*.

Plants (either Col 0 or *pin2*) were grown three to a pot and 2-3 pots used per construct.

When the plants had begun to flower and produced several mature inflorescences with a number of open flowers they were selected for dipping. Dipping transformation suspensions were prepared as above and the flowers of the plants completely submerged in this suspension for 2 minutes. After dipping, plants were placed into plastic sleeves left closed for one day and then opened to allow air flow. Seeds were harvested when siliques were all visibly dried.

Transformants were selected by growing on 1% agar media with ½ MS salts containing 50ng/ml kanamycin. Surviving plants were grown on and transformants.

2.14 Plant materials

All transgenic plant materials used in this project were generated using plasmids constructed using the green gate method as reported by Lampropoulos et al., (2013).

BIRD gene, *SHR*, *SCR* and *CYCD6* reporter lines were kindly donated by Dr Ikram Blilou, all are in the *Arabidopsis thaliana* Columbia background (Long *et al.* 2015).

The *jkd-4* mutant was kindly donated by Dr Ikram Blilou (Welch *et al.*, 2007), mutants were genotyped using primers Lba, jkd4FW, and jkd4R.

The *mgp-i* was supplied by the Nottingham arabidopsis resource centre (NASC). The SALK identification number is SALK140490, it is in the Columbia background and was genotyped using primers Lba, SALK_140490 LP, and SALK_140490 RP (Long *et al.* 2015, Welch *et al.* 2007).

The *pin1-1* mutant has been described previously (Okada, *et al.* 1991; Jones *et al.* 2005)

The *pin3-4* mutant has been isolated from the SALK T-DNA insertion library SALK_038609 (Young *et al.* 2006).

The *shr-3* mutant is the result of a transposon insertion (Helariutta *et al.* 2000).

The *arf6-1*, *arf8-2*, single mutants and *arf6-1 ar8-2* double mutant were kindly provided by Jekaterina Truskina (Okushima *et al.* 2005).

The *arf19-3* is a loss of function T-DNA insertion line kindly provided by Dr Rahule Bhosale.

The *arf7-1* is a T-DNA insertion line (Okushima *et al.* 2005), kindly provided by Dr Rahule Bhosale.

The *aux1-7* was kindly provided by Dr Ranjan Swarup and is a point mutation (Bennett *et al.* 1996).

The *eir1-1* is a diepoxybutane induced point mutation allele of *pin2* (Luschnig *et al.* 1998)

The *lax2* and *lax3* mutants were kindly provided by Dr Ute Voß

The pPIN2:PIN2:GFP reporter line was kindly donated by Agata Gołębiowska. It is in the Columbia *eir1-1* background (Xu and Scheres, 2005).

2.15 Primers

Primer name	Sequence	Associated locus
CYCD6_Fwd	TTGGCCTTTTTCATTAGTCTCTTT	CYCD6 At4g03270
CYCD6_Rev	TGGTTTAAACTCGAGGAACTGA	CYCD6 At4g03270
BIB_Fwd	CCCTAACCTAATCCTACCTCCT	BIB At3G45260
BIB_Rev	CGATGACTTCTGCATCTGGA	BIB At3G45260
JKD_Fwd	GCGAAAACCTTGTTGGTACTCGT	JKD At5G03150
JKD_Rev	CGCAGAACGCTCTATGTGTG	JKD At5G03150
MGP_Fwd	GAATATAGATGCGACTGTGGTACAAT	MGP At1G03840
MGP_Rev	TGCTAAAGCGTCACAGAACG	MGP At1G03840
NUC_Fwd	AAATCTCCCTGGAAATCCTGA	NUC At5G44160
NUC_Rev	CCTTTGCCACATACCTCACA	NUC At5G44160

Table 2.1 List of RT-qPCR primers used to analyse transcript abundance of *CYCD6*, *BIB*, *JKD*, *MGP*, and *NUC*. Primers read from 5' to 3'.

Primer name	Sequence	Associated locus
proAUX1_F	AACAGGTCTCGACCTAGGTCGATGGTTATTCGATAAGCTTG	AUX1 At2g38120
proAUX1_R	ACCAGGTCTCGTGTGTTTTTTAGCTTCTAGATCTGAGAAATAAACAGAGC	AUX1 At2g38120
axr3_1F	TTTTGGTCTCCGGCTCACAGATATGATGGGCAGTGTCGAGCTGAATCTGAG	AXR3/IAA17 At1g04250
axr3_1R	TTTTGGTCTCTGGAGCGAGCCCAATGGCATCCG	AXR3/IAA17 At1g04250
axr3_2F	GCGTTTACGTCTCATGAAAGGATCGGATG	AXR3/IAA17 At1g04250
axr3_2R	TTTGGTCTCCCTGACAAAGCTCTGCTTTGCACTTCTCCATCG	AXR3/IAA17 At1g04250
PIN2_GG_F1	TTTTGGTCTCCGGCTATGATCACCGGCAAAGACATGTACG	PIN2 At5g57090
PIN2_GG_R1	TTTGGTCTCCCTGACAAAAGCCCCAAAAGAACGTAGTAGAGTAC	PIN2 At5g57090
JKD_GG_1F	TTTTGGTCTCCGGCTCACAGATATGCAGATGATTCCAGGAGATCC	JKD At5g03150
JKD_GG_1R	CACCGTGTGCGGCTGTAGTGTTC	JKD At5g03151
JKD_GG_2F	TTTTGGTCTCTCCTTGAGATCTCACTGGGATCAAGAAAC	JKD At5g03152

JKD_GG_2R	TTTGGTCTCCCTGACAAACCCAATGGAGCAAACCTTGCGAGTTC	JKD At5g03153
BIB_GG_1F	TTTTGGTCTCCGGCTCTAAACCAATATCAGCATGATGATGCCAGATGATC	BIB At3g45260
BIB_GG_1R	CTTTAGCTTCCATGGAAGGTTATGGCCTCTC	BIB At3g45260
BIB_GG_2F	TTTTGGTCTCTTAAGAGAGGCCAGAACCTTCAGCTTCACCGG	BIB At3g45260
BIB_GG_2R	TTTGGTCTCCCTGACAACTGGTTCATGTCGGCGGTTGGTGTGCCGTCC	BIB At3g45260
NUC_GG_1F	TTTTGGTCTCCGGCTGACATGACAAGTGAAGTTCTTCAAACAATCTCAAGTG	NUC At5g44160
NUC_GG_1R	CTTCATGGAAGATTATGTCCTCGCCGATG	NUC At5g44160
NUC_GG_2F	TTTTGGTCTCTCAAAGAGATCAAACTTACAGCTTCATCGG	NUC At5g44160
NUC_GG_2R	TTTTGGTCTCCCTGAAATCCATCCATTGATAGACGATGGATGGCAC	NUC At5g44160
MGP_GG_1F	TTTTGGTCTCCGGCTGAAGAAGACATGACAACTGAAGATCAGACAATTTCAAG	MGP At1g03840
MGP_GG_1R	GGTTGTGTCCTCGACGGTGAAGC	MGP At1g03840
MGP_GG_2F	TTTTGGTCTCTTCAAAGAGATCAGAATCTACAGCTTCACCG	MGP At1g03840
MGP_GG_2R	TTTGGTCTCCCTGACAAAATCCATCCATTGATAGAGGAAGAATGGCATATTGTTG	MGP At1g03840

Table 2.2 List of primers used for cloning of specific sequences for building constructs for plant transformation. Primers read from 5' to 3'.

Chapter 3: Anatomical characterisation of the increased cortex cell file number response to low P in arabidopsis roots

3.1 Introduction

If roots are exposed to a low P stress, after one to three days, an increase in the number of cortex cell files (or cortex cell file number; CCFN) can be seen. Following this there is a corresponding increase in the number of trichoblast cells since there are now more cortex cell-cortex cell cleft positions. Some studies have looked at radial cellular anatomy on low P (Cederholm & Benfey, 2015; Ma *et al.* 2001; Zhang, Lynch, & Brown, 2003). These three studies all analysed arabidopsis roots grown on agar plates using high P or a low P treatment of either 1 μ M (Ma *et al.*, 2001; Zhang *et al.*, 2003) or 0M P (Cederholm & Benfey, 2015). They report that on low P conditions arabidopsis roots develop extra cortex cell files which results in an increase in the number of epidermal cells which take on trichoblast identity. These studies clearly report the phenomenon and provide some explanation for how it might be regulated (i.e. three ROOT GROWTH FACTOR peptides and putatively, an auxin transporter). However, in spite of their discoveries there are limitations to the current available research. Firstly, there is currently low resolution with regard

to where in the root the cortex proliferation is occurring. Moreover, very little is known about which tissues are affected; is this really cortex specific or is the low P stress having an effect on the behaviour of the meristem in a more general way? It is also not clear what the concentration sensitivity or precise time frame is for this response to occur within. Additionally, the work done by Cederholm & Benfey, (2015) focusses on young plants (5-6 days old) which is relatively early in arabidopsis root meristem maturation (Clowes, 2000). It will be interesting and more relevant to issues in crop and wild plants to explore the response of more mature roots.

With these limitations in mind, several outstanding questions must be answered; these are:

1. What is the critical low P concentration at which a proliferation of cortex is seen?

This is important because it will be crucial to subsequent experiments to identify a concentration at which the plant will respond in this way, but will maintain otherwise normal growth. Additionally, the sensitivity of the response to P concentration will enable it to be more accurately placed in the context of field conditions.

2. What is the time frame of the response?

Knowing the answer to this question will provide greater understanding of the underlying processes because it will help to elucidate whether it is a transient, local response to P concentration or a longer term systemic response. It will

also help to inform the design of future experiments and provide clues about what kinds of underlying mechanisms could regulate this.

3. Which tissues are affected by this response?

One major gap in the knowledge around this phenomenon is whether it is specific to the cortex tissue or if cell files in other tissues also undergo proliferation in response to low P. It is known that the number of trichoblasts is increased as well (but only as a result of the increased number of cortex cell cleft positions leading to more epidermal cells in the hair cell position), and that in young seedlings endodermis cell file number can also increase (Cederholm and Benfey, 2015). However, we do not know if stele, pericycle, or overall epidermal cell file numbers are also increased. Knowledge of this will reinforce the presumed function of this response. If other cell types are also affected then perhaps a more generalised root growth response mechanism is at play. This information is also crucial because it helps to decide in which tissues potential regulating factors should be investigated. It will also give clues in which cells the response may originate; e.g. if stele, pericycle and ground tissues are affected then perhaps we should see a change of regulation in the quiescent centre. If the response is specific to the cortex, then we should be looking for regulatory pathways in the cortex tissue itself.

4. Which cells respond?

The answer to this question is illusive, without witnessing a number of proliferative cortex cell divisions occurring *in vivo* in real time it is difficult to be

completely sure where they are occurring. However, by using reconstructed 3D stacks of confocal image slices (from pseudo-Schiff propidium iodide stained and cleared roots), tracking changes in cortex cell file number will help to narrow it down. Understanding this aspect of the response will clarify the specificity to the cortex low P response even further. In addition to this, it will provide greater insight into what regulating factors may be at play, and where in the root to study their activity.

In order to answer these questions effectively and be able to place the answers in context, it is fundamental to standardise and optimise a method which works under my conditions and reflects the responses reported previously. As well as addressing the holes in current knowledge of low P associated CCFN increase, this chapter will present the development of an appropriate low P treatment.

3.2 Establishing a consistent methodology for monitoring growth in P limiting conditions

Before any detailed experiments could be performed it was first necessary to optimise a standard low P treatment which would reliably produce a low P response. This section will present the variety of techniques which were used to study the effects of low P stress on plants and the exact range of responses which are found. Following this, data to explain low P responses in the media used here will be presented.

3.2.1 P sources and growth media

Literature surrounding the study of low P stress and P use efficiency in plants presents a vast array of specific techniques directed towards different aims.

Much of the literature focussing on plant-P interactions (especially with arabidopsis) uses square plastic plates filled with agar- or phytagel-gelled liquid nutrient medium. The major limitations of this are that it does not replicate the physical and chemical heterogeneity of soil, and it only allows for two-dimensional growth of the plant; thus allowing only for two-dimensional architectural observations. However it does allow for specific adjustments to nutrient concentrations and complete recovery of intact, clean tissue for microscopic observations. (Ma, *et al.* 2001) aimed to study how P availability affected root hair density in arabidopsis. (Ma et al., 2001) used a manually mixed nutrient solution consisting of 3mM KNO₃, 2mM Ca(NO₃)₂, 500μM MgSO₄, 25μM KCl, 12.5μM H₃BO₃, 1μM MnSO₄, 1μM ZnSO₄, 0.25μM CuSO₄, 0.25μM (NH₄)₆Mo₇O₂₄, 25μM Fe-EDTA, 0.55mM myo-inositol, 2.5mM MES, 29.2 M sucrose, set with 2g/L phytagel and pH adjusted to 5.7 using KOH. P levels in this medium are regulated by using NH₄H₂PO₄ which is replaced appropriately with (NH₄)₂SO₄ to reduce P concentration. Seedlings on plates were then grown in constant light at 25°C horizontally until roots grew to the bottom of the plate, at which point plates were rotated up so that roots were growing downwards at 45° to the gravity vector. Similarly, (Zhang et al., 2003) grew arabidopsis on plates using NH₄H₂PO₄ as a P source, but grew the plants under a 16/8h light/dark lighting regimen. As with the (Ma et al., 2001), this work aimed to

closely examine root hairs and also to harvest roots to use in plastic sectioning in order to observe radial anatomy. Because of these requirements, the plants needed to be grown on plates so that they could be recovered from their growth environment with little to no physical damage. Some earlier publications from this group (Bates & Lynch, 1996; Trull, *et al.* 1997) cited their nutrient solution as being based on Johnson, *et al.* (1957). The mix in the latter publication is very similar to the former but differs in some of the mineral sources, for example using H_2MoO_4 rather than $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}$ and FeSO_4 instead of Fe-EDTA. Bates & Lynch, 1996; Ma et al., 2001; and Trull et al., 1997) also use a number of additives such as myo-inositol, thiamine, pyridoxine-HCl, and nicotinic acid.

The consideration of vitamin and sugar additions are important factors- it has been shown previously that there are strong interactions between the presence of sucrose, P availability, auxin and cytokinin signalling in agar-based media. (Franco-Zorrilla, *et al.* 2005; Jain et al., 2007). Additionally, when thinking about P availability, the addition of myo-inositol, which forms the central ring of phytic acid, could have an effect on plant phytic acid metabolism and as such a knock on effect on P or Fe metabolism. Indeed *myo*-inositol and phytic acid metabolism are closely intertwined (Hegeman, Good, & Grabau, 2001; Loewus & Murthy, 2000). López-Bucio et al., (2002) grew arabidopsis in agar plate conditions using NaH_2PO_4 as a P source, but otherwise used a similar set of conditions to the above studies.

In order to create conditions which represent those found in the field, some studies employ the use of growing plants in soil when studying root architecture and other traits. This has proven particularly useful when studying crop plants with larger roots and root systems. For example Ao, *et al.* (2010) studied soybean in the field using a system where they grow the plant material in naturally P deficient soil. Some plots were enriched with P added in the form of calcium superphosphate to mimic phosphate-replete field conditions. To analyse the root traits of interest the whole plants were dug up and then certain parameters such as total root system width and depth, plus plant P content were analysed. The main limitation which this method presents is that digging up the root system is inherently destructive to smaller structures such as finer roots and root hairs. Growing arabidopsis in this way for my experiments would not be appropriate due to the loss of finer structures and inevitable presence of soil on the roots. Additionally, the entire root system architecture and many deeper and further roots will not be easily recovered. Ao *et al.*, (2010) also utilised another technique of growth roots in sand-containing rhizotron growth chambers into which transparent plexiglass tubes were inserted at a 30° angle. These tubes were used to insert an endoscope camera to photograph root architecture growth at different time points. A similar growth medium of sand was also used by (Da Silva & Gabelman, 1992) who combined quartz sand with activated alumina (Al_2O_3) which had been allowed to adsorb KH_2PO_4 at concentrations between 100mM and 600mM. This growth technique was used to assess low P (and aluminium toxicity) tolerance of maize inbred lines in a

system which is representative of the heterogeneous distribution of metal-bound P found in field conditions.

As well as growing experimental plant material in the field or artificial media designed to mimic soil in restricted growth containers, some studies use a middle ground of growing plants in soil in containers. Zoysa, *et al.* (1997) and Zoysa, *et al.* (1999) grew *Camellia japonica* and *Camellia sinensis* (tea) respectively in a “rhizosphere study container” which is packed with soil sampled from the field. P capture by roots was assessed in this work by measure P depletion within the container.

Besides growing plants in soil or soil-like media, some studies use nutrient solutions to feed specific phosphate concentrations to plants. For example (Liao *et al.*, 2004) grew common bean (*Phaseolus vulgaris*) in growth pouches consisting of two sheets of germination paper housed in a perforated polythene bag. These were suspended in nutrient solutions which provided nutrients of the same concentration but supplemented with either low P ($0.2\mu\text{M NH}_4\text{H}_2\text{PO}_4$) or high P ($1\text{mM NH}_4\text{H}_2\text{PO}_4$). This technique allowed for clear measurements of root gravitropic traits which could be combined with similar measurements in the field for a large scale genetic mapping study with a number of recombinant inbred lines (RILs). The concept of using nutrient solutions to feed growing plants in specific phosphate concentrations can be extended to hydroponic growth techniques. In a later study (Liao *et al.*, 2006) grew soy bean in compartmentalised growth chambers fed by hydroponic solution. This allowed the researchers to isolate areas of the root system (or individual roots) and

harvest samples of root organic acid exudates produced in response to specific P concentrations.

Simple hydroponic culture (i.e. immersion in to a specific nutrient solution) has proven a useful technique for examining root traits of different genotypes under low P conditions. For example, (Gong, *et al.* 2011) identified maize genotypes which were either sensitive or tolerant to low P conditions in a soil environment. They then used hydroponic culture to study root architecture in relation to specific high and low P concentrations and without the destructive consequences of digging them out of the ground. (Byrne *et al.*, 2011) demonstrate a slightly more complicated form of hydroponic cultivation whereby ryegrass (*Lolium perenne*) plants are placed in to plugs containing perlite and then submerged in nutrient solution containing either 0.31 mM or 0.016mM KH_2PO_4 . These conditions were used to expose the plants to low or high P for a series of experiments for microarray gene expression analysis and metabolite profiling.

L'taief *et al.*, (2012) used hydroaeroponics/aeroponics to provide differing concentrations of KH_2PO_4 to the roots of common bean (*Phaseolus vulgaris*). This process involves spraying a nutrient solution from nozzles at plant roots suspended in an air space. This methodology was especially appropriate to this application since the researchers wanted to apply known concentrations of specific minerals, but because they were cultivating plants with symbiotic nitrogen fixers, wanted the plants' roots to be exposed to air.

Another method of growing plants in a way in which roots could be easily imaged and harvested, but still grown in soil is the rhizobox method such as that used in Adu et al., (2017). In this method, plants roots are allowed to grow along the interface between a soil medium and the edge of a plexiglass box where they can be observed and imaged.

Not only the methodology of P application can be seen to vary greatly amongst studies, but so does the form of P depending upon the study aims. For example (Du et al., 2009) test the P capturing efficiency and mechanisms of a selection of *Stylosanthes* varieties using five sources of P to mimic soil conditions. Plants were grown in sand and supplied with KH_2PO_4 , CaHPO_4 , AlPO_4 , FePO_4 , and phytic acid ($\text{C}_6\text{H}_6\text{O}_{24}\text{P}_6\text{Na}_{12}$). This growth medium enabled the researchers to study how the plants were able to cope with a variety of stresses including P bound in metal phosphate form, P bound in organic form (phytic acid), and aluminium toxicity. (Liu et al. 2006) wanted to study the formation of root hairs in hydroponic solution, which is often not seen due to the high availability of P in standard hydroponic systems. One problem of hydroponic systems is that important traits such as root hair formation often does not occur in the same way as in soil due to the different P availability between these growth media. To combat this and create a P availability more resembling that seen in a soil environment, they buffered the P release by using $\text{Ca}_3(\text{PO}_4)_2$ (tricalcium phosphate) and additional CaCl_2 in varying concentration in order to modulate the concentration of bioavailable free Pi in solution. By doing this they were able to induce the formation of root hairs in a number of species including *Arabidopsis thaliana*, *Zea mays*, *Triticale secale*, and *Triticum aestivum*, but not

onion (*Allium cepa*) which is apparently incapable of producing root hairs under any circumstances.

3.2.2 P concentration used in agar plate studies

The concentrations of P used in these studies varies somewhat, but usually 1μM is the concentration of choice for a “low P” treatment. Some studies simply remove P altogether; Ticconi et al., (2009) make use of Murashige and Skoog (MS) salt mixtures with and without P, with 0.625mM KH₂PO₄ as the “High P” treatment. Similarly, Cederholm & Benfey, (2015) use custom MS salts without P to simulate a phosphorus stress situation. However, in the latter two studies low P treatment was administered by transferring seedlings from “+P” to “-P” plates. This method may result in a starker, sudden loss of P to elicit visible responses, compared to the former studies where plants are grown on “low P” for extended time periods. Franco-Zorrilla et al., (2002) use the same media as Ma et al., (2001) but opt to use it completely without P for their low P treatment, however, these in fact are for longer term growth experiments rather than a high to low P transfer. Where most studies have used concentrations of less than 50μM, Williamson, *et al.* (2001) do not use a concentration lower than 100μM and see visible low P responses, suggesting that a fairly broad range of P concentrations are capable of producing low P responses.

As these studies show, the different methodologies of supplying plants with different levels of P, growing the plants, and examining the responses to low P and the ways in which plants are able to capture P are vast. The ways

researchers choose to provide P and study the plants is hugely dependent on the aims of their studies and the species they are growing. Some methods would simply not be suitable for particular types of study, for example a large scale genetic association study may be easier to interpret with a more consistent nutrient solution method rather than the heterogeneity found in the field. Conversely a study which focuses on how a plant's roots deplete P from the rhizosphere requires the particulate 3D structure of a soil environment, rather than the self-replenishing environment of a hydroponic tank.

3.2.3 Selection of growth conditions and characterisation of P responses

Since the literature represents a wide range of different P environments for plants to grow in, it was also important that the specific P dependent responses which are occurring in plants are carefully characterised.

Since the work here required only two dimensional RSA analysis in the first instance, followed by detailed anatomical work and confocal fluorescence microscopy it was decided that non-soil growth conditions were optimal. For these reasons, it was decided that agar-gelled liquid medium on plates would be the growth method of choice. Not only does this method allow for recovery of completely clean, intact root tissues but the method is well established within the growth facilities used. Additionally, this technique allows for easy transfer of seedlings from one treatment to another, and it was also decided

that the rhizosphere depletion of P on plates actually more accurately represented the P depletion conditions in soil. This ruled out hydroponic growth as an option since in this environment there is constant replenishment of P.

Previous studies have mostly shown that concentrations of between 0 μ M and 100 μ M are sufficiently low to induce notable responses to reduced P in arabidopsis. Williamson et al., (2001) describe a concentration dependent decrease in primary root length and increase in lateral root density as phosphate becomes more limiting. They ascribe these changes to a reallocation of growth from primary root to lateral roots, which is suggested to have the outcome of mining P from shallower layers when in soil. However, this phenomenon is well studied and the main observations are that low P has three main effects on root architecture and anatomy. These are: shortened primary root (Ward, *et al.* 2008; Ticconi et al., 2009; Dong et al., 2017; Svistoonoff et al., 2007), increased lateral root density (López-Bucio et al., 2002), and increased root hair length/density (Bates & Lynch, 1996; Bates & Lynch, 2001; Z. Ma et al., 2001; Ma, *et al.* 2003; Zhang et al., 2003).

Although the above literature encompasses quite a broad range of P concentration (0-100 μ M), López-Bucio et al., (2002) examined the effects of varying concentrations of P in growth media on arabidopsis root growth and hormone sensitivity. Their results showed that 50 μ M P had a notable effect in increasing lateral root density and decreasing primary root length.

To establish a the optimal growth conditions for experiments where plants undergo a low P stress and whose roots can be easily recovered, wild type

Columbia 0 *Arabidopsis thaliana* seeds were sterilised using 70% ethanol and then sown on to either high P (1mM $\text{NH}_4\text{H}_2\text{PO}_4$) or low P (50uM $\text{NH}_4\text{H}_2\text{PO}_4$) containing agar-based medium before being grown at 21°C in 12 hours dark/12 hours light. After 5 days of growth, the plants were imaged at 4 hour and 40 minute intervals for 10 days using an automated system (Wells et al., 2012). This method generated sequential images sufficient to produce a dataset where primary root length and LRD measurements could be used to assess the effect of phosphorus limiting conditions on root growth over time.

Firstly, primary root elongation rate was investigated to find out whether the treatment would cause the same reductions in primary root elongation rate or even arrest of primary root elongation which had been reported before (Ticconi et al., 2009).

It was found that primary root elongation rate was reduced from around 450 $\mu\text{m}/\text{hour}$ at 15 days on 1mM phosphate to around 100 $\mu\text{m}/\text{hour}$ on 50 μM P at 15 days (figure 3.1e). Interestingly, over time primary roots elongating on 1mM phosphate increase in their elongation rate over time. At 5 days, high P roots are elongating at 200 $\mu\text{m}/\text{hour}$ and by day 15 they are elongating at a rate of ~450 $\mu\text{m}/\text{hour}$. This increase is absent or very much reduced when plants are grown on low P.

As expected, this decrease in primary root elongation rate induced by low P is followed by a decreased in final primary root length. These results show that average primary root length on 1mM at 15 days is 70mm, but this is decreased

to 30mm ($P = <0.001$; figure 3.1d) when seedlings are grown on 50 μ M P containing media.

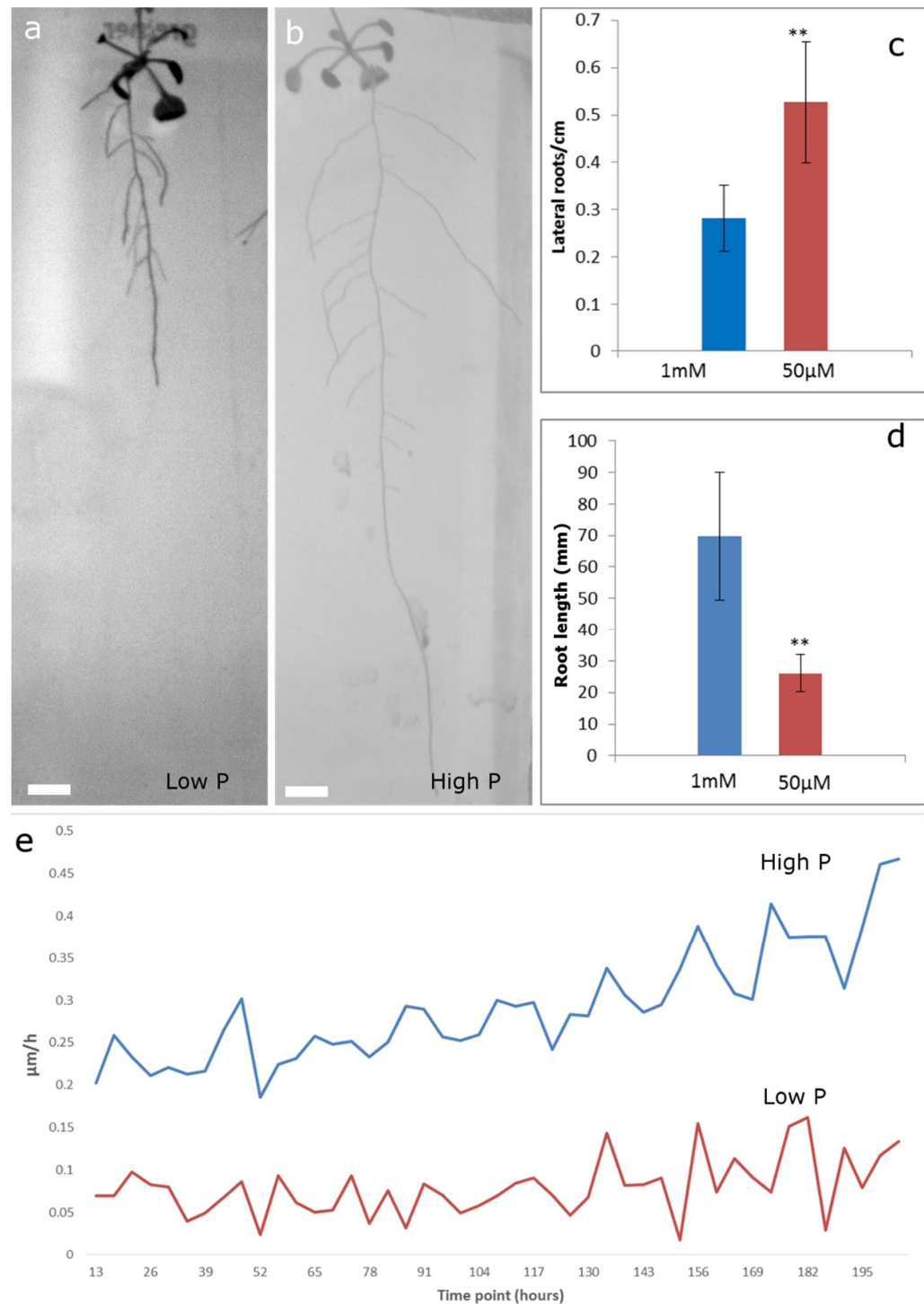


Figure 3.1 Primary root elongation rate and final length of Arabidopsis roots are reduced and lateral root density is increased by low P treatment

Root architecture and primary root growth rate are affected in response to low phosphate. (a, b) Images taken using an infra-red camera for IR illuminated imaging in darkness. Images were taken over 10 days at 4 hour, 20 minute intervals to monitor root growth over time. (c) bar chart showing lateral root density for 50uM and 1mM P treated roots expressed (y-axis) as lateral roots per centimetre of primary root length. (d) Bar chart showing final primary root length at 15 days after germination for roots grown on 50uM and 1mM P, expressed (y-axis) in millimetres. (e) Line graph showing primary root elongation, expressed as micrometres per hour (y-axis) for roots grown on 50uM (red line) and 1mM (blue line). Visual differences in root architecture are apparent under low (50uM; a) and high (1mM; b) phosphate conditions. Lateral root density increases under 50uM phosphate treatment (c) and primary root length is decreased. Under sufficient phosphate conditions (1mM), primary root elongation rate is higher than under low phosphate (50uM) and increases over time. Low phosphate treated primary roots do not increase growth rate over time (e). ** indicates $p < 0.001$.

The final aspect of root system architecture investigated in relation to low P was lateral root density. These data revealed that the low P treatment used increases lateral root density from around 0.3 lateral roots/centimetre to around 0.5 LR/cm ($P = <0.001$; figure 3.1c).

3.3 Root cortex response to low phosphate is concentration dependent

Previous work studying low P stress in plants has used a variety of P concentrations to mimic a stress scenario. Trying to determine a specific concentration of P which a plant will respond to as a deficiency is complicated by several factors.

P is largely insoluble and immobile at most soil/media pH ranges, and is affected by other minerals (i.e. precipitating in the presence of iron oxides; (Lin, *et al.* 2014; Raghothama, 1999). In terms of direct uptake, plants primarily obtain P as orthophosphate anions, whose concentration in solution is typically 1-5 μ M (Richardson, *et al.* 2009), the rest of the P exists in a variety of insoluble mineral and organic forms. Thus a given concentration of P does not necessarily reflect its availability when other chemical factors are considered.

The physical nature of the media also affects the availability of P, for example in clay containing soils P is less bioavailable when compared to a more sandy soil. It has also been shown that P will become immobilised and “trapped” in the gel matrix of agar growth media, depleting around the root growth region. It is important to consider this when thinking about agar plate-based low P

experiments, since the predetermined concentration in the media is not necessarily the concentration the plant root will be exposed to throughout its growth cycle.

As well as soil physicochemical properties, microbes, fungi and other plant roots present in soil will all have an impact on the solubilisation and delivery of P to plant roots in an active soil environment. It is estimated that around 70-80% of land plants are capable of forming associations with arbuscular mycorrhizal fungi and that in many cases this is because these associations are essential for sufficient P acquisition in soils (Smith, *et al.* 2011). In addition to fungi, some bacteria are capable of increasing the availability of P in soil by solubilising some metal-phosphates such as iron, calcium, and aluminium phosphates (Richardson & Simpson, 2011; Richardson *et al.*, 2009).

For these reasons trying to replicate any or all field P deficiency scenarios in the laboratory, whether using soil, agar, or hydroponic media, is very difficult to do precisely. Not least because P dynamics are not fully understood, but also because the P availability to plants in any one soil-plant environment will vary greatly. This renders any low P experiment (in the laboratory or field) somewhat abstract and only an approximation of the dynamic soil-nutrient interactions which are found in non-experimental systems. Even in the laboratory several other factors may affect P availability in a given system: phosphorus present in the agar, growth environment temperature, iron source, P source, water source, and media pH will all affect the availability of P in a given experiment.

For this reason it is important that prior to a set of P experiments, a working P concentration is determined which provides a P deficiency under the present conditions.

Therefore, a critical aim of this study was to test a range of P concentrations in the media and choose a concentration which resulted in a low P response as seen in previous studies (Dong *et al.*, 2017; López-Bucio *et al.*, 2002; Svistoonoff *et al.*, 2007; Ward, *et al.*, 2008). I.e. where the roots exhibit reduced primary elongation enhanced lateral root length but at which the plants still remained green and capable of sustained root and shoot growth.

In the interest of consistent P concentration in the media, one source of agar gelling medium was acquired and used for every experiment in this study. Supplier data sheets were obtained and a batch of agar which was determined as having a low P concentration by HPLC was tested and then a large quantity purchased to last for the entire study.

To find the lowest concentration at which a CCFN response was seen, but plants were still growing, a concentration range from 0M-1mM P was tested.

Columbia 0 seeds were sown on to plates containing different P concentrations. These were placed at 4°C for 1 day to stratify seeds then growth in 12h light, 12h dark. Roots were harvested at 12 days after germination and fixed before being pseudo-Schiff Propidium Iodide stained. Unless stated otherwise, all plant growth and imaging in this chapter is performed as described above.

The pseudo-Schiff propidium iodide (pS-PI) technique uses the treatment of plant tissues with periodic acid which oxidises the bond between adjacent carbon atoms in polysaccharide chains within the cell wall. This leaves two

aldehyde residues which can be stained by propidium iodide (PI). This binding of PI is strong, and thus will resist tissue clearing with chloral hydrate unlike live staining where PI accumulates in the cell wall matrix. Chloral hydrate tissue clearing allows for greater resolution of root cell anatomy using fluorescence confocal microscopy.

Specific concentrations 0M, 50 μ M, 100 μ M, 200 μ M, 400 μ M, 800 μ M, and 1mM were used to identify an optimal “low P” condition for work here. Seeds were sown on to plates containing these concentrations of P and then harvested at 12 days, pS-PI stained, and imaged using confocal microscopy to count the number of cortex cell files found under each of these conditions. The hypothesis of this experiment was that lower P concentrations would be associated with higher CCFN.

The overarching finding from this experiment is that concentrations between 0-400 μ M P elicit an increase in CCFN in arabidopsis roots. At 0M P, CCFN was ~11; at 50 μ M P there were also ~11 cortex cell files, this value was ~10 at 100 μ M, ~11 at 200 μ M and then ~10 at 400 μ M. These values were all statistically significant at $P < 0.005$, except for the 400 μ M treatment which was statistically significant at $P < 0.05$ (figure 3.2b). Figure 3.2a shows how this general trend appears in root tissue with yellow dots and numbers to indicate the cortex cell files and how many there are.

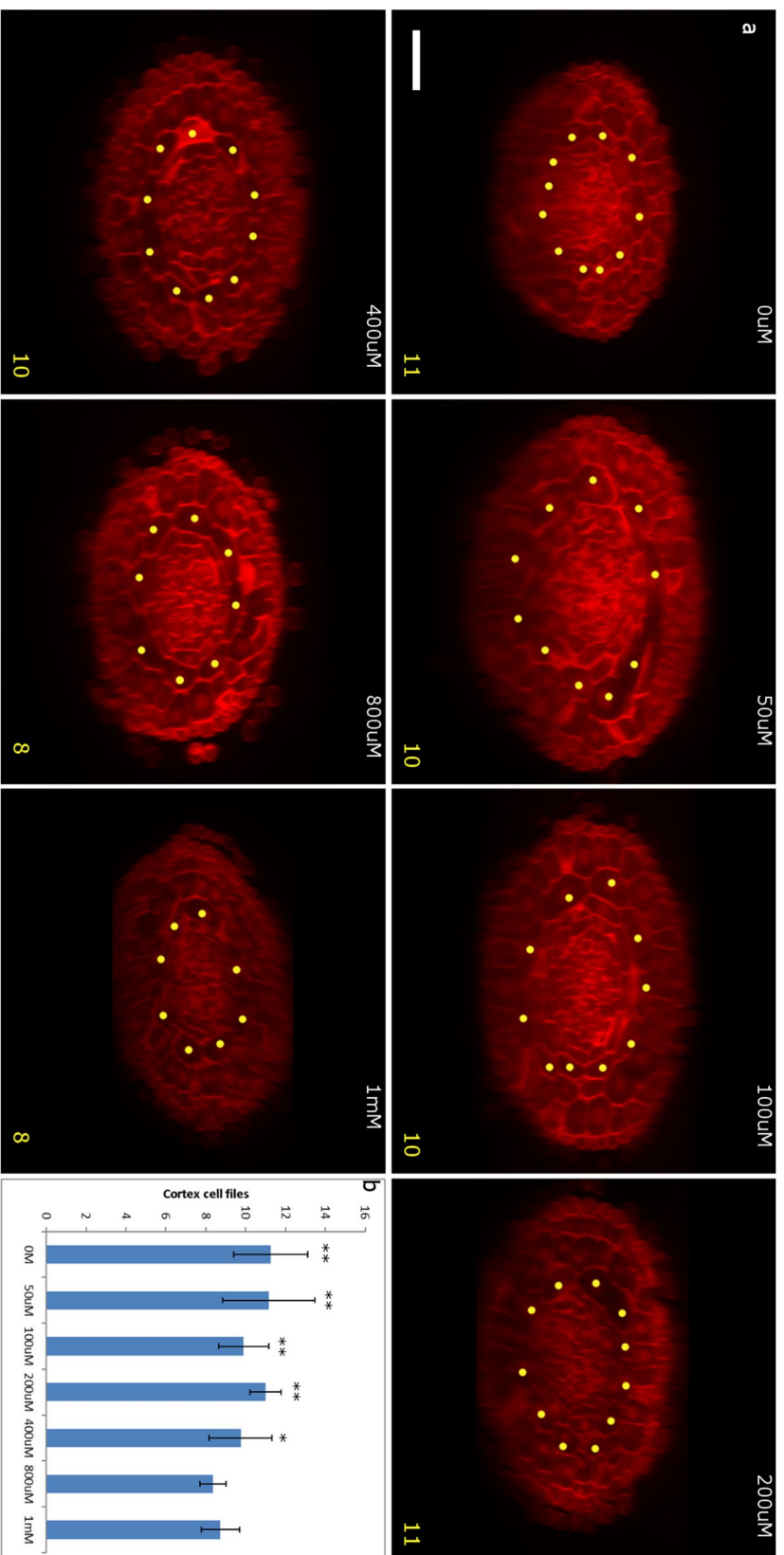


Figure 3.2 CCFN response to low phosphate occurs at concentrations of less than 400μM

Optical confocal cross sections of pseudo-Schiff propidium iodide stained roots (a) showing the effect of phosphate concentration on radial cortex cell file number. Cortex cell file numbers (yellow dots; yellow numbers) begin to increase when roots are exposed to medium containing 400μM or lower. At lower concentrations the number of cortex cell files is as high as 13, but at 800μM-1mM the number of cortex cell files remains at 8 (b). Error bars represent standard deviation. Scale bars= 25μm. Cortex cell file counts 0M: n=8; 50μM: n=12; 100μM: n=11; 200μM: n=10; 400μM: n=12; 800μM: n=11; 1mM: n=11. ** indicates statistically significant difference to 1mM P (p<0.005); * indicates p<0.05. n= number of seedlings.

In addition to the data produced for aspects of primary root elongation and RSA, it was also found that plants grown on less than 50 μ M phosphate typically exhibited severe stress symptoms than those grown on lower concentrations. These included dramatic inhibition of growth, anthocyanin accumulation and chlorosis in above ground tissues, and visible dying off of organs. These observations echoed those of Trull et al., (1997) and (Bates & Lynch, 1996) who both noted anthocyanin accumulation and stunted growth at a P concentration of $\sim$ 1 μ M. The results in the present study show that the highest P concentration at which a response in the cortex tissue is seen is 400 μ M P (average of $\sim$ 10 cortex cell files; figure 3.2a; 3.2b). This is an interesting result as this concentration is far higher than most estimates for typical soil concentrations, and is also far higher than concentrations used in most low P studies.

However, the strongest response is seen at 50 μ M P (average $\sim$ 11 cortex cell files, maximum 16 cortex cell files; figure 3.2a, figure 3.2b). When growing arabidopsis seedlings on this agar for 12-14 days this concentration elicited a strong CCFN response to low P under present conditions. However it was found that plants still appear green, capable of growing whilst maintaining these visible low phosphorus responses (figure 3.1a, figure 3.1b). Because of these factors it was chosen as the concentration to go ahead with for all following experiments. Based on these considerations, 50 μ M P was used as a low P treatment for the first set of experiments and tested plants for a range of low P responses more closely.

The visible responses of the root to low phosphate also act as an important indicator that low phosphate is being responded to by the plant. Under

phosphate limiting conditions, the two most conspicuous changes to root growth and development are an increase in lateral root density (Ward et al., 2008), and a decrease in primary root length (and elongation rate; Ticconi et al., (2009). From this point on, all plants are grown at 21°C under a 12h light/12h dark day length regimen on low (50µM) or high (1mM) P media adjusted to pH 5.7.

3.4 Changes to radial cortex anatomy occur after 24 hours exposure to low phosphate

Previous work (Cederholm & Benfey, 2015; Ma et al., 2001; Zhang et al., 2003) compared growth on low and high P, but didn't show if this was a dynamic process capable of changing rapidly. If this cortical repatterning is to confer adaptive advantage, then it should occur quickly as a root grows through a patch of soil low in P. The question still remains what time-frame this response occurs within. To answer this question, arabidopsis seedlings were sown on to 1mM P containing agar plates. Plants were then transferred to 50µM P containing medium after 4 days, 7 days, 10 days, 11 days, 12 days, and 13 days. The day after the final transfer, seedlings were harvested from all the 50µM P plates and the remaining 1mM P plates. This meant that roots could be analysed which had been exposed to low P for 0, 1, 2, 3, 4, 7, and 10 days. Root tissue from the root tip to 5mm along the length of the root was removed before being pS-PI stained and imaged using confocal microscopy. CCFN was then counted visually using resulting cross-sectional images.

The most striking aspect of this data is that a CCFN response to low P can be detected within 1 day (24hr) of exposure to low P (figure 3.3a; 3.3b). In this time period, there is an increase in average CCFN from 8.6 to 10.5 which is statistically significant ($P = <0.005$).

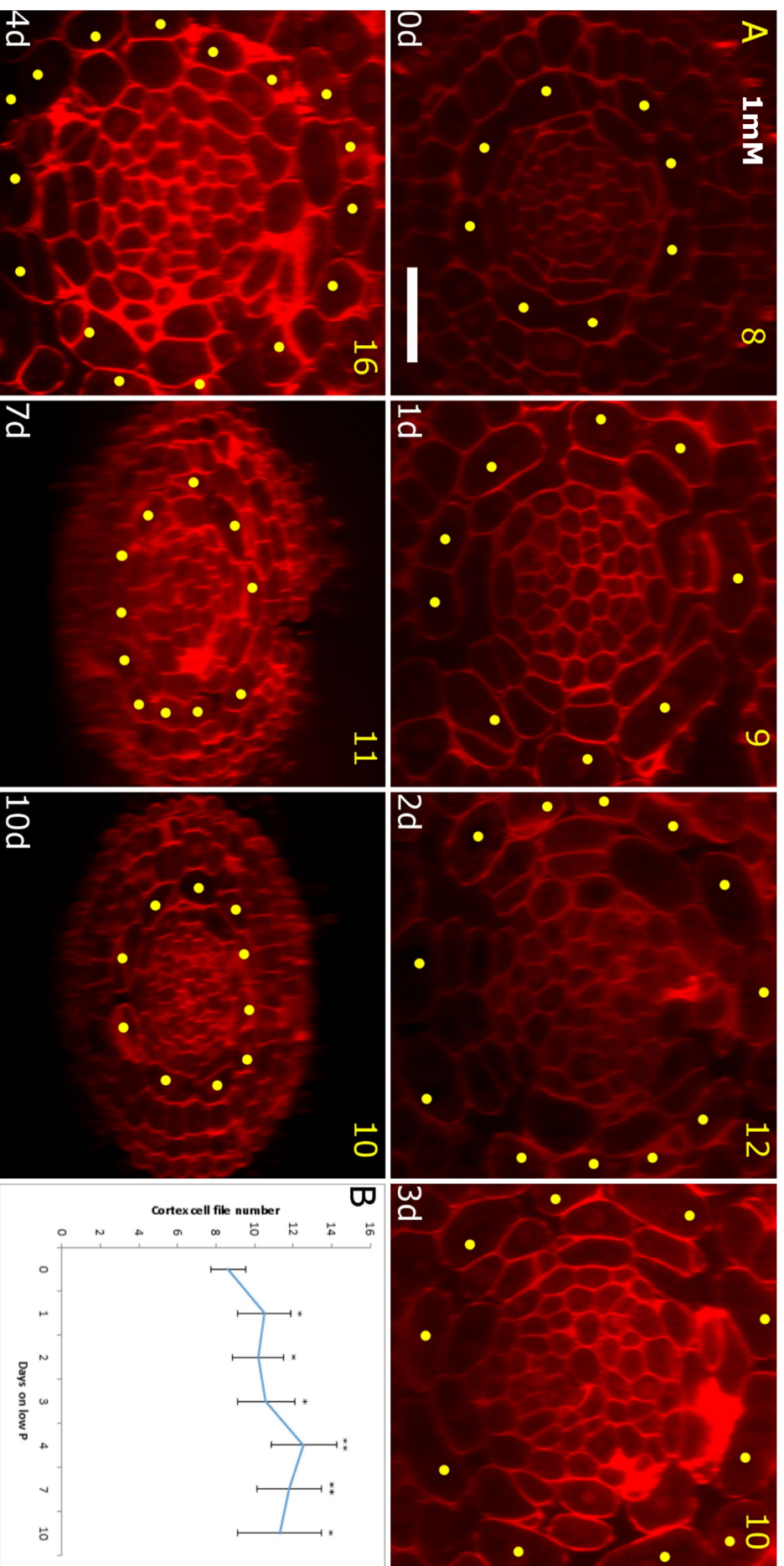


Figure 3.3. Cortex cell file number starts to increase after 1 day of exposure to low phosphate

Optical confocal cross sections of pseudo-Schiff iodide stained roots show that cortex cell file response to low phosphate can occur as soon as one day after exposure. Roots of the same age grown on 1mM phosphate or 50 μ M phosphate for different lengths of time are shown (A), with their cortex cell file numbers shown (yellow numbers, yellow dots). Cortex cell file number can be seen to increase to an average of 10 after one day and continuing to increase to an average of 12 after 4 days. Interestingly a small reduction can be seen between 4 and 10 days of exposure to low phosphate (B). Scale bar=25 μ m. Error bars represent standard error. Cell file counts 0 days: n=11, 1 day: n=10, 2 days: n=11, 3 days: n=10, 4 days: n=9, 7 days: n=10, 10 days: n=10. ** indicates statistically significant difference to 0 day treatment (p<0.005); * indicates p<0.05.

time point, average CCFN is at around 9.5 at 2 days which goes up to around 10 after 3 days. At 4 days CCFN then increases to an average of 12.5 which and then is 11.3 by 10 days low P (figure 3.3a; 3.3b). However, the latter difference is not statistically significant ($p=0.104$; not shown in figure). (Representative images of these changes over time can be seen in figure 3.3a, where cortex cell files are denoted by yellow dots and the CCFN in that root is indicated by a yellow number). These changes in CCFN through time are all statistically significantly different to the 0 days on low P control treatment where roots had an average of ~8.5 cortex cell files.

3.5 Changes to root radial anatomy are restricted to cortex and hair-identity epidermal cells

In this section, the work aims to answer the question of which root tissues undergo changes to radial anatomy in response to low P, since this response has been reported before, but not described in any more detail.

Arabidopsis Col. 0 seeds were sown on media containing 1mM (high P), 50 μ M (med P), or 1 μ M (low P) for 12 days. After this root tips were harvested before being stained using the pS-PI protocol and imaged Cell file numbers of major root tissues within the primary root were counted to investigate whether the low P response was cortex specific or just a general effect on cell division in the root meristem. The cell types counted were stele (which includes xylem, phloem and vascular precursor/procambium cells), pericycle, endodermis, cortex, and epidermis (trichoblasts and atrichoblasts separately).

At this point it is important to further clarify the tissues which are being counted; specifically in relation to cortex and epidermis cells. Baum, *et al.* 2002; Lee et al., (2016) show that as arabidopsis plants begin to mature around 14 days and onwards in age, maturation of the root ground tissue begins to occur. This begins with extra layers of ground tissue; middle cortex, which can be seen longitudinally as a layer of cells parallel to the epidermis-adjacent cortex but in addition to the endodermis. These can be seen developing as periclinal divisions of the epidermis which may occur close to the apical meristem or further towards the basal meristem zone. The roots analysed in this study are 12 days old so are at the point where some of these changes to ground tissue are beginning to occur. In these experiments, the middle cortex is not included in the counts of cortex cells. This is because only the cortex cell files which are in contact with the overlying epidermis have a role in trichoblast patterning and so are the only cortex tissue cells considered relevant to this phenomenon as a functional low P response. For additional clarity it is also important to distinguish between an increase in the number of cells within a tissue such as the cortex, which results from proliferative divisions originating in the meristem and an increase in the number of trichoblast cells. Increases in the number of trichoblast cells originate not from specific meristematic divisions giving rise to this cell type, but from position dependent respecification of atrichoblast cells in to trichoblast cells. The latter is a process of epidermal patterning rather than *de novo* tissue generation such as that which occurs at the quiescent centre and in initial cells.

It was found that the number of cell files in the stele (vascular bundle; xylem, phloem, procambium), pericycle, endodermis, and total epidermal cells did not change significantly in response to phosphate deficiency (figure 3.4b). Vascular bundle (stele) had an average of 30-35 cell files, pericycle an average of 14-16 cell files, endodermis an average of 12-14 cell files and epidermis an average of 26-28 cell files.

The only tissues which exhibited a change in cell file number were cortex, and as a result, epidermal cells which lay in the H (trichoblast) position. These results confirm that the response to low P is specific to cortex and trichoblast cells and is not just an effect on cell division throughout the root meristem.

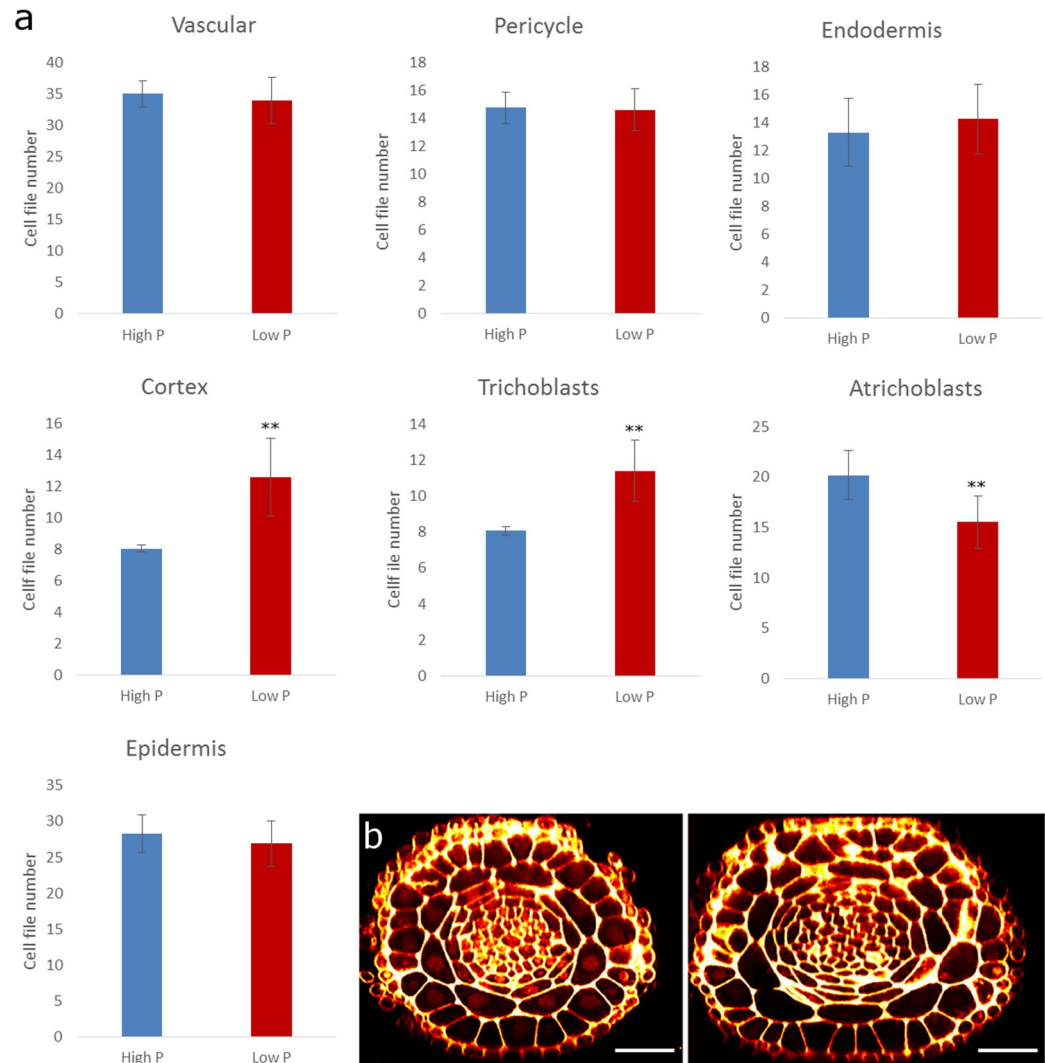


Figure 3.4 Changes to radial anatomy under low phosphate are limited to changes in cortex and hair-cell number.

(a) Bar charts showing number of cells files in each tissue visualised in confocal microscope cross sections for three phosphate treatments: 'high P' (1mM), and 'low P' (50µM). Roots of seedlings growing on high P (1mM) or low P (50µM) have, on average, 8 and 12 cortex cell files respectively ($p < 0.001$; a). (b) Images showing confocal microscope imaged pseudo-Schiff propidium iodide stained tissue in cross section from roots grown on high (1mM) P (left) and low (50µM) P (right). This change corresponds to a change in the average number of hair position epidermal cell files (H cells) of 8 to 11 respectively ($p < 0.001$; b). Error bars= standard deviation, scale bars= 25µm.

3.5.1 Trichoblast cell length is decreased under low P conditions

As discussed previously, it seems that the increase in CCFN in arabidopsis roots under P limiting conditions is a response to increase the number of trichoblast position cell files in the overlying epidermis. If the number of cortex cells increases from 8 to ~14 then this will typically result in the specification of extra trichoblast cell files. This therefore represents a mechanism for the plant to increase the density of root hairs in the radial dimension specifically. Numerous studies demonstrate the importance of root hairs in P acquisition and the response of root hairs to low P; this includes contributions of root hair length (Bates & Lynch, 1996; Gahoonia & Nielsen, 2004), density (Bates & Lynch, 2001; Ma et al., 2001; Ma et al., 2001; Vandamme et al., 2013), longevity (Nestler & Wissuwa, 2016), and root hair-soil interactions (Keyes et al., 2013) to the efficient capture of P from the soil in a number of species. In addition to root hair density being increased in the radial dimension, a reduction in the length of epidermal cells would also increase in the density of trichoblasts in the longitudinal dimension.

To test this arabidopsis Columbia seeds were sown on to media containing either 50 μ M (low P) or 1mM (high P). Plates were then grown for 12 days. Roots were live-stained with propidium iodide and imaged using confocal fluorescence microscopy. Trichoblast cells were measured in the resulting images using image analysis software imagej.

The presented images show that there is a visible difference in the morphology of trichoblast cells between low and high P treated roots; under low P there

were significantly shorter trichoblast cells fitted in to the longitudinal space (figure 3.5a).

Data presented here show that the average trichoblast cell length on high P media is 212 μ m, but grown on low P media roots have trichoblast cells with an average length of 99 μ m. This difference is statistically significant ($p = <0.001$) (figure 3.5b).

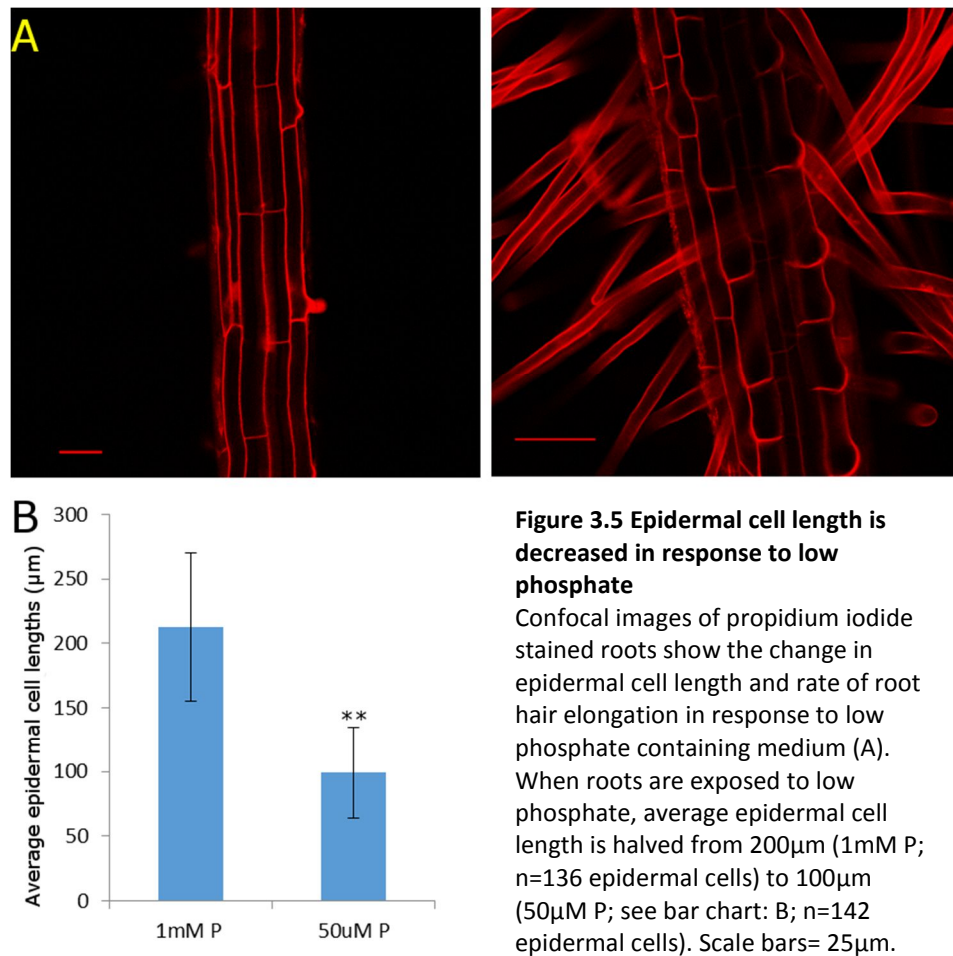


Figure 3.5 Epidermal cell length is decreased in response to low phosphate

Confocal images of propidium iodide stained roots show the change in epidermal cell length and rate of root hair elongation in response to low phosphate containing medium (A). When roots are exposed to low phosphate, average epidermal cell length is halved from 200 μ m (1mM P; $n=136$ epidermal cells) to 100 μ m (50 μ M P; see bar chart: B; $n=142$ epidermal cells). Scale bars= 25 μ m.

3.6 Changes to radial cortex anatomy occur in the first cortex cell behind the cortex/endodermis initial

Although it has been noted several times that low P can elicit a radial proliferation of cortical cells (Cederholm & Benfey, 2015; Ma et al., 2001; Zhang et al., 2003), it remains unclear exactly where in the root tissue this occurs.

Cederholm & Benfey, (2015) present a figure which shows a cortex specific yellow fluorescent protein (YFP) marker line grown in low P conditions, but without time-lapse imaging of these roots it is difficult to determine the precise site of divisions. They note “bifurcation” points in a cortex cell file where it appears that any one file can switch between dividing to produce two files or not dividing and flux from the meristem as one file. Feasibly, this opens the possibility that the divisions could be occurring after the first differentiated cortex cell which are sustained for some time and then cease. However it seems more likely that, as the authors suggest, these divisions are stochastically controlled at the meristem and additional divisions may be promoted or inhibited at a particular radial position in the meristem at any one time or another.

To test this, arabidopsis Col. 0 seed was sown on to low or high P plates and were grown until 12 days of age before roots were harvested and stained with the pS-PI method. Confocal microscopy was then used to generate high resolution Z-plane image stacks of whole root tips. These image stacks could then be rendered as 3D reconstructions and viewed in imagej, enabling precise optical sectioning at desired points in the root tip. This technique allowed for examination of radial organisation at specific cell tiers through the meristem.

In this experiment, this method was used to precisely optically section and observe cortex cell file anatomy at the first differentiated cortex cell (i.e. behind the CEI cell) and then each following cortex cell tier basally from there.

These data show that the number of cortex cell files usually increases closer to the quiescent centre. Throughout all biological replicates, high P treated roots consistently exhibited 8 cortex cell files at each tier observed (figure 3.6a, c).

This pattern can be seen in figure 3.6a where cortex cell files in transverse section are marked with yellow dots and the number thereof written in yellow.

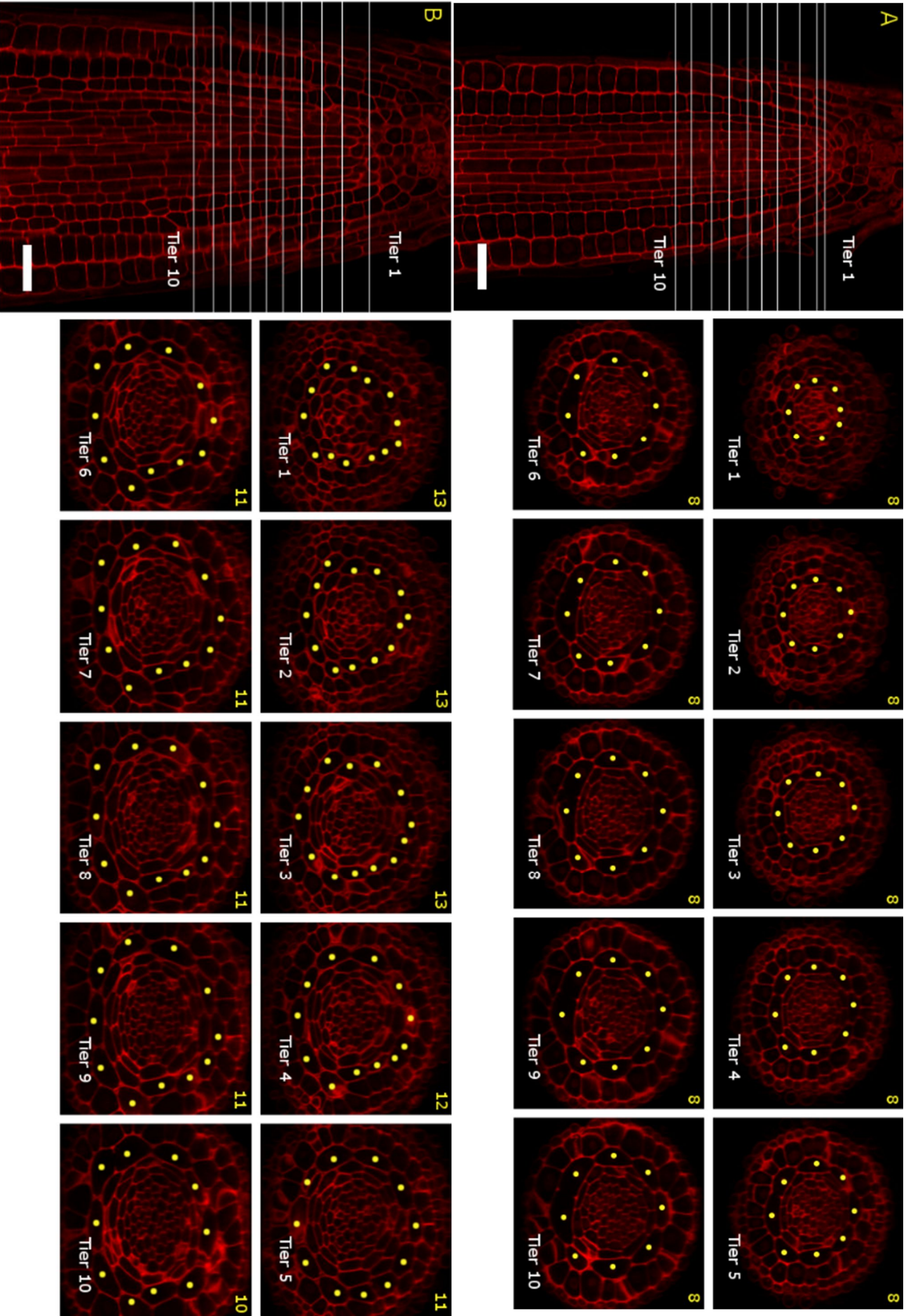
Longitudinal sections indicates where each optical slice was taken (a). This pattern was different in roots grown on low P. Referring to the example in figure 3.6b, it can be seen that CCFN here starts at 13 in the first cortex cell tier; at the fourth tier this decreases to 12, 11 at the fifth tier and then finally down to 10 in the tenth tier. The actual number of cortex cell files at each tier varied between seedlings but this pattern was consistent for 25 out of 30 roots.

From these results it can be asserted that the proliferative cortex cell divisions most likely occur very close to (or indeed at) the first cortex identity cell after the cortex/endodermis initial division (CEID). It can be seen that the number of cell files in the cortex usually only increases as you observe closer to this position (figure 3.6a; 3.6b). This suggests that as roots are exposed to low P for longer, they generate more cortex cell files at the meristem. Hence why the higher tiers (i.e. older rings) have fewer cortex cell files in 25 out of 30 roots.

The possibility that this is occurring at the time of the CEID is ruled out by the fact that no significant increase in endodermal cell files can be observed under low P treatment. (Cederholm & Benfey, 2015) do indeed report an increase in

endodermal cell file number under their conditions. However, since this is not observed in the present material, there could be a pathway for endodermal cell file proliferation in both high and low P treated plants which is independent of that which controls CCFN.

Having said this, in the experiments presented here, five out of thirty low phosphate treated roots do not follow the same pattern as the other 25 roots and exhibit a lower CCFN at the root tip than in older cell tiers. For example, one root showed an increase in CCFN of 8 at tier one, to 10 at tier 10. This could mean that the response is capable of stochasticity as suggested by Cederholm & Benfey (2015), whereby the divisions which give rise to additional CCFs are not necessarily maintained at an increasing or constant rate. It is possible that this stochasticity is a mechanism to respond rapidly to local changes in P concentration in soils. However, it is unclear why this would be occurring on an agar plate with a homogenous concentration of P.



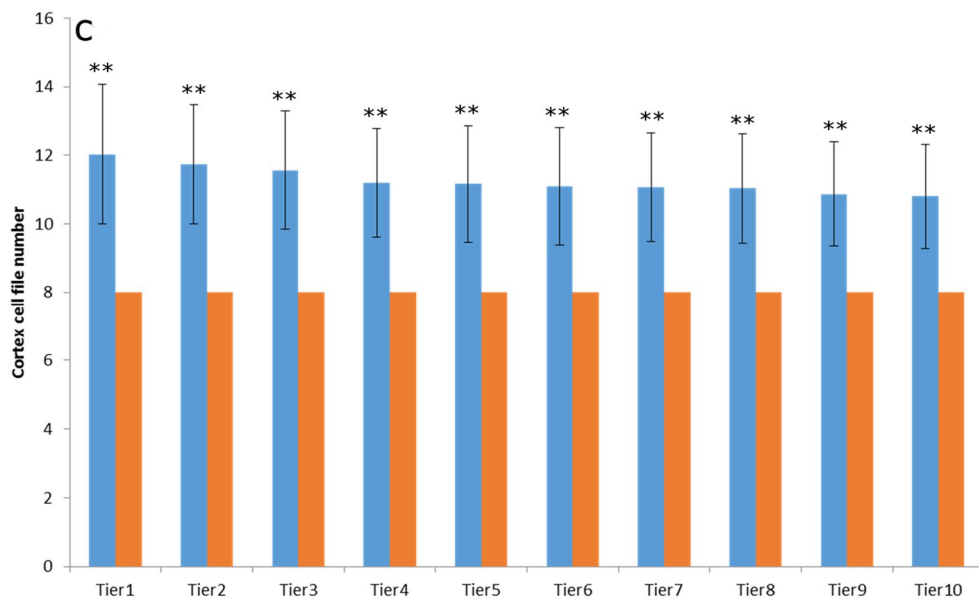


Figure 3.6 Cortical cell proliferation occurs in close proximity to the root apical meristem

Reconstructed confocal z-stacks of imaged pseudo-Schiff propidium iodide stained roots show that cortex cell file (yellow spots; yellow numbers) numbers are highest in proximity to the growing tip/cortex endodermis initial (CEI) cells in roots grown on 50 μ M phosphate (B). Plant roots grown on 1mM phosphate containing medium exhibit 8 cortex cell files from 1-10 cell tiers (yellow lines) behind the CEI (A). (C) Bar chart showing average cortex cell file numbers at cell tiers 1-10 over a number of roots for 1mM (Orange) and 50 μ M (blue) phosphate containing medium. Error bars represent standard deviation. Scale bars= 25 μ m, same scale for all images. Cell file counts high P: n=17: low P n=30.

3.7 Iron concentration in media affects root architectural responses, but not cortex cell response, to low phosphate

Recent studies (Dong et al., 2017; Gutiérrez-Alanís et al., 2017; Hirsch et al., 2006; Svistoonoff et al., 2007; Ward et al., 2008) indicate that under low P conditions roots can accumulate high concentrations of free iron in the root meristem. This accumulation seems to be particularly high in the QC and surrounding initials and it has been suggested that this has a toxic effect which can lead to meristem disorganisation, mis-localisation/expression of patterning regulator proteins such as SHR and SCR. According to these studies, Fe

accumulation under low P leads to iron toxicity and eventual termination of meristem activity within 8 days. It is highly possible that iron toxicity is responsible for some of the extreme responses which people see under phosphate limiting conditions, i.e. very short primary root and meristem differentiation/consumption (leading to growth arrest). Many of these studies are performed using media with $<1\mu\text{M}$ P and iron levels which were not reduced concomitantly to this P reduction. Therefore it was proposed that these published responses do not always represent those which a plant might display in a field condition where phosphate is not completely absent. For this reason, “low P” treatment was adjusted to $50\mu\text{M}$ and this section discusses the effect of reducing iron availability accordingly.

It was hypothesised that reducing the iron concentration in the media would limit some of the extreme low P responses which have been reported, such as dramatic shortening of the primary root and termination of the root apical meristem. It was proposed that by reducing iron concentration, it would be possible to isolate responses which were more associated with a lack of P than an excess of iron. This aimed to answer the question of which responses can be attributed to low phosphate and which responses can be attributed to an excess of iron (and therefore potential iron toxicity).

To test this Col. 0 seeds were sown on to agar plates containing media with high P high Fe (1mM $\text{NH}_4\text{H}_2\text{PO}_4$, $50\mu\text{M}$ Fe-EDTA), high P low Fe (1mM $\text{NH}_4\text{H}_2\text{PO}_4$, $5\mu\text{M}$ Fe-EDTA), low P high Fe ($50\mu\text{M}$ $\text{NH}_4\text{H}_2\text{PO}_4$, $50\mu\text{M}$ Fe-EDTA), or low P low Fe ($50\mu\text{M}$ $\text{NH}_4\text{H}_2\text{PO}_4$, $5\mu\text{M}$ Fe-EDTA). Plants were grown for 12 days after germination. Root tip tissue was removed and stained the pS-PI protocol and

imaged using confocal fluorescence microscopy. Cortex cells were then counted by eye.

The data here show that under reduced iron exposure (i.e. 5 μ M) and P limiting conditions (50 μ M P), the P responsive CCFN increase is still observed. On HPHFe medium, an average CCFN of 8.75 is seen, whilst as before, an increase to 10 is seen on LPHFe which is statistically significant to $P < 0.05$ (figure 3.7a, 3.7b). However, when plants are exposed to low P, but also 10-fold less iron (5 μ M Fe-EDTA) a CCFN increase is still seen. On this media, roots have a CCFN of 11; which is statistically significantly different to HPHFe ($P < 0.001$). This suggests that it is highly probable that the increase in CCFN seen in P limiting conditions is associated with low P and not an increase in Fe and/or Fe toxicity in the root.

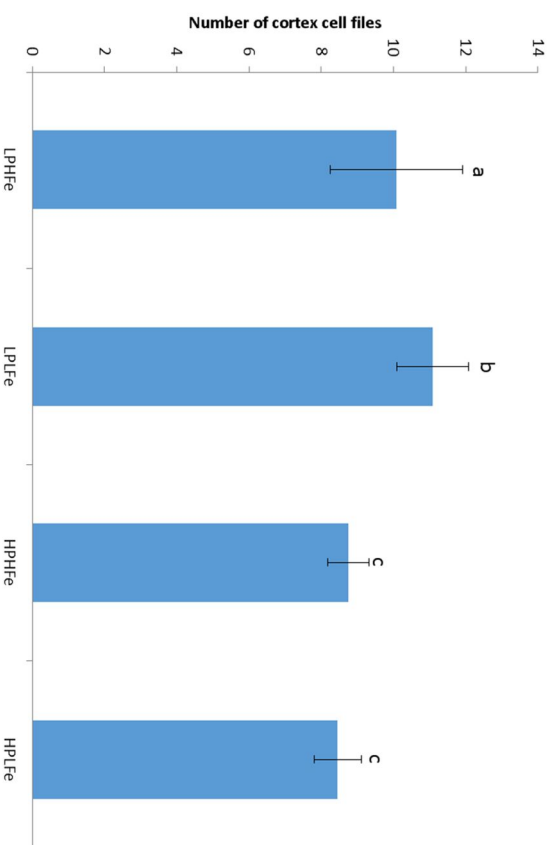
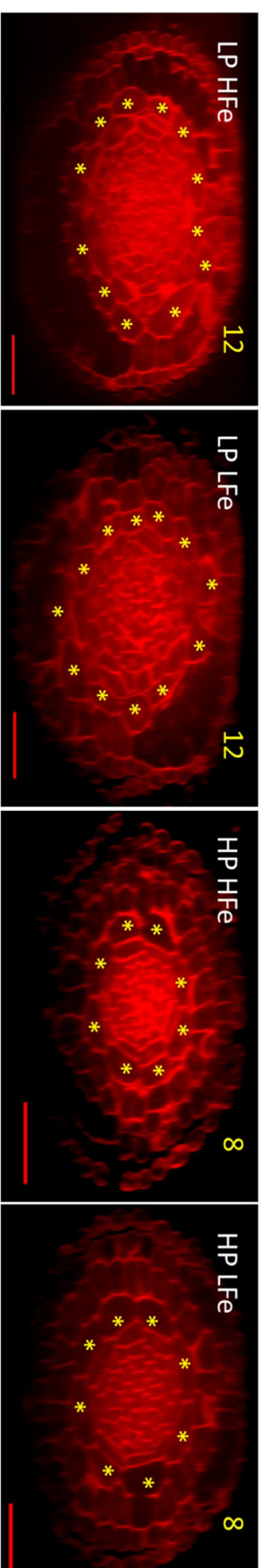


Figure 3.7 Exposure to low P but low iron maintains the CCFN response of Arabidopsis roots to low P
Pseudo-Schiff propidium iodide stained roots imaged using confocal microscopy to generate optical transverse cross sections. (a) yellow asterisks indicated cortex cell files; yellow numbers indicate number of cortex cell files present. Treatments (LP HFe, low P high Fe; LP LFe, low P low Fe; HP HFe, high P high Fe; HP LFe, high P low Fe) are in white text. Scale bars= 50µm. (b) bar chart showing average CCFN for each treatment: LP HFe, CCFN=10, n=11; LP LFe, CCFN=11, n=11; HP HFe, CCFN=8.75, n=12; HP LFe, CCFN=8.45, n=11. Error bars represent standard deviation. a= statistically significantly different from c (p=0.05), b= statistically significantly different from c (p=0.001).

3.8 Discussion

This chapter originally set out to answer four main questions- these were:

1. What is the critical P concentration at which a proliferation of cortex cells is seen?
2. What is the time frame of this response?
3. Which tissues are affected by this response?
4. What is the location of the response?

To summarise the answers to these questions, it can be said that increased CCFN occurs at P concentrations of less than 400 μ M and that this response can be seen within 24 hours.

From my results presented in sections 3.2, 3.3 and 3.4, it can be concluded that my low P growth conditions reflect the typical responses which have been reported previously (Bates & Lynch, 1996; López-Bucio et al., 2002; Svistoonoff et al., 2007). There are some differences between my results and previous studies but these are most likely reflected in the great variety of methodologies used. The studies summarised in chapter 3 use a great variety of specific media, growth environments, P concentration and sample tissue at various ages. These factors generate a degree of variability in the reported responses and highlight the need for me to characterise these responses under my own conditions before proceeding. The results presented in figure 3.1 show that 50 μ M phosphate reflect changes seen in response to low P in previous literature.

These results for primary root elongation rate are in line with previous findings by Ma *et al.* (2003) who reported that 9 day old seedlings growing on 1mM P elongate at a rate of 450µm/hour which can be seen at this phase in the present work (figure 3.1e) However, when grown on low P media primary root elongation rate does not increase over time, remaining at ~1µm/hour throughout the time course (figure 3.1e). This result represents a more dramatic reduction in primary root elongation; Ma *et al.*, (2003) report an elongation rate on low P of around 250µm/hour). However, both sets of results indicate a strong decrease in elongation associated with P deficiency.

As mentioned previously, Ma *et al.*, (2003) report that they grew seedlings by germinating on horizontal plates so that the root tips reached the underside of the agar layer. These plates were then turned to 45° so that the roots would grow along the bottom of the plate towards the gravity vector. It could be possible that this growth method imposes a different set of factors, such as a wetter environment and the fact that the roots must penetrate the agar, which influence the rate of root elongation.

Nacry *et al.*, (2005) also report on the effect of low P on primary root elongation in arabidopsis. This increase is from around 0.25cm/day to around 1cm/day. Despite the lack of difference between low and high P for 12 days of growth, low P treated plants start to significantly slow their growth compared to high P after this time point. Similarly, primary root length does not significantly differ between the two treatments until day 14, at which point low P primary roots are around 7cm and high P roots are around 8cm.

Not all studies have examined the primary root elongation rate, however a number of previous studies have also reported a decrease in primary root length on low P. López-Bucio et al., (2002) report a decrease in primary root length from 6cm to 1cm between 1mM and then 1 μ M and 10 μ M P concentrations in 17d old seedlings.

The LRD found in the present study at low P is fairly consistent with those of Williamson et al., (2001) who found that LRD was on average around 0.6 LR/cm when plants were grown on 100 μ M P. However, the increase they see from high to low P is not as large as is seen in this study- an increase in LRD of only around 0.1 was reported. Conversely, López-Bucio et al., (2002) report an almost 10-fold increase in lateral root density between 1mM and 1 μ M P which is even greater than the increase seen here. Nacry et al., (2005) also report an increase in LRD between 1mM and 3 μ M P treated plants from 0.75 LR/cm to 1LR/cm. This increase is on a similar magnitude to that found in the present study, although both low P and high P LRDs are much higher than those presented here.

This response is not only consistent with other findings in arabidopsis but also mirrors those which seem conserved in some other plants. Borch, *et al.* 1999) describe an increase in LRD from 4.5 LR/cm to 5.5 LR/cm between 50 μ M and 1 μ M P in *Phaseolus vulgaris*.

The experiments in section 3.3 aimed to develop some temporal resolution of the CCFN response to low P. To do this, a time-course experiment was performed whereby the response to low P could be tracked along the course of 1, 2, 3, 4, 7, and 10 days. The findings from this experiment are not completely in line with previous studies, although it does fall in to a similar time frame. Cederholm & Benfey, (2015) use the method of germinating seedlings on P replete media until 4 days old and then transferring them to plates which provide a P limiting environment. They found that at 1 day post transfer (DPT) there was no significant change in CCFN, but at 2 DPT the CCFN increases to 1.5x that of the high P control. (Ticconi et al., 2009) investigate changes to ground tissue patterning in seedlings transferred from high P to -P. They demonstrate that -P conditions elicit extra divisions in ground tissue 1 DPT in a mutant of *PHOSPHATE DEFICIENT ROOT 2 (PDR2)*, *pdr2*, which is hypersensitive to low P and seems to have a role in regulating SHR movement/localisation. However, their WT controls do not appear to show any changes in ground tissue patterning at this point. It is unclear from the paper at which point they would start to see changes occurring in WT roots. Despite this, they state that *CYCB1;1::GUS* (a marker of cell division in the meristem and differentiation zone) expression is reduced after 5 days on -P. Nevertheless, a complete loss of *CYCB1;1::GUS* expression is visible at 10 DPT; but it is important to consider that in their experiments, QC identity also appears to be lost at this point. Zhang *et al.* (2003) report that low P induces increase in CCFN, but the only time frame that they give is that the plants were grown on low P for 12 days. On a shorter time scale than other studies considered here, Devaiah *et al.* (2007) report

transcript changes in *WRKY75*, a low P responsive transcription factor, within 3 hours of P starvation. Wang et al., (2014) does not provide quite the temporal resolution as the former study, but they do show that *WRKY45*, an arabidopsis low P responsive transcription factor, is upregulated transcriptionally after 1d low P treatment. Its expression increases each day up to 5d low P treatment-up to around three times that after 1 day. On the same time scale Gutiérrez-Alanís et al., (2017) show that the small signalling peptide CLE14 is expressed in response to low P at 24h post transfer from high to low P; a seemingly low P specific response. Del Pozo et al., (1999) show that after two days on – P, AtACP5 protein, an acid phosphatase, is induced considerably, later showing that this protein is expressed specifically in response to low P when tested against a number of nutrient and other abiotic stresses.

These previous studies suggest some variability in the time frame of low P responses in arabidopsis, but the overall picture appears to be that anatomical changes do begin to be seen within 1-2 days of low P and that the plants begin to respond to low P at the molecular level within hours. Ma *et al.* (2003) report a cell flux of cortex and epidermal cells through the arabidopsis root meristem of around 2 cells per hour. This being the case, within 24 hours it is feasible that assuming a rapid P sensing and signalling cascade is in place, then enough cell cycles would have passed in this time frame for the anatomical changes to be made along a considerable length of root. The data presented here shows that the response of interest in this study (CCFN increase) occurs at 1 day of low P treatment. This is also critical to know, because if changes in cell anatomy occur

within 24 hours, then regulatory changes in gene expression and hormone signalling must occur on a much shorter time scale.

In section 3.5, it was shown that the only tissue whose cell file numbers increased in response to low P as a result of increased proliferative divisions was the cortex. However, these data are at odds with the other most comparable study; Cederholm & Benfey, (2015) show that after 2 DPT from high to low P, there is in fact a statistically significant increase in the number of endodermal cell files. The latter is the only existing previous study which examines this specific response in that much detail, however Zhang *et al.* (2003) show images wherein the number of endodermal cell files does not seem to differ between low and high P treated roots (although this is by comparing two individual cross sections by eye).

This discrepancy could be due to the age of the plants in question; the roots studied in Cederholm & Benfey, (2015) would be most likely no more than six days old. They transfer seedlings to low P at “5 days post imbibition” and then analyse at “1 and 2 days post transfer”. Considering that the seeds take at least one day to germinate the analysis can only be done at 6 days of growth at most. Other papers which have reported on radial anatomy of ground tissues such as Welch *et al.*, (2007) and Long *et al.*, (2015) describe the presence of around 8-9 endodermal cell files, but these are on 3 and 4 day old seedlings respectively- still much younger than presented here.

Regardless of age it does seem, based on available evidence, that in young seedlings (<7 days) it is possible for low P stress to induce proliferation of endodermal cell files. However since such a phenomenon is not detected in the

present study, at the age of plants used, it will not be considered as part of the response in the work carried out here.

Following the identification of tissues which increased their cell file number, it was also identified that trichoblast length was shortened in response to low P (figure 3.5), which it was hypothesised may reflect a response to increase the longitudinal density of root hairs.

Interestingly, on the occasions that trichoblast length has been assessed previously, findings and conclusions seem to contradict the present observations and presumed function of this response to low P. Ma, *et al.* (2003) investigated the dynamics of root elongation under P limiting conditions, which involved measuring the length of cells being produced at the root tip and their flux (i.e. number of cells produced per unit time). They report no significant difference in trichoblast cell length between low and high P treated plants. However, they do show that there is a reduction in the flux of trichoblast cells under low P conditions but this is may be an effect of the reduced root elongation rate caused by low P stress. In addition to this study contradicting the present results, Ma et al., (2001) use a mathematical modelling approach to assess how effective various root hair adaptations are for P acquisition. Their model predicted that increasing root hair density by increasing the number of trichoblast files increased P acquisition efficiency (PAE) to a higher degree than by decreasing trichoblast length. The model predicted a benefit from shortened trichoblast cells under certain conditions. These findings are difficult to conclude from in the real-world context, but could reinforce the idea that

different root hair patterning mechanisms work together to enhance root hair area and therefore nutrient uptake.

Under the conditions used in this study the only tissue whose cell file number changes in this response to low P by means of proliferative divisions within the meristem is the cortex. As a result of this increase there is a concomitant increase in the number of epidermal cell files in the trichoblast position. The results also indicate that this is most likely occurring in the cortex cell closest to the quiescent centre (i.e. immediately after the cortex/endodermis initial division). However it was suggested that the process of CCFN increase under low P is stochastic in nature, made apparent by the fact that CCFN varies amongst roots; this is seen more in low P treated roots but also in high P treated roots. It could simply be that CCFN is not that tightly regulated, which could be an age related feature of meristem maturation (Baum *et al.* 2002). Additionally, based on the presumption that this is a response to increase the frequency of trichoblast specification, it would fit the working hypothesis for the cortex divisions to occur early in meristem activity since trichoblast specification begins early in the meristem (Grebe, 2012; Lee & Schiefelbein, 1999). The finding that the proliferative divisions are cortex specific and therefore most likely a stochastic response to increase RH density also suggests that the changes seen are unlikely to be a direct result of metal toxicity or critical P starvation. Despite these considerations, it remains open whether divisions in the cortex could be occurring in cortex cells rootward to this point. However even if this is the case, the available evidence and arguments compel that most of the divisions but be occurring in the first cortex cell. For this reason,

experiments in following chapters will focus on the first 1-5 cortex cells after the cortex/endodermis initial division.

Taken together, these observations suggest a suite of changes through which the plants can adapt root hair development in response to low P. It will be interesting to consider if the same genetic pathway can control these process.

One prominent question is whether one signalling mechanism is capable of collectively orchestrating this suite of responses all at once or whether they are activated by distinct pathways. It is clear (at least in terms of phytohormones) that auxin and ethylene have interacting effects on root hair development. For example Zhang et al., (2003) showed that ethylene is essentially required for root hair elongation under low P conditions. Jones et al., (2009) demonstrate the requirement of auxin flow through non-hair cells to sustain root hair outgrowth by providing trichoblast cells with auxin. While Zhang et al., (2003) failed to find a contribution of ethylene signalling towards CCFN increase under low P, the one mutant which they found to influence this response was *eir1*. *eir1* is a mutant of the polar auxin transporter PIN2, which is required for generating basal-apical and apical-basal auxin flux in the cortex and epidermis respectively. This function has largely been researched as a significant contributor to root gravitropism (Rahman et al., 2010; Sukumar, *et al.* 2009) and has been identified as playing a role in RSA responses to low P (Nacry et al., 2005). The question of whether these responses are intrinsically linked remains open, but it seems that if a plant hormone is involved in regulating CCFN increase under low P then auxin is a more prominent candidate than ethylene at this stage.

Aside from phytohormones, another possible regulatory pathway for controlling this response is that of the *BIRD* genes, which are associated with the *SHORTROOT (SHR)/SCARECROW (SCR)* radial patterning mechanism. *BIRD* genes are a family of zinc-finger transcription factors which interact with the SHR/SCR complex which is required for determining endodermal cell fate (Helariutta et al., 2000; Paquette & Benfey, 2005). The *SHR/SCR* pathway is also required for the CEID event and specifying the endodermal/cortex tissue boundary; *BIRD* genes are involved in this specification. They are also required for cortex cell identity and, most relevantly here, controlling periclinal divisions which determine CCFN (Long et al., 2015; Welch et al., 2007). There is no current link in the literature between low P and *BIRD* genes as a family, although Ticconi et al., (2009) suggest connections between low P and *SHR* expression. Despite this, the strong influence of *BIRD* genes on CCFN make them a clear candidate for factors which may be at play during the low P response in cortex tissue.

Collectively, the data in this chapter provide the anatomical foundations to identify possible regulators of low P induced CCFN increase which are at play in the first few cortex cells in the root meristem.

Chapter 4: Identification of factors regulating the increased cortex cell file number response to low P in arabidopsis roots

4.1 Introduction

Few studies have shed light on what components may regulate low P dependent cortex cell proliferation in Arabidopsis roots. Zhang, *et al.* (2003) indicate that polar auxin transport, and not ethylene, is likely to regulate CCFN increase under low P conditions. They describe how two ethylene insensitive mutants, *ein2* and *ein4* do not display a deficiency in low P. They also show how ACC (ethylene precursor) and AVG (ethylene synthesis inhibitor) fail to affect the CCFN increase response to low P. *eir1* (an allele of *pin2*) did produce a phenotype whereby it does not exhibit an increase in CCFN in response to low P.

This is significant as PIN2 is required for auxin transport within the cortex and epidermis. In the cortex, PIN2 protein is localised to the rootward (apical) membrane of cells and the shootward (basal) membrane of cells in the epidermis (figure 4.1a, yellow arrows); this localisation pattern is also seen using immunolocalisation (figure 4.2b, left panel; Vieten et al., 2005). By means of this differential polar localisation PIN2 facilitates the flux of auxin rootward in the cortex and shootward in the epidermis, thus contributing to auxin reflux

at the root tip (Blilou et al., 2005a). Figure 4.1b shows a model of auxin flux through the root facilitated by a number of PIN proteins in the root tip. This localisation pattern and its role in polar auxin flux is required for gravitropic responses by regulating asymmetric root growth via mediating differential auxin flux (Jones et al., 2009; Rahman et al., 2010b; Schlicht et al., 2008). PIN2 also regulates root hair growth via its control of auxin flux through epidermal cells (Ganguly et al., 2010).

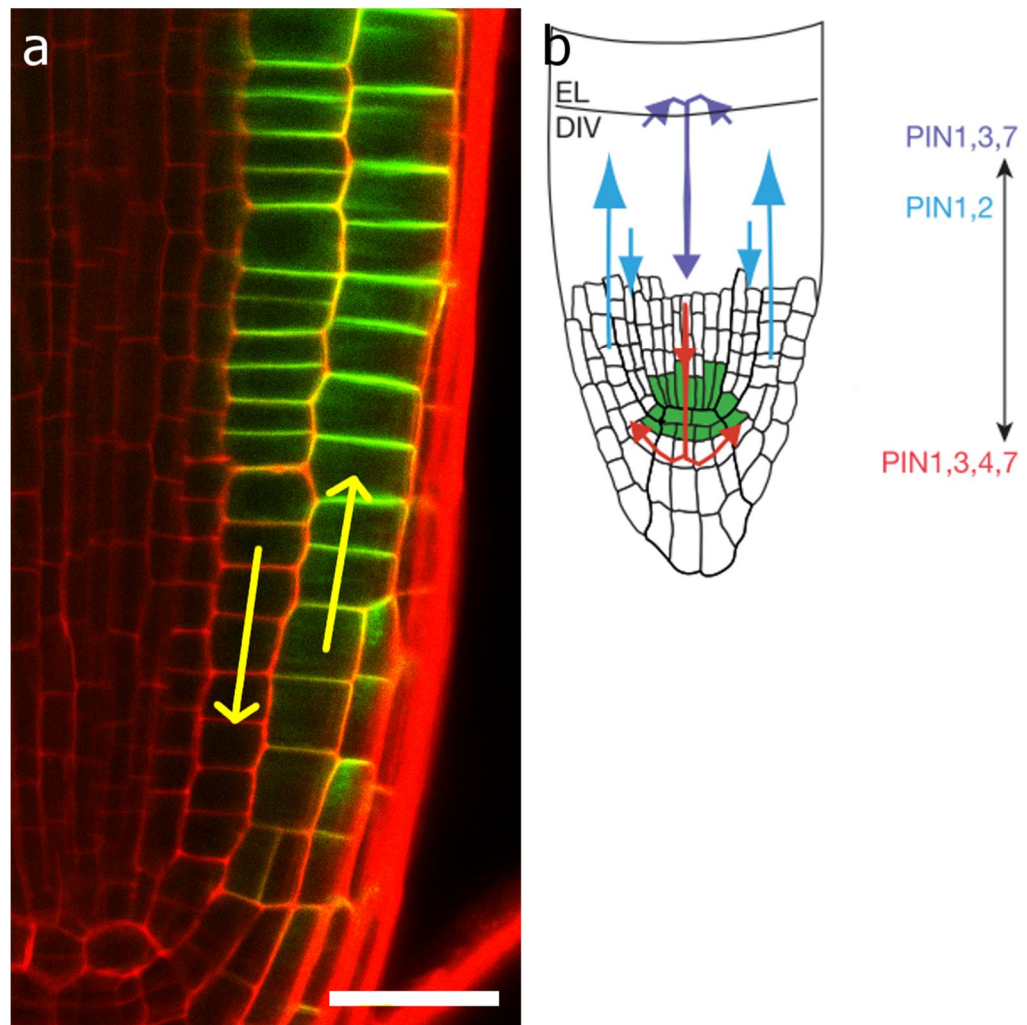


Figure 4.1 PIN2 protein is localised in a polar fashion in cortex and epidermis cells

PIN2 protein fused to green fluorescent protein expressed under *PIN2* promoter which displays the characteristic rootward/shootward polarisation seen between cortex and epidermis (a). Yellow arrows indicate the general direction of the polar localisation of PIN2:GFP domains in cortex and epidermis; PIN2:GFP is seen in green and propidium iodide stained cell walls in red. (b) shows a model of auxin distribution through the root tip as mediated by PINs 1, 2, 3, 4, and 7; contribution of PIN2 (in concert with PIN1) can be seen in blue arrows (taken from Blilou et al., 2005). PIN2 is proposed to effect the rootward flux of auxin in the cortex and shootward flux in epidermis. (EL= elongation zone, DIV= division zone) Scale bar= 25µm.

PIN2 is clearly an important component of the PAT system and is obviously important in low P CCFN increase, but it is not the only PIN which plays a role in this. Other PIN proteins which are found in the root tip are PIN1, 3, 4, and 7. However, these are all localised in the plasma membrane to facilitate cell-to-cell flux. Some PINs however are not localised in the plasma membrane: PIN5 and PIN8 are localised intracellularly and function to control intracellular auxin levels. These proteins however are so far only found in the male gametophyte and regulate pollen development and pollen tube growth; although their overexpression does cause auxin defects in a number of organs (Dal Bosco et al., 2012; Ding et al., 2012; Mravec et al., 2009). Figure 4.2d shows images of the subcellular localisation of PIN5 and PIN8 compared to that of plasma membrane localised PIN3.

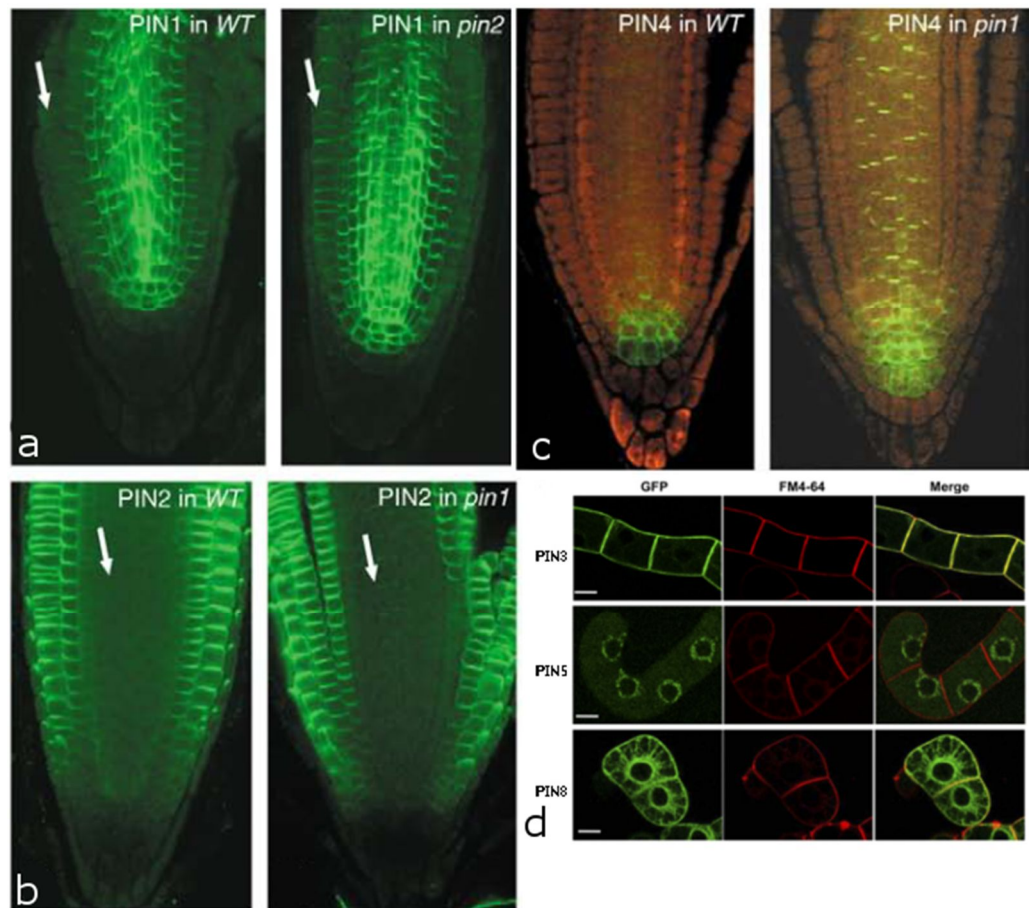


Figure 4.2 Localisation and functional cross-action amongst PIN proteins in the root tip

Immunolocalisation (a-c) and green fluorescent protein fusion localisation (d) of PIN1, PIN2, PIN3, PIN4, PIN5, and PIN8 in Arabidopsis root tips (a-c) and in tobacco BY-2 cultured cell lines (d). PIN proteins can expand their domain of expression in mutant scenarios: PIN1 is shown to occupy some of the PIN2 domain in *pin2* background (a), PIN2 can be found in PIN1 domain in *pin1* background (b), PIN4 can be found in PIN1 domains in *pin1* background (taken from Vieten et al., 2005)(c). PIN5 and PIN8 exhibit intracellular localisation in tobacco BY-2 cells, which differs from the plasma membrane localisation of PIN3 (d) (GFP= green fluorescent protein, FM4-64= a lipophilic, red fluorescent dye; taken from Ganguly et al., 2010).

Cederholm, *et al.* (2012) suggest a role for ROOT GROWTH FACTOR signalling peptides in regulating cortex cell division during low P response. Another recent study approaches the role of increased root hair density in roots which are exposed to $-P$ and how a transcription factor, *WRKY6*, may function in modulating cortex cell diameter. They suggest that this has a follow-on effect on trichoblast specification. However, they report no data to explain whether this is concomitant with increased CCFN or not. Therefore this transcription factor may or may not influence cortex divisions under low P.

One promising pathway which could regulate this response, but which has not been investigated in the low P response context, is the *SHORTROOT/SCARECROW* and *BIRD* gene radial patterning mechanism. This is a well-studied network which functions around the GRAS (GIBBERELLIC ACID INSENSITIVE [GAI], REPRESSOR OF GAI3 [RGA], and SCARECROW [SCR]) family transcription factor *SHORTROOT* (*SHR*). The basic principle of this pathway is that SHR protein is expressed in the stele, but lacking a nuclear localisation signal, remains in the cytoplasm. However, SHR protein is then transported from the cytoplasm of the stele cells, in to the QC, CEI, and endodermis where it associates with SCARECROW protein. The complex of SHR and SCR is then capable of nuclear localisation and activates a series of downstream gene expression which promotes the division of the cortex/endodermis initial cell and specifies endodermal cell fate. SCR also functions here to sequester SHR in the endodermis and ensure a single layer of endodermis. Several negative and positive regulators work to maintain QC, CEI, endodermal, and cortex identities and orchestrate the divisions which occur between these cell populations in

order to maintain meristem activity and pattern the tissues within the root (Cruz-Ramírez et al., 2012; Helariutta et al., 2000; Sozzani et al., 2010). In addition to SCR and SHR this protein complex also involves JACKDAW (JKD) and MAGPIE (MGP), which are C2H2 zinc-finger transcription factors. *jkd* mutants exhibit a phenotype of having additional CCFs compared with WT control, which is further enhanced by the *bib* mutation (figure 4.3e-f). This phenotype makes the role of JKD potentially interesting for the CCFN increase in response to low P.

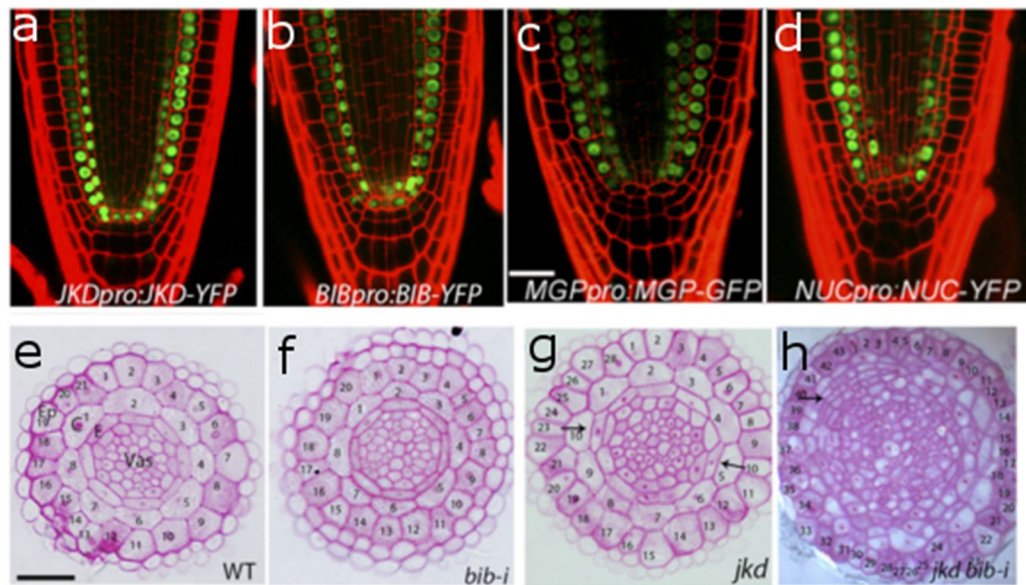


Figure 4.3 Localisation of BIRD proteins in the root tip and *jkd* and *bib* mutant radial organisation phenotypes

Localisation of protein-yellow/green fluorescent protein fusion tags of JKD (a), BIB (b), MGP (c), and NUC (d) under control of native promoters. Protein of each of these genes is found localised in the endodermis and cortex; MGP and NUC are excluded from quiescent centre (c, d). *jkd* mutant exhibits a phenotype of having higher CCFN compared with WT (10 compared to 8; g compared with e), which is added to by *bib* mutation whose phenotype is like that of WT (h, f compared with e). Taken from Long et al., (2015) Scale bars= 25μm, green signal= fluorescent protein tag, red signal= propidium iodide stained cell walls.

Ultimately this complex acts to promote division of the CEI involving the D-type cyclin *CYCLIN-D6*; *jkd* mutants exhibit an extended domain in *CYCD6* promoter activity (Long et al., 2015; figure 4.4a, b). *MGP* and *JKD* introduce a self-limiting mechanism whereby *JKD* promotes *SCR* activity, but where *MGP* inhibits *JKD* activity. Outside of the endodermis and CEI, *JKD* and *MGP* appear to have a similar function to each other in cortex cells.

In addition to *JKD* and *MGP*, this complex also recruits several other IDD (INDETERMINATE-DOMAIN) proteins. Members so far identified are *BALDIBIS (BIB)*, *NUTCRACKER (NUC)*, *BLUEJAY (BLJ)*, and *IMPERIAL EAGLE (IME)*; figure 4.3a-d show the localisation of *JKD*, *BIB*, *MGP*, and *NUC* proteins. Although they are most likely involved in cortex identity specification, they are also important for regulating periclinal divisions giving rise to new cortex cell files (Long et al., 2015; Welch et al., 2007). Moreno-Risueno et al. (2015) demonstrate the overlapping and antagonistic activities of this family of transcription factors, and propose that this multifaceted mechanism enables plasticity in the patterning of ground tissue. It was speculated that this system could act as a fine-tuning mechanism whereby the plasticity enables dynamic responses to environmental stimuli such as P deficiency.

The main objective of the work presented in this chapter was to find components which regulate the increase in CCFN in response to low P.

After consideration of the available literature the following conclusions can be drawn:

1. *pin2* mutants exhibit a deficiency in CCFN increase under low P; this indicates a very strong possibility for the requirement of PAT and therefore auxin signalling in this response
2. A prominent role for ethylene at this point seems unlikely, despite its importance in other root hair responses. Ma, *et al.* (2003) showed that a number of ethylene insensitive mutants, and treatment with ethylene precursor (ACC) and ethylene inhibitors did not affect CCFN. These ethylene related experiments did however affect root hair specific responses.
3. BIRD genes and the SHR mechanism have been shown to have a very strong influence over both cortex cell specification and CCFN determination. Since this is an early tissue patterning pathway it would make the SHR/BIRD pathway a good candidate for modulation during low P response.

In the context of the questions answered in the previous chapter and the available literature, five main questions arise:

1. *Are other components of the auxin transport/signalling pathway besides PIN2 required for the increase in CCFN which is observed under low P?*
This question is important because it may further reveal tissue specificity (i.e. via tissue specific auxin response elements), or whether auxin response or transport through other tissues is required.
2. *Are prominent components of the SHR/BIRD pathway required for this response?* If this is the case then it would be quite clear that this

pathway makes an obvious line of inquiry for the origin of CCFN increase under low P.

3. *Do any of these factors exhibit transcriptional changes in response to low P?* Studying the phenotype of simple (i.e. single or double) mutants will not always reveal the involvement of a given component in a particular response. Genetic and molecular redundancy could mask the role of a particular gene. Therefore RT-qPCR provides a simple technique to give a clue as to whether genes may be regulated in an alternative manner under certain conditions.

4. *Do any of these components undergo smaller or more localised (i.e. in smaller subsets of cells) changes in expression at the pre- or post-translational level?* RT-qPCR can shed light on the question of whether a given transcript is up- or down-regulated under given circumstances, thus suggesting whether it plays a role or not. However, there are two situations in which this technique alone may not provide an accurate understanding of function. Firstly if the change in expression of a component is specifically in the first cortex cell but not the rest of its domain, then this change may be masked or diluted by collecting transcripts from the whole root tip. Secondly this level of analysis does not consider regulation of proteins themselves. Therefore, investigating with higher resolution using transcriptional and translational

fluorescent protein fusion reporters will provide single cell specific information.

5. *Are ectopically expressed BIRD genes capable of disturbing the low P CCFN response?* This question is important because if BIRD overexpression has no effect on the low P response, then it would seem clear that the BIRD genes do not act as prominent regulators of CCFN under low P.

4.2 Screening of mutants for identification of candidate factors involved in low P CCFN increase

The auxin efflux carrier PIN2 has previously been shown to be required for increased CCFN in response to low P in Arabidopsis roots (Zhang et al., 2003). This pointed towards a role for auxin but as of yet no other auxin transport or signalling component has been assessed for its requirement in this response. Indeed a number of studies have investigated a role for auxin in low P responses, indicating that it is an important step in plants' adaptations to P limiting conditions. However these have focussed on root architectural responses and systemic physiological and biochemical responses (López-Bucio et al., 2002; Rogg, et al., 2001). These studies reveal that various components of the auxin signalling pathway control specific aspects of P response, whilst others are not required. For example, López-Bucio et al., 2002 demonstrate how the auxin resistant mutants *axr1-2*, *axr2-1*, and *axr4-1* all show normal auxin sensitivity in response to low P. However, they find that *iaa28-1* (a gain-of-function mutant of an *AUX/IAA* gene; Rogg et al. 2001) lacks a P induced

auxin dependent lateral root response. It is important to note here though that *iaa28-1* is also lateral root defective under normal P conditions. Nevertheless, this study helps to clarify the specific signalling components via which low P stress is perceived by plants in enacting particular responses. Conversely to the previous study, Nacry et al., (2005) found that *axr4-1* (a mutant defective in AUX1 localisation; Dharmasiri *et al.* 2006) is indeed required for architectural responses to low P. However, they focus more on the requirement of *axr4-1* for slowing primary root elongation which then leads to an increase in LR primordia initiation; they also suggest that AUX1 and PIN2 are required for this flux of auxin to the apical meristem. While these results seem at odds, it could be that they begin to untangle the network of different auxin pathways which orchestrate the different aspects of changes to RSA under low P. Narise et al., (2010) show that *IAA14*, *ARF7*, and *ARF19* are required for auxin responsive membrane remodelling in response to low P. Taken together with the results of Ruíz-Herrera & López-Bucio, (2013) this latter study shows how there are overlaps in the components between different low P responses. Ruíz-Herrera & López-Bucio, (2013) show that *ARF7* and *ARF19* are required for AI induced low P responses in lateral root and primary root elongation. These are quite different responses, but it seems that signal transduction via some of the same waypoints in the auxin network are required between responses. These studies all refer to arabidopsis, but Talboys, *et al.* (2014) demonstrate how low P results in changes to auxin transport in wheat (*Triticum aestivum*) roots which increases lateral root branching.

4.2.1 PIN2 and no other assessed auxin transporters are required for CCFN increase under low P

Zhang, *et al.* (2003) identified a role for PIN2 in the increased CCFN response to low P suggesting that differential auxin distribution, and therefore auxin response, are likely to be involved in this. However since the publication of this research nobody has revisited this data and further probed the role of auxin in this response. In this chapter auxin components were systematically screened for their role in the low P CCFN response using a range of mutants.

Arabidopsis has eight identified (*PINFORMED*) *PIN* genes (*PINs* 1-8), which are so-called due to the pin-like malformation of the inflorescence in the *pin1-1* mutant (Okada, *et al.* 1991). The encoded proteins are all auxin carriers, which essentially allow one-way flow of dissociated ($\text{IAA}^- + \text{H}^+$) auxin ions from the cytoplasm either out of the cell (PIN1, PIN2, PIN3, PIN4, PIN7) or in to intracellular compartments of the cell (PIN5, PIN6, PIN8) (Blilou *et al.*, 2005a; Ganguly *et al.*, 2010). This process is not active but occurs along a concentration gradient in an IAA^- specific manner. Of these PIN proteins, PIN1, PIN2, PIN3, PIN4, and PIN7 are expressed as plasma membrane localised carriers in root tips (Ganguly *et al.*, 2010; Křeček *et al.*, 2009). These PINs have all been shown to be active in various aspects of root growth and development. For example PIN7 is involved in directing auxin to form auxin maxima in vascular patterning in the stele, during ethylene-auxin cross-talk in lateral root initiation and outgrowth, and determining tissue patterning and polarity during embryo development (Friml *et al.*, 2003; Lewis, *et al.*, 2011; Muraro *et al.*, 2014). PIN3

has been found to be required for asymmetric growth in hypocotyl gravitropic responses (Rakusová et al., 2011). The latter study shows the versatility and flexibility of PIN function throughout the plant and different organs, since PIN2 is classically the primary PIN associated with asymmetric auxin flux and gravitropic growth in the root (Luschnig, et al. 1998).

The interaction of PINs with other hormone signalling pathways acts as to mediate hormone crosstalk. For example, PIN7 is activated by cytokinin signalling in the developing stele to direct auxin and generate auxin maxima at xylem poles in *Arabidopsis* (Bishopp et al., 2011). Interactions between ethylene and auxin transport via PINs has also been demonstrated; Lewis, et al. (2011) investigate how ethylene regulates the expression of PIN3 and PIN7 in roots by showing that ACC application inhibits lateral root formation via these transporters.

Collectively the data presents a web of interactions whereby hormone cross-talk is integrated to influence the flux of auxin and also where the flux and accumulation of auxin can influence the effects of other hormones. From this it can be seen that PIN proteins represent a central framework in the directionality and polarity of growth, development and spatial patterning of the root. Therefore an understanding of which auxin transporters affect CCF proliferation could help identify a mode of auxin action and a pathway of auxin through the root. Additionally, although many of the auxin transporter proteins appear to have fairly specific roles in development, collectively they contribute to the polar flux of auxin through the root tip and so could potentially have

effects on root auxin distribution. For this reason every root-specific, plasma-membrane PIN in the *A. thaliana* genome which is involved in polar auxin flux, particularly in and around cortex tissue was assessed.

As well as the PIN family of proteins, there are several auxin influx carriers denoted as the (AUXIN1/LIKE-AUX1) AUX1/LAX plant specific amino-acid permease-like plasma membrane proteins. It has been shown that these proteins enable the flow of auxin in to cells to regulate auxin flux through roots to control growth and development (Bennett et al., 1996; Peret et al., 2012; Swarup et al., 2008). There are three LAX proteins, LAX1, LAX2, and LAX3. LAX2 and LAX3 are both found to be expressed in the root tip (Peret et al., 2012). AUX1 has classically been a factor considered important for root gravitropism (Bennett et al., 1996), and has more recently also been shown to have an influence on root hair elongation by generating an auxin flow canal through non-hair cells which feeds hair cells (Jones et al., 2009). As well as having similar roles in root growth to PIN2, *aux1* is also ethylene resistant (like *pin2*). This could mean that PIN2 and AUX1 are operating through a similar ethylene associated auxin transport system. If so, then it also seems possible that AUX1 may have an impact on CCFN increase under low P conditions. It has been shown that ethylene can impact auxin mediated developmental processes, such as lateral root development (Lewis et al., 2011), and *pin2* and *aux1* both exhibit insensitivity to ethylene induced inhibition of root elongation (Luschnig et al., 1998; Roman, et al., 1995).

In order to test the involvement of AUX1/LAX proteins and PINs in the low P CCFN response, seeds of mutants *aux1*, *lax2*, *lax3*, *pin2*, *pin1*, *pin3*, and *pin3pin7* were surface sterilised, sown on to either 50µM P, 5µM Fe or 1mM, 50µM Fe and grown for 12 days. After this, roots were harvested, live-stained with propidium iodide and imaged using confocal microscopy. CCFN was then counting using imagej image processing software.

The findings presented here show that all of the mutants studied exhibit a WT-like response in CCFN to low P stimulus, except *pin2* (figure 4.4).

Line	High P CCFN	Low P CCFN
<i>aux1</i>	8-9	12-13
<i>lax2-1</i>	9	11-12
<i>lax2-3</i>	9-10	10-12
<i>lax3-1</i>	8-9	10-11
<i>lax3-2</i>	8	11
<i>pin2</i>	8	8-9
<i>pin1</i>	9-10	13-14
<i>pin3</i>	8	14
<i>pin3pin7</i>	9	11-12
Col 0	8	11-12

Table 4.1 CCFN for auxin transporter mutants under high or low P conditions

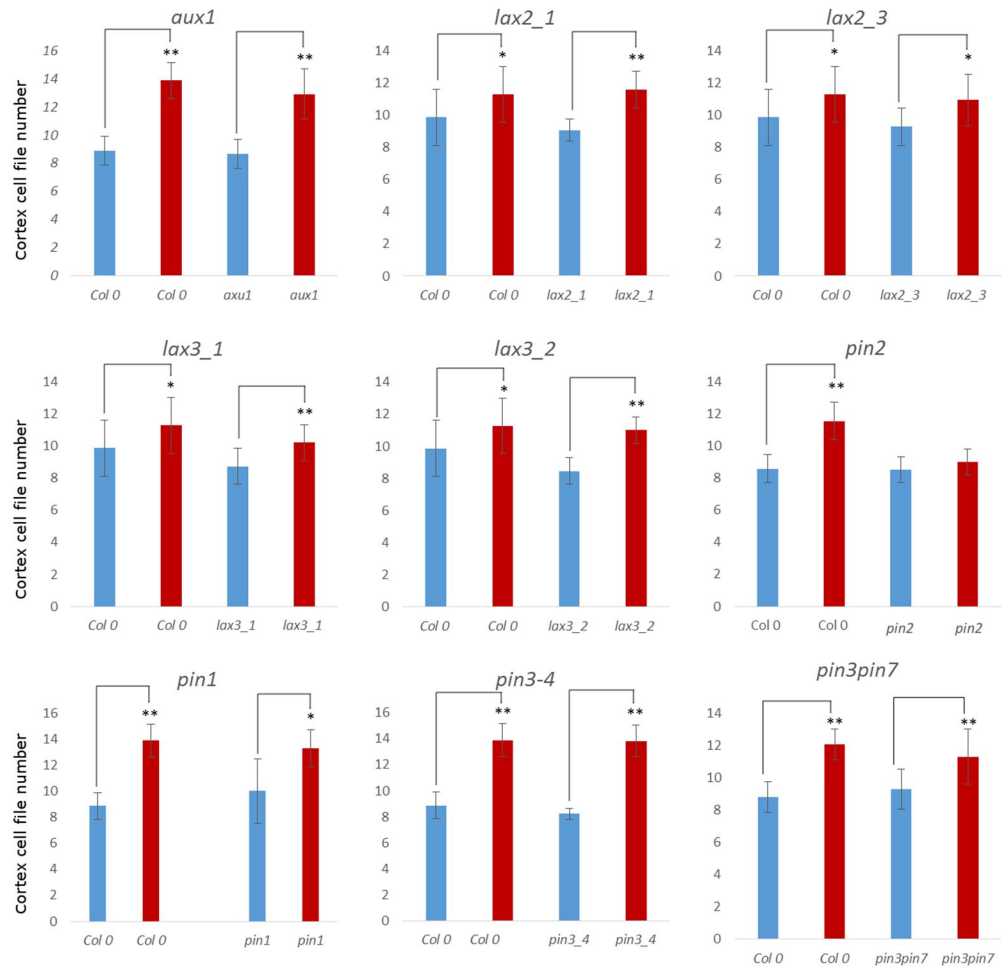


Figure 4.4 *aux*, *lax*, and *pin* mutants have wild type CCFN responses to low P, but *pin2* does not.

Bar charts showing cortex cell numbers for low and high P treated roots of mutants *aux1* (n=12, and 12, for high and low P respectively), *lax2-1*, *lax2-3*, *lax3-1*, *lax3-2* (n=15 for all), *pin2* (n=12, and 15), and *pin1* (n=7, and 14), *pin3-4* (n=13, and 12), and *pin3pin7* (n=18, and 17). Bars on the left of each chart represent values for Columbia 0 wild type control plants; blue bars are values under high P conditions and red bars are values under low P conditions. The only mutant which shows a change in CCFN under low P conditions is *pin2*, with other mutants exhibiting a wild type-like increase in CCFN on low P. Bars on the right of each chart show values for the mutant lines. Error bars are standard deviation. Differences between values statistically significant to $p < 0.001$ (**) or $p < 0.05$ (*); probabilities based on Student's t test.

All of the differences between low and high P for each mutant line are statistically significant to at least $p < 0.05$, except for *pin2*.

The results of this investigation in to the possible roles of PINs in low P CCFN response show that PIN2 remains as the one PIN which seems to be essential to the response. Out of all the auxin carrier mutants studied: *aux1*, *lax2*, *lax3*, *pin2*, *pin1*, and *pin3*, the only mutant which had no statistically significant difference in CCFN between low and high P was *pin2*.

There are explanations for this outcome. Firstly, it may be that because PIN2 is the only PIN which is actually expressed in the cortex and is directly required for auxin flux through this tissue, then only the loss of this transporter has a significant impact on auxin accumulation in the cortex. While other PINs are important for polar flux of auxin through the root, such as PIN1 bringing auxin to the root apex through the stele, the disturbance to global auxin flux by these mutations may not be enough to significantly perturb PIN2 mediated distribution of auxin across the cortex cells. This is related to the functional redundancy of PINs in the root. Several of the PINs in the root tip have overlapping domains of expression and activity, whereby they essentially exert the same effect on auxin flow through those tissues. For example, *PIN3* and *PIN7* are both expressed in roughly the same cells within the columella; this also overlaps with the region in which *PIN4* is found, which is in the early columella, QC, and CEI. Both of these PINs appear to have a significant effect on auxin flux through this part of the root meristem, although *PIN3* appears to be more important for gravitropism (Friml, *et al.* 2002; Rakusová *et al.*, 2011),

whereas PIN4 seems to affect QC activity as well as embryo and meristem patterning (Friml, et al., 2002). This shows that although PINs may be expressed in a similar domain, they may be contributing unequally to different processes.

It has also been shown that some *PINs* are capable of extending their expression domains in mutants of other *PIN* genes (Blilou et al., 2005b; Vieten et al., 2005). The latter two studies show how *PINs* are plastic in their expression and localisation under conditions of the loss of other *PINs*. For example, a particularly remarkable case is that reported by Vieten et al., (2005) whereby exclusively *PIN1* and *PIN7* are expressed in the first few divisions after zygote division. If either of these *PINs* are mutated there are aberrations to embryo development, however the *pin1pin7* double mutant exhibits a phenotype no stronger than the single mutants. However, a *pin1pin3pin4pin7* quadruple mutant has a very strong defect at this stage, even though *PIN3* and *PIN4* are not expressed this early in these cells. Upon further investigation it became apparent that in the *pin1pin7* double mutant, PIN4 is ectopically expressed in the *PIN1/PIN7* domain and even shows the same polar membrane localisation as *PIN7*. This showed that there is strong cross regulation of PIN expression which is most likely controlled through differential auxin gradients via AUX/IAA-dependent signalling. More relevant to the present work, they also showed that a similar phenotype occurs in roots; for example, in *pin1* mutants *PIN4* is seen expressed strongly throughout the *PIN1* domain when usually it is only restricted to a far more apical domain around the columella and QC.

Some possible further work which may help to untangle and remove some of these possibly confounding effects could be experiments in which more auxin transport activity in the root is abolished. A fairly rough way of doing this would be to treat roots with naphthylphthalamic acid (NPA) on low and high P media and then see if this has any effect on radial patterning in the cortex. NPA has a strong blocking effect on auxin flux/transport and should create the same effect as all auxin efflux/influx carriers being inactive. Although this is quite a non-specific way of looking at the effects of auxin carriers on particular processes which could have pleiotropic effects, it is a very rapid and easy way of determining if auxin transport will be an interesting avenue to follow. Indeed many published studies which investigate auxin transport begin by testing for NPA sensitivity. However due to the non-specific, widespread effect of NPA, it would have an effect on PIN2 making the results hard to interpret since it does not isolate the roles of other PINs. An alternative to this could be to study the effect of multiple mutations. Although these are often hard to propagate due to embryo lethality (Vieten et al., 2005) and, similarly to NPA treatment, have widespread effects which do not allow the isolation of any particular PINs.

4.2.2 Root-specific activating ARFs are not required for CCFN under low P

As well as major root auxin transporters, the roles of root specific activating *AUXIN RESPONSE FACTORS* (*ARFs*) were investigated. The majority of *ARFs* function by transcriptional repression (i.e. they are repressor *ARFs*), which act to block transcription of target genes by binding to their promoter regions.

Thus, when released from their bound AUX/IAA proteins, they will downregulate sets of genes in response to auxin. However, auxin can activate downstream target genes by functioning via activating *ARFs*. In arabidopsis, just five of the twenty three identified *ARFs* are known to function as activators (Tiwari *et al.* 2003; Ulmasov *et al.* 1999); these are: *ARF5*, *ARF6*, *ARF7*, *ARF8*, and *ARF19*. The promoters of all of these *ARFs* are active in the root tip; Rademacher *et al.*, (2011) used promoter-nuclear localised GFP fusion lines to visualise this activity in the root. Their data show that they have quite different expression domains- *ARF5* is expressed in vascular cylinder, the apical-most layer of the columella and in lateral root cap cells. *ARF6* appears to be expressed in all tissues, *ARF7* in just three cell tiers in the stele just basal from the QC, and *ARF8* in stele, epidermis and faintly in early cortex or CEI. *ARF19* is found in all tissues but appears to be absent from QC and early meristematic cells. Based on the expression domains of these *ARFs* and published reports of their roles in development/environmental responses four *ARFs* were selected to investigate. *ARFs* 6, 8, and 19 whose expression domains all appear to include cortex tissue were chosen for screening, plus *ARF7* which is not expressed in cortex but has previously been reported to exhibit some redundancy with *ARF19* (Kang *et al.* 2013; Okushima *et al.* 2007a). Viable mutants of these *ARFs* are available, however the use of *arf5* mutants was not possible since this mutation leads to dramatic embryogenic defects which does not allow growth of viable plants. Studies of these mutants have led to important findings about embryo patterning but would not be useful for

primary root anatomy studies on low P (Hardtke & Berleth, 1998; Krogan *et al.* 2014).

The roles of these *ARFs* in nutrient stress conditions is not well investigated to date. However, Gifford *et al.* (2008) found that *ARF8* is up-regulated in pericycle in response to nitrogen availability which plays a role in mediating auxin-controlled root architecture plasticity. As mentioned above, it was also shown by Narise *et al.*, (2010) that *ARF7* and *ARF19* contribute to changes in lipid metabolism under P stress. Besides this knowledge it is well established that *ARF7* and *ARF19* collectively are important for initiating and patterning lateral root initiation in roots (Goh *et al.* 2012; Ito *et al.*, 2016; Kang *et al.*, 2013). Given the involvement of lateral root patterning/density in response to low P, it seems reasonable that *ARF7* and *ARF19* could be involved in this response.

To test whether any of these auxin signal transduction and transport elements are involved in low P induced CCFN increase, mutants of these genes were grown on low or high P for 12 days. Root tips were then stained with propidium iodide and imaged using confocal microscopy.

The results presented here show that none of the activating *ARFs* which were assessed had a significant effect on CCFN increase under low P. All of the mutants, *arf6*, *arf8*, *arf6arf8*, *arf7*, and *arf19* exhibit a CCFN statistically significantly different between low and high P treatments (Figure 4.5).

Line	high P CCFN	low P CCFN
<i>arf6</i>	8-9	10-11
<i>arf8</i>	8	11
<i>arf6arf8</i>	9	11
<i>arf7</i>	8	10
<i>arf19</i>	8-9	10-11
Col. 0	8	10-11

Table 3.2 CCFN of mutants of activating ARFs on low or high P

The differences between low and high P for each of these lines is statistically significant to at least $p < 0.05$ (figure 4.5).

These findings indicate that no individual *ARF* is absolutely required for the response. However, there are other explanations for this outcome.

Firstly, it could be that auxin mediated action on the cell division activity of early cortex cells could be working via repressor *ARFs*. Some of these are indeed expressed in the root, including *ARF1*, 2, 9, 10, 11, 16, and 18 which are all canonical repressor *ARFs* (Guilfoyle & Hagen, 2007; Rademacher et al., 2011). Since mutants of these factors have not been assessed, it remains a possibility that they are involved in this response.

Secondly, it is possible that genetic redundancy between *ARFs* occurring in the root may be masking the collective role of *ARFs* in the low P CCFN increase response. This should have been overcome by the *arf6/arf8* double mutant whose expression domains in the root overlap and have been shown to have synergistic effects on development previously (Nagpal, 2005). Similarly, *ARF19* and *ARF7* are known to exhibit redundancy with each other- this is especially

prominent in lateral root development (Okushima *et al.* 2007b; Kang *et al.*, (2013).

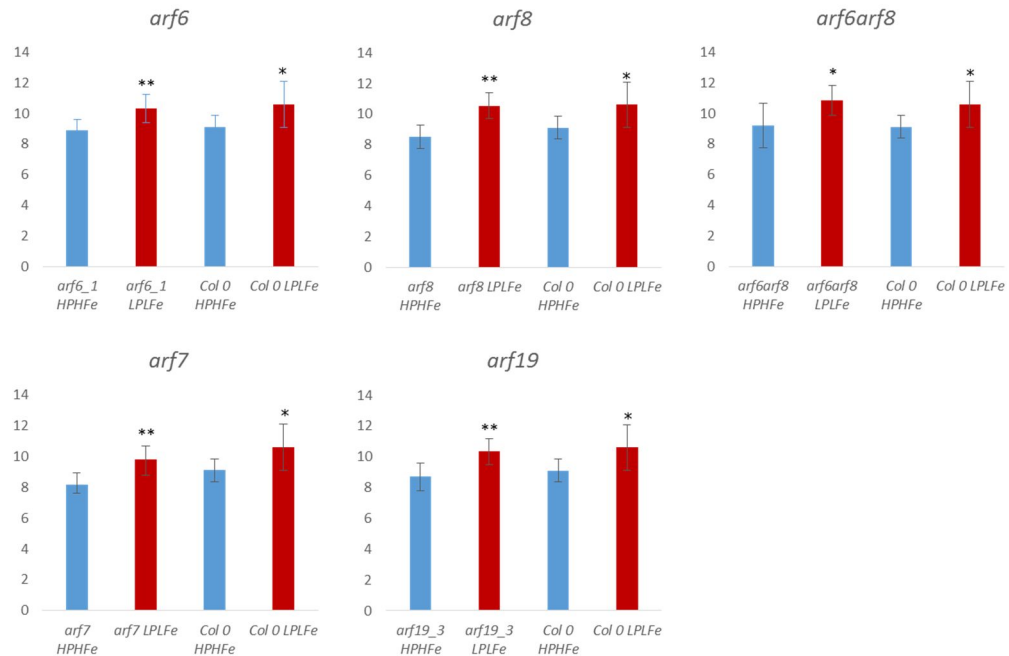


Figure 4.5 *arf* mutants do not respond differently to wild type in their CCFN on low P

Bar charts showing cortex cell numbers for mutants *arf6* (n= 15 for high P, 16 for low P), *arf8* (n= 12, and 13), *arf6arf8* (n=10, and 12), *arf7* (n=12, and 12), and *arf19* (n=14, and 13). Bars on the left of each chart represent values for mutant lines; blue bars are values under high P conditions and red bars represent values under low P conditions. Bars on the right of each chart show values for wild type Columbia 0 plants. None of the *arf* mutants show a pattern of CCFN different to the Col 0 wild type control. Error bars represent standard deviation. Differences between values statistically significant with $p < 0.001$ (**) or $p < 0.05$ (*); probabilities based on Student's t test.

Not only can genetic redundancy play a part, but functional redundancy has also been shown to occur with other proteins. It has been shown with PIN proteins that the loss of one PIN protein by mutation can lead to a change in expression of other PINs to make up for this mutation (Blilou *et al.*, 2005a). This phenomenon is not studied in regard to ARFs here, but is an additional possibility which could lead to redundancy in ARF activity even between tissues where a particular ARF may not “normally” be expressed.

Thirdly, it could be that the auxin response here may not be acting through the traditional TIR1/AFB signalling pathway which the activity of most ARFs is based upon. The traditional conception of auxin signalling is based on the binding of auxin to TRANSPORT INHIBITOR RESPONSE1 (TIR1) or AUXIN RECEPTOR F-BOX (AFB) proteins. Once bound with auxin, these proteins are then capable of associating with the SCF ubiquitin ligase complex to ubiquitinate AUX/IAA proteins and target them for proteolytic degradation. These AUX/IAA proteins bind to ARFs at two C-terminal domains III and IV (forming the PB1 domain), which share sequence similarity both between and within the two families and enable homo-dimerisation. By binding ARFs, AUX/IAA proteins are capable of repressing their function; destruction of AUX/IAAs by the TIR/AFB-auxin-SCF-proteasome complex then enables ARFs to function.

However, not all ARFs contain these homo-dimerisation domains and so do not function via the AUX/IAA repression/de-repression pathway. Until recently it was unclear how these ARFs functioned without the classical model of AUX/IAA interaction. Simonini et al., (2016) identified and describe how the protein ETTIN (ETT)/ARF3 functions in a non-canonical auxin signalling pathway. In this model ETT/ARF3, which lacks a PB1 domain, associates with bHLH transcription factor INDEHISCENT1 (IND). Together, at low auxin concentrations, these two proteins are required to repress the expression of *PINOID* (*PID*) in specific domains in the gynoecium. However, at high auxin concentrations, the IAA molecule binds at a site within the complex causing ETT and IND to dissociate; this dissociation leads to derepression of the *PID* locus and changes in auxin distribution via PINs follow. This auxin receptor complex is important for

modulating auxin flux and, so, polarity within the developing gynoecium; however, these factors have also been shown to play a prominent role in lateral root development. Simonini et al., (2016) suggest that this mechanism may offer a faster return to the pre-auxin-response state of receptivity since its auxin sensitivity need not wait upon the replenishment of AUX/IAA proteins. With this in mind it would seem a suitable signal transduction pathway for a local, rapid-onset, transient alteration to anatomy which must respond dynamically to spatial changes in P concentration.

While this non-canonical mechanism seems to be active in lateral root initiation and patterning, there seems to be no evidence for the expression of *ARF3* in root tips; in mature lateral roots its expression diminishes in the tip and only remains in the stele (Rademacher et al., 2011; Simonini et al., 2016). Because of this, there is no strong reason to believe that *ARF3* is active in the root tip and playing a role in the CCFN increase under low P. Although *ARF3* is not expressed in the root tip under “normal” (i.e. P replete) conditions (at least this is not detected using current reporter constructs), there is no detailed study in to the effect of low P on its expression. In lieu of detailed study however, data available on the *Arabidopsis* *eFP Browser* expression data resource website (Brady, 2007) also shows very low expression of this gene in root tips, including cortex tissue under normal and low P conditions.

Although *ETT/ARF3* does not seem to be found in the root tip, it is not the only ARF to lack a PB1 domain- *ARF13* has a similar arrangement of domains to *ARF3* and is expressed in the root (Guilfoyle & Hagen, 2007; Rademacher et al., 2011).

Given the complexity of auxin perception and signal transduction, and that such a non-canonical perception mechanism has been found, it remains a possibility that there could be as-yet unknown pathways via which auxin may exert its effects. Therefore, while the *arf* mutant screen in this chapter did not reveal a role for any of the *ARFs* tested here, this does not mean that auxin signal transduction is not important. It could be that the specific response of CCFN increase during low P stress acts via an undiscovered or currently poorly understood pathway.

4.2.3 BIRD gene and SHR mutations do not affect CCFN increase under low P

Besides PIN2 and RGFs, no other components have been investigated in relation to this CCFN increase low P response. However, there are mutants known to affect cell identity in the ground tissue in general. Since the *SHR/SCR* pathway is already well known for its function in regulating endodermal cell identity, division, and spatial boundaries it is logical to consider factors which interact within this network. For this reason, this section will focus on the *BIRD* genes- this family of genes encodes a family of C2H2 zinc-finger proteins which are part of the INDETERMINATE DOMAIN (IDD) transcription factor group.

The action of *JACKDAW* (*IDD10/JKD*) and *MAGPIE* (*IDD3/MGP*) were first identified by Welch et al., (2007) in a GUS promoter-trap screen for genes expressed in the *SCR* domain which exhibit ground tissue defects. One

promoter trap mutant stood out as being expressed in this domain and having aberrant periclinal divisions of the ground tissues (i.e. contrasting the single indeterminate ground tissue layer found in the *scarecrow* mutant; attracting the name '*jackdaw*'). This phenotype can be seen in figure 4.3g, which is enhanced by the *bib* mutation (another BIRD gene, *baldis* [IDD9]; Long et al., 2015; figure 4.3h). A mutation in the related *MAGPIE* was found to complement the *jkd* phenotype, thus leading the authors to conclude that the two genes had antagonistic roles within the *SHR/SCR* pathway during ground tissue development. They further demonstrate using yeast-two-hybrid that both MGP and JKD physically interact with SCR and SHR. Ever since this finding, further research has ensued into the involvement of *IDD/BIRD* genes in the radial patterning of ground tissues in roots. Long *et al.*, (2015) investigated the interactions between JKD, MGP and two other *IDD/BIRD* transcription factors, BALDIBIS (IDD9/BIB) and NUTCRACKER (IDD8/NUC). They found that JKD and BIB act to retain SCR/SHR in the nucleus and restrict this complex to the endodermis, and that MGP and NUC promote periclinal divisions within cortex and endodermis. It seems from genetic evidence that the pairs *MGP* and *NUC*, and *JKD* and *BIB* appear to interact partially antagonistically with each other. Hassan *et al.*(2010) show how *JKD* is also important as a signal originating from cortex cells to provide positional information in patterning of trichoblast (root hair) files in the epidermis. *JKD* functions in this patterning mechanism by acting upstream of *SCRAMBLED* (*SCM*) to modulate *SCM*-mediated regulation of *WEREWOLF* (*WER*) to establish a bias in the stabilisation of alternating H/N cell

fate in the epidermis. They found that this activity of *JKD* is independent of *SHR*, *SCR*, and *MGP*, suggesting a novel function of *JKD* in radial patterning of roots.

As well as the radial patterning model involving *SHR* and *SCR*, (Miyashima, *et al.* 2009) describe a role for *ARGONAUTE1 (AGO1)*, a central component of miRNA processing machinery. They show that *ago* mutants exhibit a phenotype characterised by extra layers of ground tissue and aberrant periclinal divisions in these cells. They demonstrate that these mutations are epistatic to *scr* mutants, which they argue suggests that the two pathways act independently. The expression of *MGP* and *NUC* is reduced in *ago* mutants and very strongly reduced in *ago scr* double mutants; at first making it seem that the two pathways could be related. However, the authors postulate that this is due to a loss of endodermal cell identity caused by the dramatic disturbance to ground tissue patterning. While the authors claim this to be a separate pathway to *SHR/SCR*, it would be logical to put forward that the miRNA processing function of *AGO1* could well be an important factor in the fine tuning of *SHR/SCR/BIRD* activity. This connection however, may indeed not be direct and experiments have not yet been published that test this possibility.

While there are numerous players involved in ground tissue patterning in *Arabidopsis*, *JKD* and *MGP* both appear to have potent effects on the divisions which specify the number of cortex cell files. Because of the strong phenotype of the *jdk* mutant (increase in CCFN; figure 4.3g) and the ability of the *mgp* mutant to abolish this phenotype, these two *BIRD* genes were focussed on to test whether *BIRD* proteins may be involved in the CCFN response to low P. Also

chosen for testing was *shr-3* (a mild loss of function allele of *SHR*), because although it is not expressed in cortex, Welch et al., (2007) found that another *shr* loss of function allele was capable of blocking proliferative divisions in the *jkd* mutant, which *scr* was not capable of doing.

Seeds of *jkd-4*, *mgp-i* and *shr-3* were sown on to low P/low Fe or high P/high Fe and grown in conditions described previously. After 12 days of growth the root tips were live-stained with propidium iodide and imaged using confocal microscopy. The data from this experiment show that both *shr-3* and *mgp-i* have a statistically significant difference in CCFN between low and high P treated roots ($p < 0.001$). *shr* has a CCFN of 8 under high P and 11-12 under low P; *mgp* has a CCFN on 8 under high P and 12-13 under low P. However, *jkd-4* shows a higher CCFN (statistically significantly different to high P treated Col.0, $p < 0.001$); under both low and high P; *jkd* mutants exhibit a CCFN of 13-14, showing low P response (figure 4.6).

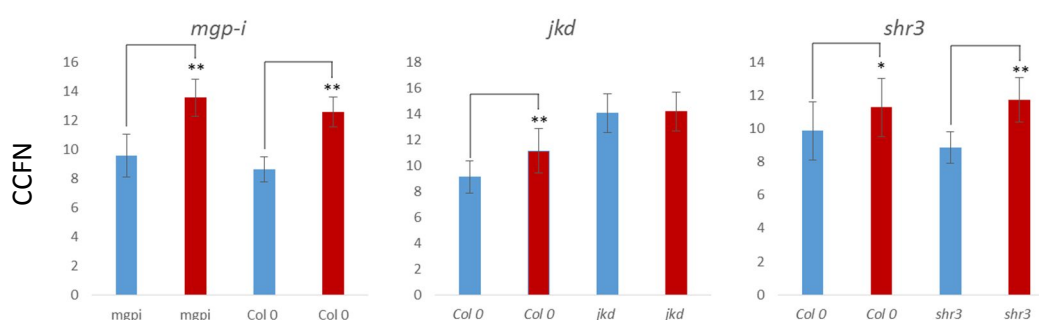


Figure 4.6 *mgp*, and *shr* mutants do not respond differently to wild type in their CCFN on low P, but *jkd* does

Bar charts showing cortex cell numbers for low and high P treated roots of mutants *mgp-i*, *jkd-4*, and *shr-3*. Bars on each chart represent values for Columbia 0 wild type control plants or for the mutant lines; blue bars are values under high P conditions and red bars are values under low P conditions. *mgp* and *shr* mutants do not show a significant reduction in CCFN under low P conditions, suggesting that neither of them are resistant to low P induced CCFN increase. However, *jkd* exhibits a constitutive low P CCFN response. Error bars are standard deviation. Differences between values statistically significant with $p < 0.001$ (**) or $p < 0.05$ (*); probabilities based on Student's t test.

These results would suggest that neither *MGP* nor *SHR* have a strong influence on the CCFN response to low P since their loss of function does not override the proliferative cortical divisions.

It is hard to determine from the response of the *jdk* mutant whether *JKD* is likely to be a prominent factor in regulating this low P response since there is no increase in CCFN from low to high P. Previous studies have shown that the *mgp-i* mutant is capable of rescuing the *jdk-4* phenotype (Long et al., 2015; Welch et al., 2007); considering this, if *JKD* were to be downregulated under low P (i.e. mimicking the *jdk* mutant) then one might imagine that a *mgp* mutation would block the resultant CCFN proliferation. Although it is difficult to glean a conclusion about *JKD* from the *jdk-4* response to low P, the fact that *mgp-i* does not have a defective low P response could suggest that *JKD* may well not be involved. Additionally, *shr* is capable of blocking the proliferative effect of the *jdk* mutant on the indeterminate single ground tissue layer in *shr*. However, a proliferation in this tissue is still seen in the *shr-3* allele on low P in this study, suggesting further that the CCFN increase could well be occurring via a different pathway to *SHR/SCR/BIRD*.

Another confounding factor however, is the redundancy which is seen between the two pairs of *BIRD* genes discussed above. As has been shown in previous work, *JKD* and *BIB* appear to work synergistically to suppress additional periclinal divisions which give rise to additional CCFs. *bib* loss of function alone has no obvious phenotype, whereas the *jdk bib* double mutant has an extreme proliferation of ground tissue. Presumably, since *mgp* complements the *jdk*

phenotype, then *MGP* is a positive regulator of these periclinal divisions and is suppressed by *JKD* and *BIB*. *MGP* and *NUC* also appear to act synergistically in promoting division in the ground tissue, since in the *mgp nuc jkd* triple mutant there is only a mutant ground tissue layer as opposed to there being one cortical and one endodermal layer. Although it is interesting that *mgp nuc* does not have the single mutant ground tissue layer phenotype and so *MPG* and *NUC* seem to rely on *JKD* to promote these divisions, suggesting positive as well as negative interactions between these proteins. It could be that different complexes are formed with different combinations of these regulators which enact different regulatory effects. Unfortunately, the radial phenotype of a *jkd nuc* double mutant has not been published so it is not known whether *nuc* would complement the phenotype of *jkd* or not. However, Moreno-Risueno et al. (2015) do show the phenotype of *jkd nuc*, and *jkd nuc mgp* mutants in longitudinal sections. According to their data *nuc* and *mgp* are capable of suppressing the *jkd* phenotype of an extra ground tissue layer when combined, but *nuc* alone cannot do this. Again this suggests antagonistic and synergistic behaviours between these proteins. Therefore a role for *JKD* in the CCFN low P response could be hidden in the results presented here by the fact that the *mgp-i* mutation may be made up for by the presence of functional *NUC* allele.

Despite the unknowns in this pathway, and the confounding factors of possible redundancy and the masking quality of the constitutive nature of the *jkd-4* phenotype, the data here generally point to there not being a central role for *BIRD* genes. However, the possibility does remain open; it may be that not just one pathway may be involved and/or that fine tuning of various components

of the *SHR/SCR/BIRD* pathway is required. The next sections of this chapter aim to address whether the latter possibility could be at play.

To determine whether *jkd* has any low P specific root architectural phenotypes which may be associated with its constitutive high CCFN phenotype, *jkd-4* seeds were sterilised and sown on to low P low Fe and high P high Fe plates and grown for 12 days. After this time the plates were photographed to assess whether there was an obvious difference between the root system architecture between *jkd-4* and Col. 0.

This experiment revealed that *jkd* roots do not display a root length or lateral root phenotype which visibly indicates a whole-root-system low P-like response (figure 4.7). *JKD* is therefore unlikely to be responsible for controlling primary root elongation or lateral root initiation under low P (indeed neither of these processes have previously been associated with *JKD* previously), but could be that its mutation constitutes a constitutive CCFN low P response.

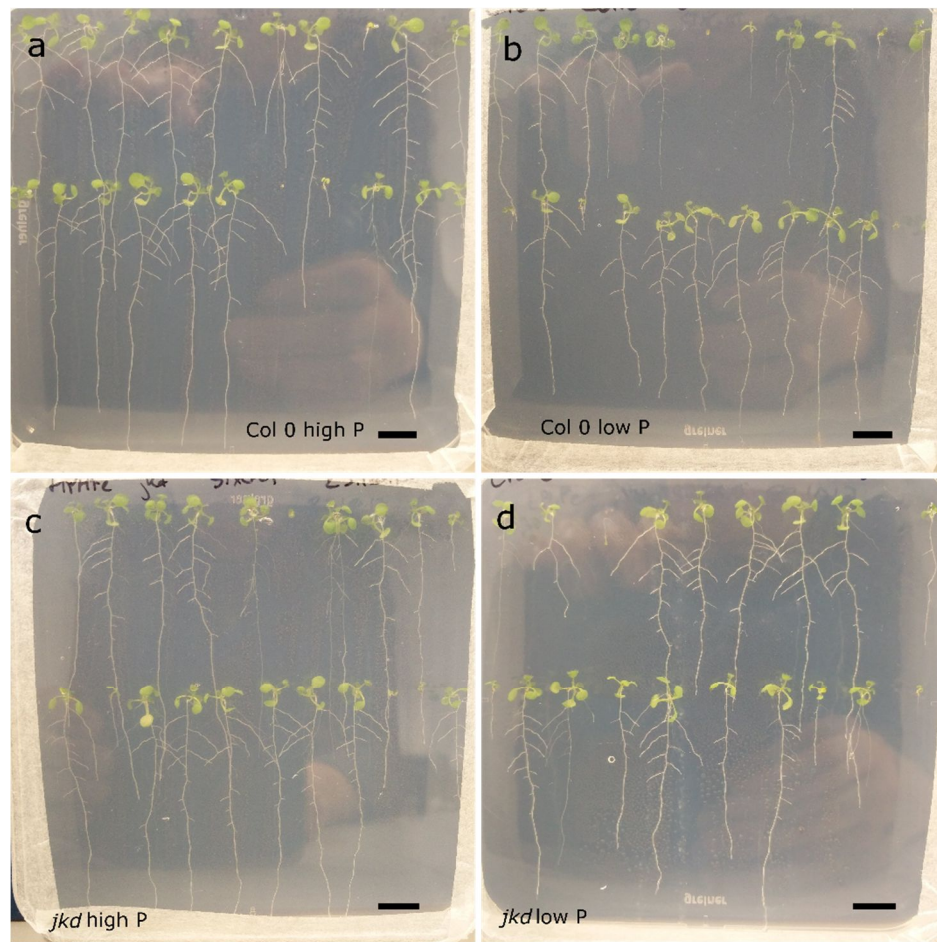


Figure 4.7 *jkd* mutant does not exhibit an constitutive low P response like-root architecture phenotype

jkd-4 mutants grown on high (c) and low (d) P containing media for 12 days alongside wild type Columbia on high (a) and low (b) P containing media. *jkd* mutants appear to exhibit a similar response to high and low P, respectively, as Columbia wild type. This indicates that *jkd* does not express a whole root system low P phenotype. Scale bar= 1cm.

It can be concluded from these results that *PIN2* is required for the response which strongly resists low P induced CCFN increase. However, *jkd* has no low P specific phenotype and its high CCFN phenotype is not exaggerated by low P. This raises the question of whether *jkd* may be exhibiting a constitutive low P response and that its activity may define a maximum number of cortex cells under low P.

4.3 Reverse transcription quantitative real-time PCR reveals differential regulation of some *SHR/SCR/BIRD* components

The findings so far in this chapter have left it unclear whether there is a role for the *SHR/SCR/BIRD* pathway in the CCFN increase response to low P. The previous section suggests that they are not likely to be central regulators of the response but it cannot be ruled out. Therefore, it was tested whether the expression of any of these components are differentially regulated under low P- if this is the case then it would seem possible that this pathway is contributing to the change in CCFN.

Seeds of Col.0 were sown on low P high Fe or high P high Fe and grown for 12 days as described previously. 5mm of root tip was then harvested from ~50 seedlings for each of three replicate samples for low and high P. Tissue was frozen quickly in liquid N, ground, and RNA extracted using trizol/chloroform method followed by RNA purification using an RNA wash column. cDNA was then synthesised using a reverse transcription reaction based on poly-T primer

complementation and used as template for quantitative PCR. Primers for *CBP20* (*CAP BINDING PROTEIN20*; *At5g44200*) were used as an internal standardisation control. Genes analysed were *CYCLIN D6* (*CYCD6*; *At4g03270*), *BALDIBIS* (*BIB*; *At3G45260*), *JACKDAW* (*JKD*; *At5G03150*), *MAGPIE* (*MGP*; *At1G03840*), *NUTCRACKER* (*NUC*; *At5G44160*), *PIN-FORMED 2* (*PIN2*; *At5g57090*), *SCARECROW* (*SCR*; *At3g54220*), and *SHORTROOT* (*SHR*; *AT4G37650*).

The results showed a significant change for the following genes: *SCR* expression is increased around four-fold between high and low P ($p < 0.001$); *SHR* expression is decreased by around half between low and high P ($p < 0.05$), and *MGP* expression is approximately doubled by low P treatment ($p < 0.05$). *JKD*, *CYCD6*, *BIB*, *NUC*, and *PIN2* do not show any difference in expression between low and high P (figure 4.8).

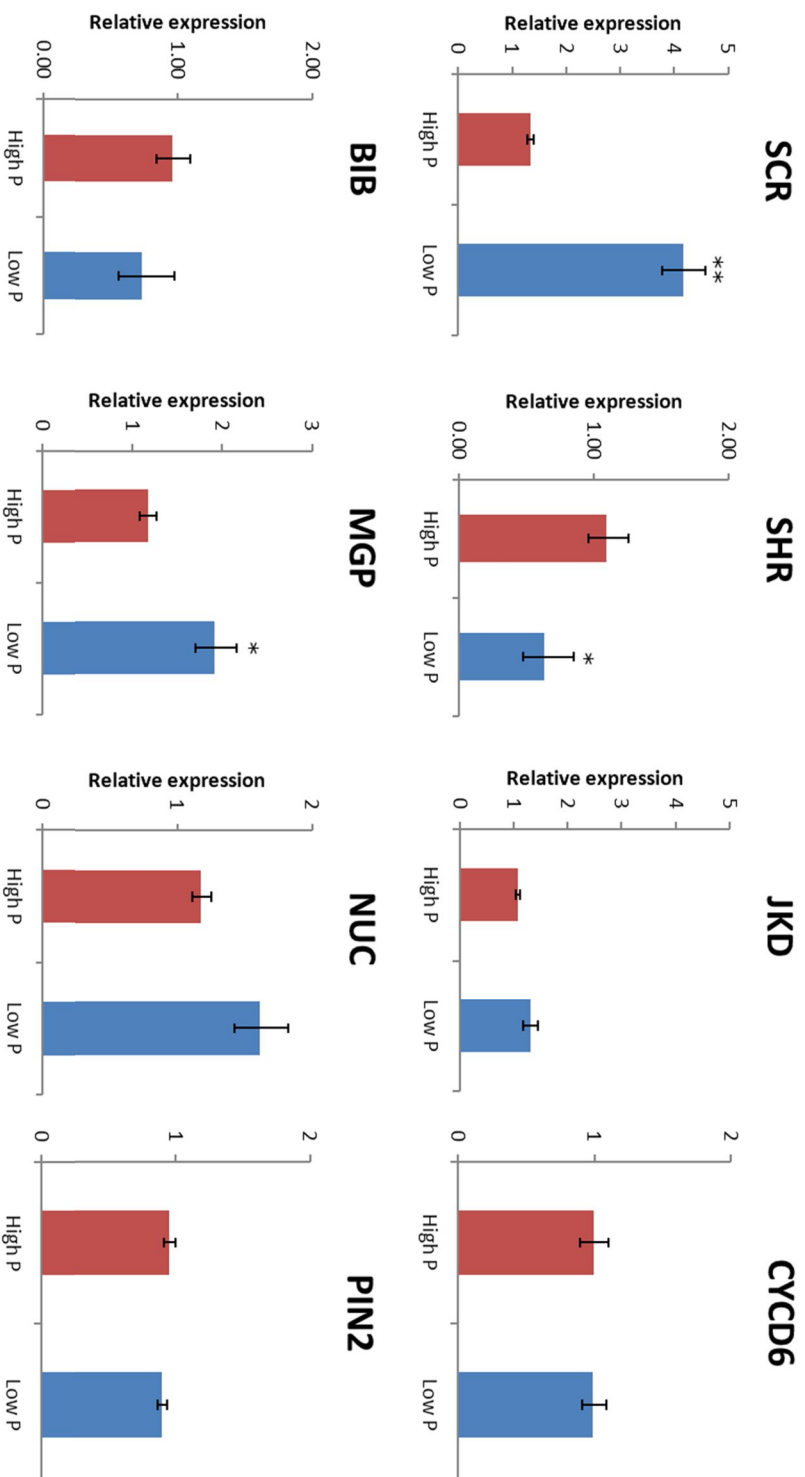


Figure 4.8 Some components of the SHR/SCR/BIRD pathway have altered mRNA transcript levels in root tips exposed to low P conditions

Bar charts showing values for relative expression in 12 day old Colombia 0 plants of (a-h) (*SCARECROW*) *SCR*, (*SHORT ROOT*) *SHR*, (*JACKDAW*) *JKD*, (*CYCLIN D;6*) *CYCD6*, (*BALDIBIS*) *BIB*, (*MAGPIE*) *MGP*, (*NUTCRACKER*) *NUC*, (*PIN FORMED2*) *PIN2* from reverse transcription quantitative PCR. Values represent an average from three biological replicates each of four technical replicate reactions. Red bars represent high P treatments and blue bars represent low P treatments. Transcript levels for *SCR*, *SHR*, and *MGP* all change significantly ($p < 0.05$ or $p < 0.001$), but the remainder of the tested genes do not change in mRNA expression to a significant degree. Error bars represent standard deviation. Differences between low and high P values statistically significant with $p < 0.001$ (**) or $p < 0.05$ (*); probabilities based on Student's T-test.

The finding that *MGP* is upregulated is interesting as it would support the initial hypothesis that the CCFN increase is regulated by *BIRD* genes; particularly as an increase in *MGP* activity could feasibly be associated with an increase in cell division activity in the cortex (Long et al., 2015; Welch et al., 2007). However, this change is not accompanied by a significant increase or decrease in the activity of other *BIRD* genes which might be expected. Although given what is known about this model and the apparent complexity and plasticity of the protein complexes between cell types and developmental gradients, changes in other components would not necessarily be required. However if this change is associated with functional activity then it would be a surprise that *CYCD6* transcript shows no change whatsoever in low P treated roots, since this gene has been shown to be a downstream target of *BIRD* associated complexes to regulate cell division (Long et al., 2015). This activity is usually specific to the endodermis, except in *BIRD* mutants, but such 'aberrant' activity of regulators is something which is speculated to be at play during the low P CCFN response.

Despite other *BIRD* genes remaining relatively static upon low P stress, both *SHR* and *SCR* undergo significant changes in expression. It is especially interesting that *SHR* expression should go down while *SCR* expression increases, since classically *SHR* migration to surrounding tissues is the primary driving factor in regulating *SCR* expression (Helariutta et al., 2000). However it is also known that *SCR* is expressed (albeit at a low level) in other root tissues besides the endodermis; i.e. cortex and epidermis. Therefore it must be that under standard (i.e. nutrient replete) conditions either *SCR* is upregulated by low levels of *SHR* activity in these tissues or, there are other factors which are

capable of initiating *SCR* transcription independently of *SHR*. Alternatively to these possibilities, it may be that *SCR* protein is capable of migrating between these cell layers.

It is also interesting that *PIN2* transcript level is unchanged in low P treated roots. Given the requirement of *PIN2* for CCFN increase on low P, it is surprising that no change is seen at all. This finding suggests that any changes in *PIN2* activity under low P conditions could be post-translational and may be related to subcellular trafficking or phosphorylation-mediated activation of *PIN2* protein. Alternatively, they could be localised to small subsets of cells making them hard to detect in a sample taken from a relatively large (5mm) piece of root. Such subcellular targeting dynamics are central to PIN activity and have been shown to be crucial to the fluidity and plasticity of plant growth. (Rakusová et al., 2016) show how during a gravity response *PIN3* directs auxin flow to the lower side of the hypocotyl, where it accumulates to promote growth to bend the hypocotyl upwards. This auxin accumulation simultaneously promotes the subcellular targeting of *PIN3* proteins to the opposite side of the cell, directing auxin away from the growing side, and bringing the response to a steady stop at the desired gravity vector. Similar dynamics are seen in *PIN2* during gravity response in the root, and *PIN2* is differentially localised on the shootward or rootward membranes of cells in the epidermis or cortex respectively. Due to this dynamic nature of *PIN2* localisation it arises as a possibility that *PIN2* may be being differentially targeted during the response to low P, which could lead to differential auxin maxima and minima (Abas et al., 2006; Rahman et al., 2010b). This targeting is

regulated in part by phosphorylation by kinases PINOID and others including CALMODULIN DEPENDENT KINASE RELATED KINASE RELATED KINASE 5 (CDPK-RELATED KINASE 5; CRK5) and D6PKs which have been shown to be required for correct PIN activity via this pathway (Barbosa *et al.* 2014; Rigó *et al.*, 2013; Sukumar *et al.* 2009).

4.4 Analysis of reporter lines reveals differential regulation of SHR/SCR/BIRD complex related genes

The previous section (4.3) addresses whether transcript levels of genes postulated to be involved in the low P CCFN increase response change when root tips have been exposed to low P for 12 days. The most striking change in the level of transcription was that of *SCR*, whose transcript abundance was amplified four-fold in response to low P. The only other two genes whose average transcript abundance changed significantly were *SHR* whose expression reduced by around half, and *MGP* whose expression approximately doubled (figure 4.8). While these results are informative about potential regulators or downstream targets, they provide no tissue specificity and therefore are not informative for looking at the cell scale. In order to investigate whether any of these factors were altered in their expression pattern within the cortex, the effect of low P on GFP and YFP fusions with candidate regulators' promoters and proteins was assessed. *pJKD:HIS2B:YFP*, *pMGP:HIS2B:YFP*, *pBIB:HIS2B:YFP*, *pCYCD6:GFP*, *pJKD:JKD:YFP*, *pNUC:NUC:YFP*, *pMGP:MGP:YFP*,

pBIB:BIB:YFP, *pSCR:SCR:GFP*, and *pSHR:SHR:GFP* were used to investigate. By using this approach the following issues could be addressed:

Firstly, despite some of these factors not having changed to a significant degree in terms of transcript abundance as detected from whole root tip extractions, it could be that there are changes to transcription but these are on a small scale (i.e. single cell) and not detected easily in bulk tissue analysis. Thus the use of promoter-fluorescent protein fusions can provide single cell resolution of changes in promoter activity; while not a direct indicator of transcript abundance, this technique nonetheless gives an idea about the expression amplitude at this level. Secondly, a change in transcript abundance of a particular gene does not imply a change in that gene's rate of translation, protein abundance or functionality. Protein-fluorophore fusions allow a more informative indicator of protein abundance and can help to suggest (in combination with qPCR data) whether regulation could be occurring at the translational, or post-translational level.

Based on finding that anatomical changes in response to low P are observed after one day (figure 3.3), seeds of *pJKD:HIS2B:YFP*, *pMGP:HIS2B:YFP*, *pBIB:HIS2B:YFP*, *pCYCD6:GFP*, and *pSHR:SHR:GFP* were sterilised prior to being grown on high P media for seven days. Plants were then transferred to either high or low P media and imaged after exposure to the new media for 24 hours, using live-staining with PI and confocal microscopy.

Measurement areas were selected using a circular selection tool to encircle fluorescent nuclei in images. The results show that YFP expressed under the control of the *JKD* promoter does show some significant change in response to low P after 24 hours.

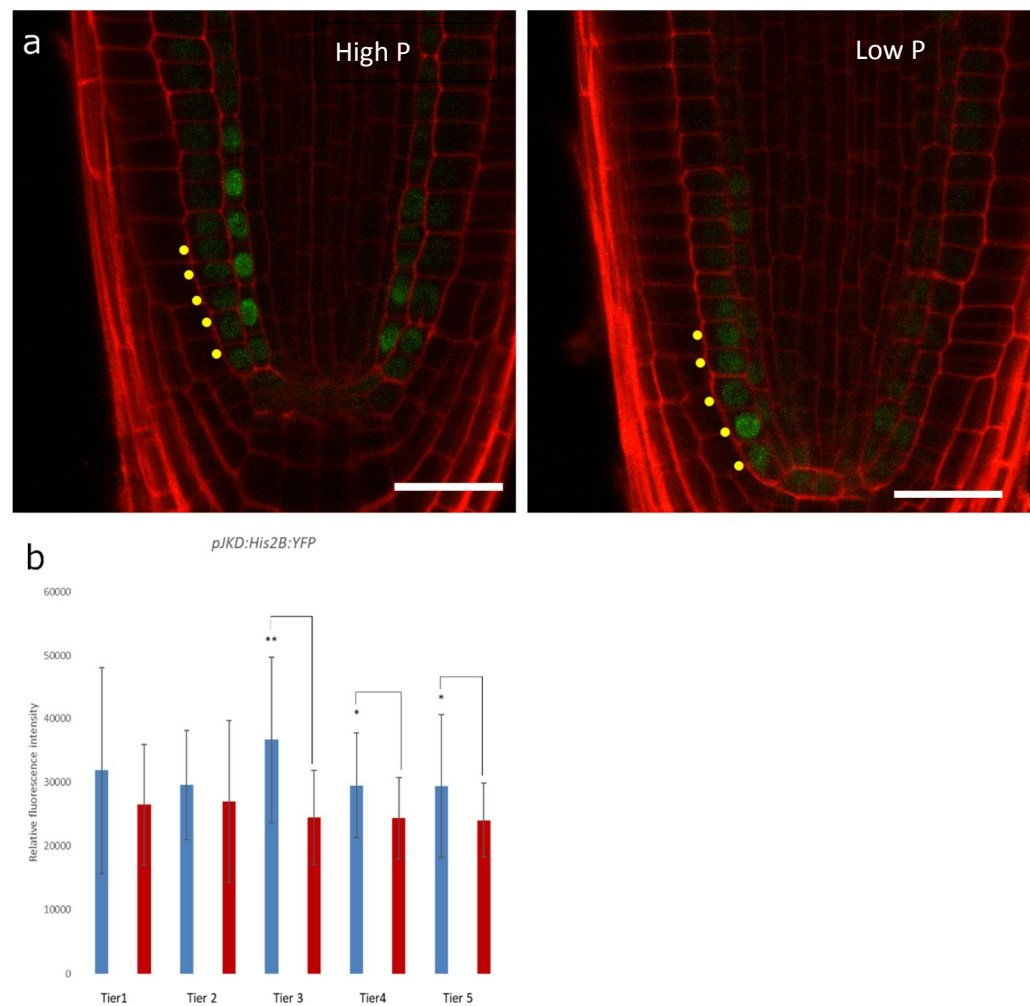


Figure 4.9 YFP expressed under the JACKDAW promoter exhibits a decrease in fluorescence intensity in cortex under low P conditions

Confocal microscopy images showing root tips from *pJKD:HIS2B:YFP* transformant plants grown on high P (left) and low P (right) (a). Green signal represents YFP fluorescence measured using the same excitation and detection settings for each treatment, red signal represents propidium iodide stained cell walls. Yellow dots mark cortex cells within the 5 tiers shootward from the root apex which were measured for fluorescence intensity. Scale bars= 25 μm. (b) bar chart showing average fluorescent intensity from 25 and 28 root tips from high P (blue bars) and low P (red bars) treatments respectively. The first two cortex cell tiers shootward from the QC show a general decrease in mean but this is not significant. A significant decrease in fluorescence is seen in the third, fourth and fifth cell tiers. Error bars represent standard deviation. Values are statistically significant with $p < 0.001$ (**) or $p < 0.05$ (*).

The first two cortex cell tiers do not exhibit a significant change in the *JKD* promoter activity low P. However, in cell tiers three, four, and five the average relative fluorescence intensity lies between 30000-40000 under high P and between 20000 and 30000 under low P, which is statistically significant ($p < 0.001$, $p < 0.05$, and $p < 0.05$ respectively). Overall in these tissues, this data suggests a drop in promoter activity between one quarter and one eighth when root tips are exposed to low P media.

In contrast to the findings for the *JKD* promoter, no significant *MGP* changes were seen under low P conditions (figure 4.10).

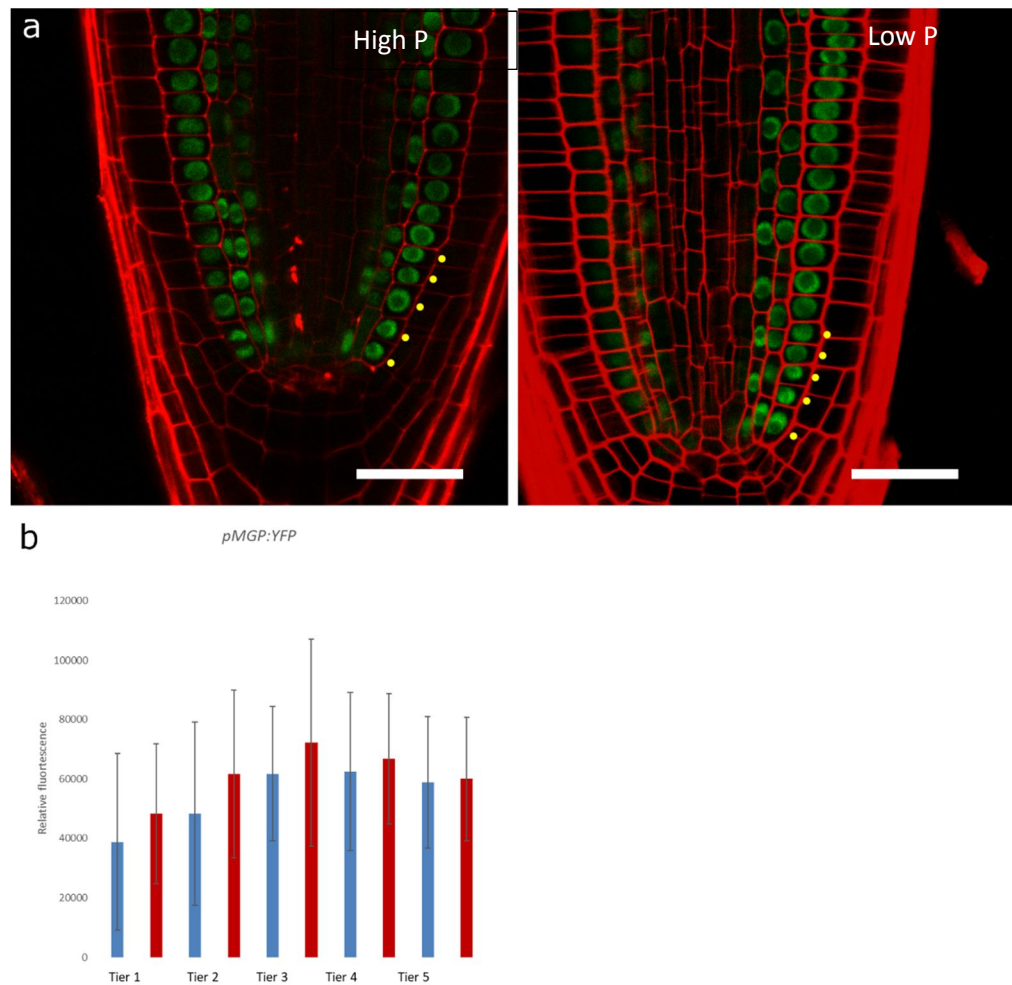


Figure 4.10 YFP expressed under the MAGPIE promoter exhibits no change in fluorescence intensity in cortex under low P conditions

Confocal microscopy images showing root tips from *pMGP:HIS2B:YFP* transformant plants grown on high P (left) and low P (right) (a). Green signal represents YFP fluorescence measured using the same excitation and detection settings for each treatment, red signal represents propidium iodide stained cell walls. Yellow dots mark cortex cells within the 5 tiers shootward from the root apex which were measured for fluorescence intensity. Scale bars= 25 μ M. (b) bar chart showing average fluorescence intensity from 24 and 25 root tips from high P (blue bars) and low P (red bars) treatments respectively. These measurements show no significant change in fluorescent signal in any cell tier between low and high P conditions. Error bars represent standard deviation.

Examination of the *pBIB:HIS2B:YFP* reporter line showed that activity of the *BIB* promoter was absent in the cortex under both high and low P conditions (figure 4.11). YFP signal can be detected in the endodermis layer within the root tip but seems to be excluded from both middle cortex and cortex tissues. This is at odds with previous work which shows that *BIB* promoter activity can be seen in the cortex (Long et al., 2015). This result suggests that *BIB* transcription itself is not changed in response to low P, and therefore at this stage of regulation seems unlikely to be regulated as part of CCFN increase under low P conditions. This result is in agreement with qPCR results presented in this chapter (figure 4.8) which showed no significant change in transcript abundance for *BIB* in low P treated plants.

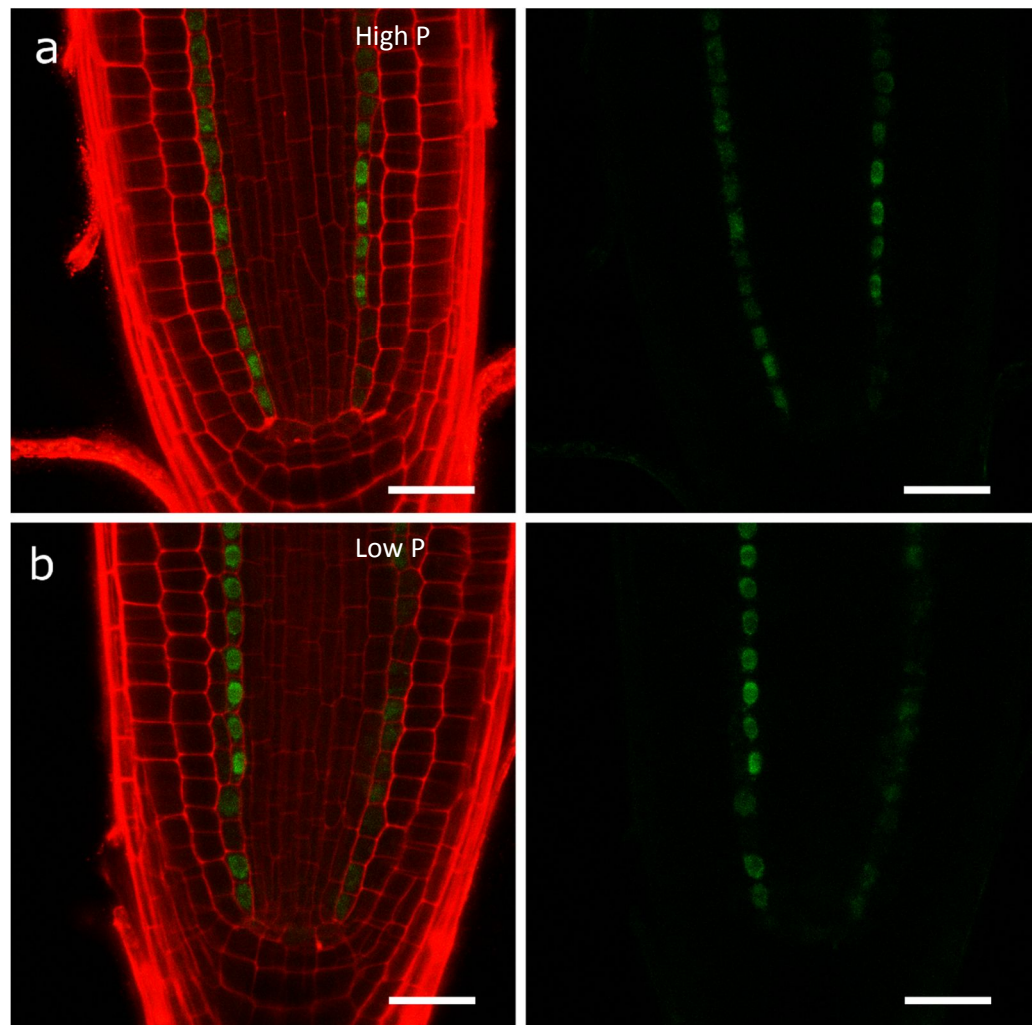


Figure 4.11 YFP expressed under the BALDIBIS promoter does not exhibit localisation in the cortex under low or high P conditions

Confocal microscopy images of root tips of high (a) and low (b) P treated *pBIB:HIS2B:YFP* transformant plants. Green signal represents YFP fluorescence measured using the same excitation and detection settings for each treatment, red signal represents propidium iodide stained cell walls. Images are representative of patterns of *BIB* promoter activity in low and high P. Under both low and high treatment, no activity was found in the cortex cells. Scale bars= 25µm.

Observation of pCYCD6:GFP revealed fairly sporadic signal can be seen in longitudinal sections of the root, suggesting that the activity of *CYCD6* is not constant in each cell file at all times. Signal from the GFP is seen in CEI, long files of endodermis (i.e. 5-10+ cell tiers above the CEI) and occasionally in the first 1-2 cortex cell tiers. However, this pattern is not noticeably different between high and low P- GFP signal in all of these tissues was noted between both treatments (figure 4.12).

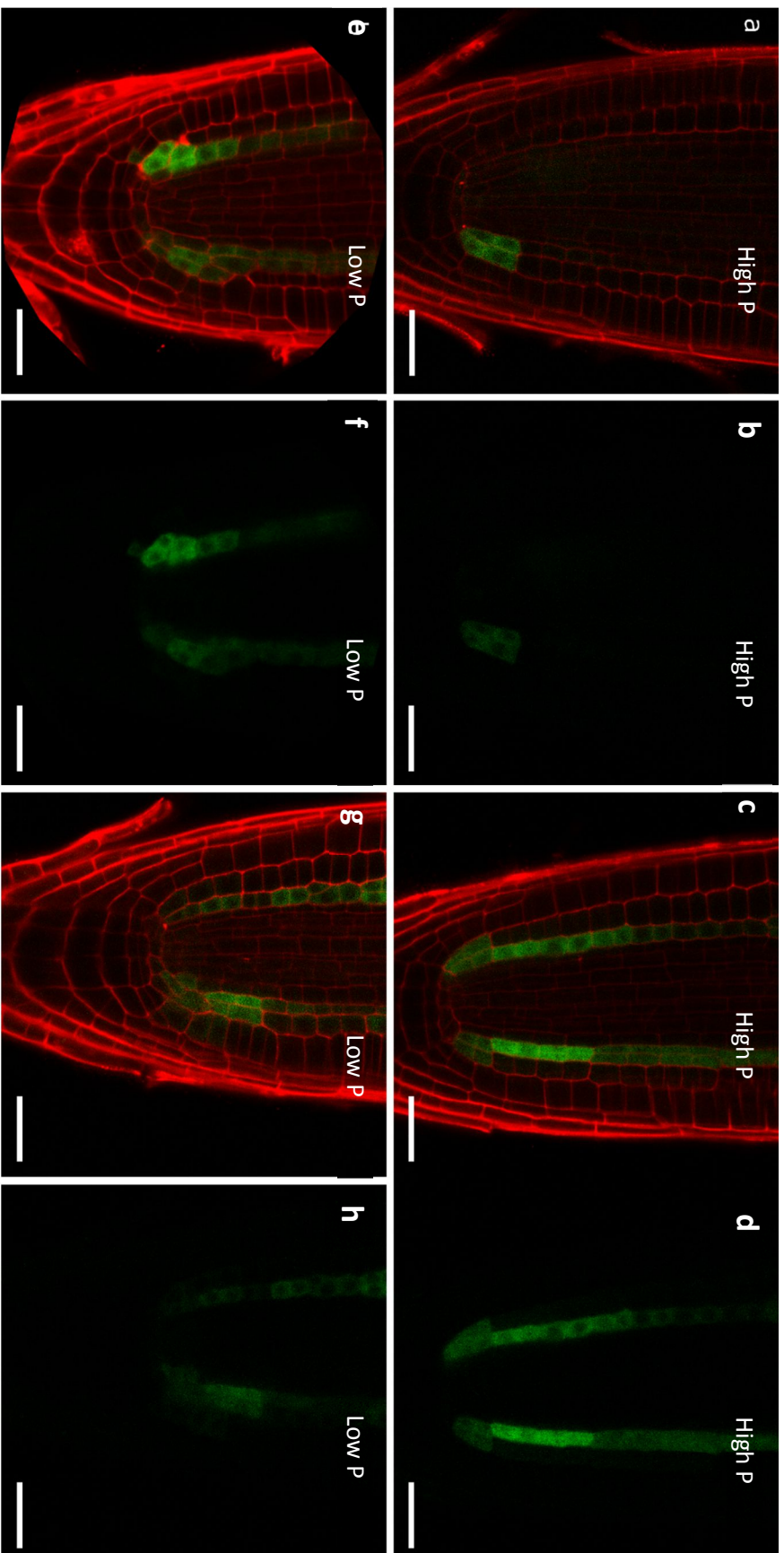


Figure 4.12 CYCLIN D6 promoter driven GFP does not exhibit differences between low and high P treatments

Confocal microscopy images of root tips of high (a-d) and low (e-h) P treated pCYCD6:GFP transformant plants. Green signal represents GFP fluorescence measured using the same excitation and detection settings for each treatment, red signal represents fluorescence from propidium iodide stained cell walls. Sporadic activity of CYCD6 promoter activity is observed under both low and high P treatments and no particular difference in pattern was noted between the two treatments. Scale bars= 25µm.

After investigating the effects of low P on the activity of promoter-fluorophore fusions, some promoter-protein-fluorophore fusions were tested to observe their response to low P.

Seeds of *pJKD:JKD:YFP*, *pBIB:BIB:YFP*, *pNUC:NUC:YFP*, and *pSCR:SCR:GFP* were sterilised and sown on to either high or low P media. After 12 days of growth roots were live-stained with propidium iodide and visualised using confocal microscopy.

Observations of *pJKD:JKD:YFP* showed decreased average relative fluorescence intensity in all five tiers measured- each was statistically significant to $p < 0.05$ besides the second tier (Figure 4.13). These findings suggest a significant decrease in the protein abundance of JKD under low P conditions which is somewhat in line with the promoter reporter line but at odds with the qPCR data in section 4.3 (figure 4.9; 4.8). However, what this could mean is that there is a stronger influence of post-translational regulation on the level of JKD activity under low P than there is (post-) transcriptional. Alternatively, it could be cell-type specific regulation.

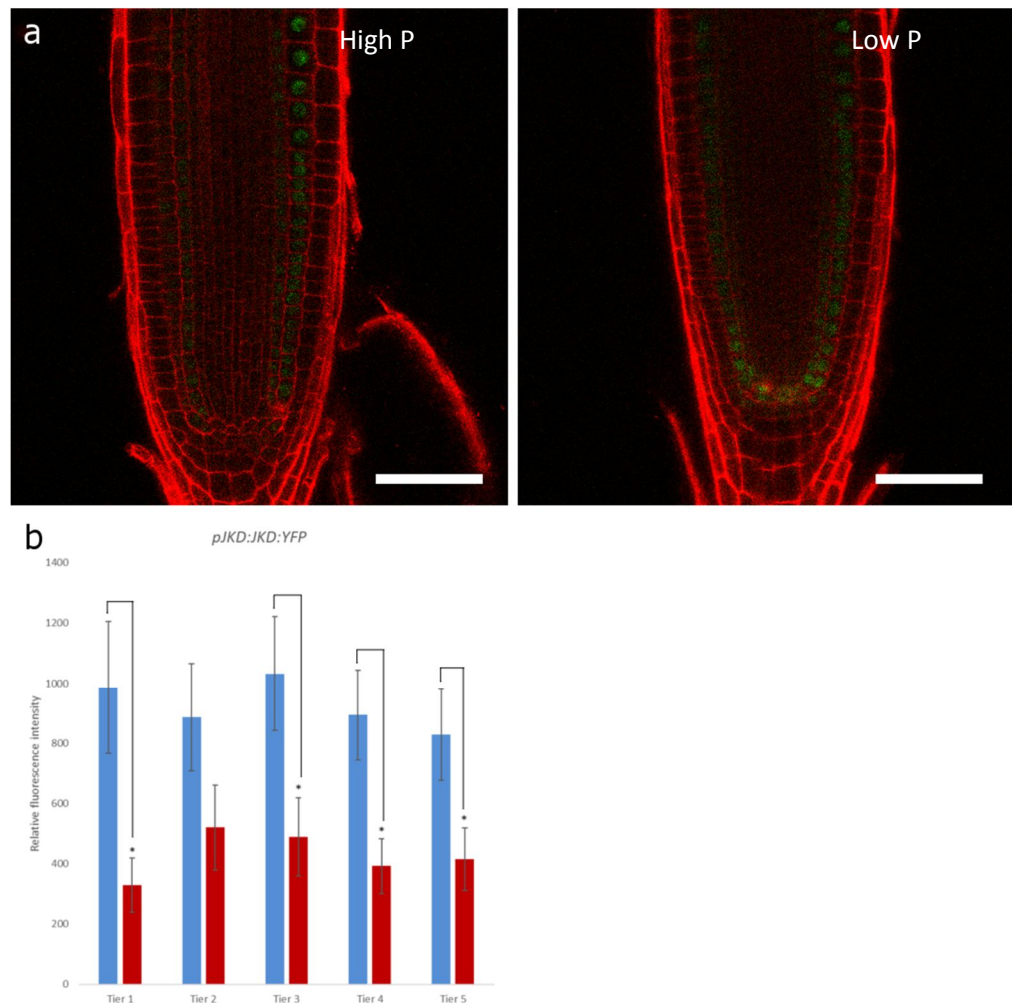


Figure 4.13 YFP fused to JACKDAW protein expressed under the JACKDAW promoter exhibits a decrease in fluorescence intensity under low P conditions

Confocal microscopy images showing root tips from *pJKD:JKD:YFP* transformant plants grown on high P (left) and low P (right) (a). Green signal represents YFP fluorescence measured using the same excitation and detection settings for each treatment, red signal represents propidium iodide stained cell walls. 5 cell tiers shootward from the root apex were measured for fluorescence intensity in the cortex. Scale bars= 25 μ M. (b) bar chart showing average fluorescence intensity from root tips from high P (blue bars) and low P (red bars) treatments respectively. A significant decrease in fluorescence intensity was observed in each but the second cell tier measured. Images are representative of level of signal seen amongst root tips (n=14 [high P] or 13 [low P]). Error bars represent standard error of the mean. Values are statistically significant $p < 0.001$ (*).

p*NUC*:NUC:YFP showed no statistically significant decrease in fluorescence signal in 4 out of the 5 cell tiers measured; only tier 3 showed a statistically significant decrease in fluorescence signal in response to low P ($p=0.001$) (figure 4.14). Since these data are not significant, the results are inconclusive. While there is a significant change in the third tier in response to low P, this is unlikely to be directly relevant to the proliferative divisions since these have been shown in chapter 3 to be most likely occurring in the first cortex cell tier.

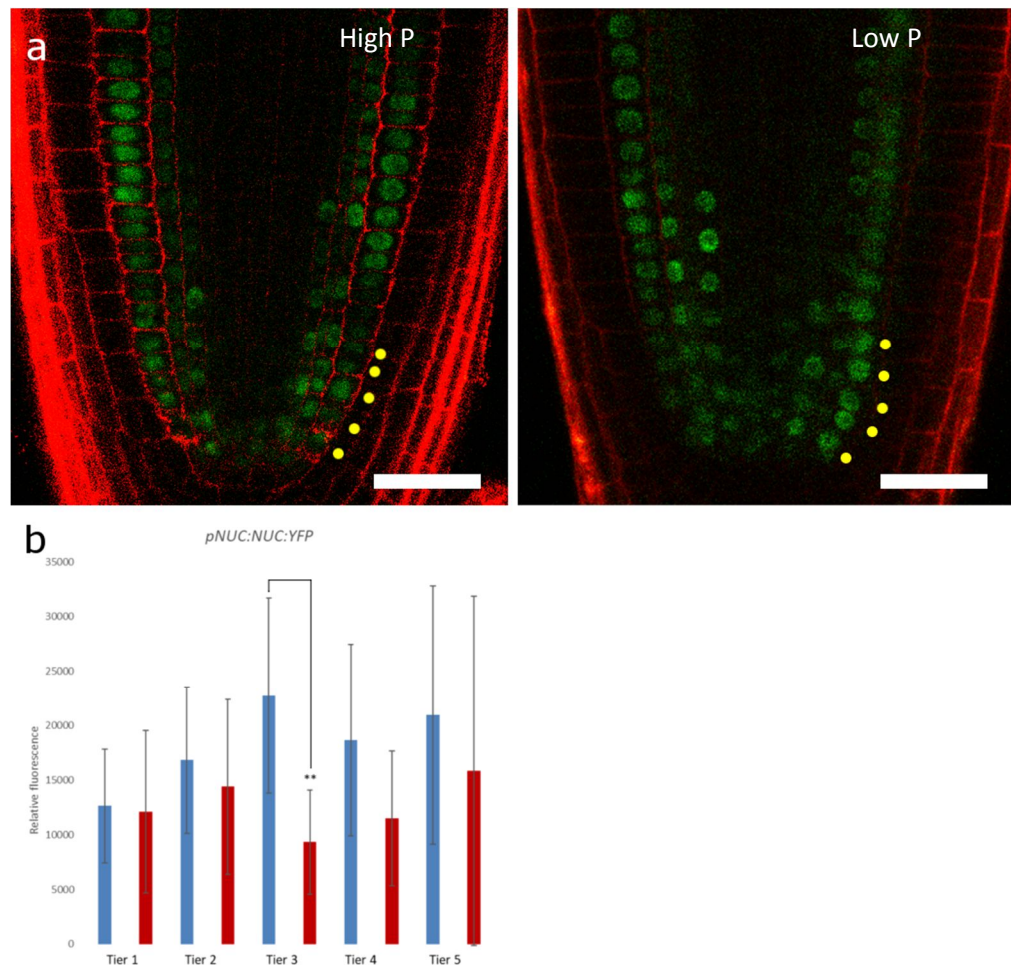


Figure 4.14 YFP fused to NUTCRACKER protein expressed under the NUTCRACKER promoter exhibits a small change in fluorescence intensity in cortex under low P conditions

Confocal microscopy images showing root tips from *pNUT:NUC:YFP* transformant plants grown on high P (left) and low P (right) (a). Green signal represents YFP fluorescence measured using the same excitation and detection settings for each treatment, red signal represents propidium iodide stained cell walls. Yellow dots mark cortex cells within the 5 tiers shootward from the root apex which were measured for fluorescence intensity. Scale bars= 25 μm. (b) bar chart showing average fluorescence intensity from 17 and 9 root tips from high P (blue bars) and low P (red bars) treatments respectively. Images are representative of the level of signal observed amongst root tips: only the third cell tier in the cortex of roots exhibits a statistically significant decrease in signal under low P. No other cell tiers undergo any significant change. Error bars represent standard deviation. ** denotes values statistically significant with $p < 0.001$.

pBIB:BIB:YFP lines showed a dramatic decrease in fluorescence signal in the root tip when exposed to low P. The majority of roots exhibited no discernible signal whatsoever (figure 4.15e and f). However, two of the roots observed did show some visible signal under low P, and in each of these roots the expression domain was seen in cortex cells (figure 4.16g and h). BIB presence in the cortex is seen under high P also but not in every case (compare roots in figures 4.16a-d). These findings suggest that low P does have a strong effect on the level of BIB protein (but not transcription), which is similar to the case of JKD protein compared with *JKD* promoter and transcript abundance.

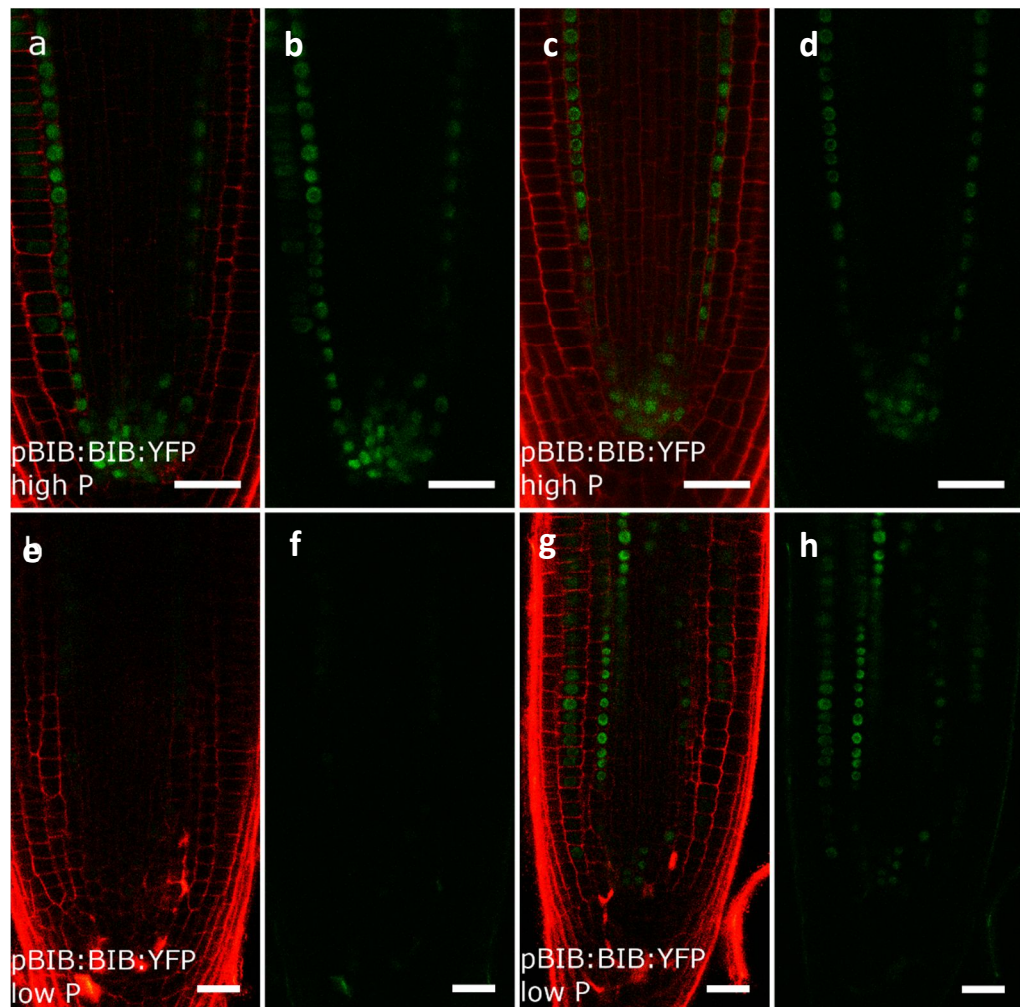


Figure 4.15 YFP fused to BALDIBIS protein expressed under the BALIBIS promoter exhibits a reduction in fluorescence intensity under low P conditions

Confocal microscopy images showing root tips from *pBIB:BIB:YFP* transformant plants grown on high P (a-d) and low P (e-h). Green signal represents YFP fluorescence measured using the same excitation and detection settings for each treatment, red signal represents propidium iodide stained cell walls. Images are representative of pattern and signal level observed under low and high P. Only three out of twelve low P treated roots showed any signal, but the ones which did show an extension of domain to the cortex. Scale bars= 25 μ M.

pSCR:SCR:GFP showed different expression patterns under low and high P.

Under high P, SCR:GFP was almost always restricted to the endodermis, except

in a small number of cases where it was found in the cortex (figure 4.17a, left two panels). Under low P SCR:GFP was seen in the cortex in every root, and sometimes it was even found to be extended to the epidermis (figure 4.17b, right two panels). This expression pattern follows with the findings in the present qPCR experiment where *SCR* transcript signal was enhanced by fourfold (figure 4.8a). This could mean that the composition of the SCR/BIRD complex in these tissues is altered, with additional SCR activity having a positive effect on CCFN increase under low P.

Although it is clear that more SCR protein is present and more *SCR* transcript is produced under low P, the source of the extra SCR protein is unclear. There are two possibilities: firstly it could be that extra SCR is translated in the epidermis and then moves outwards intercellularly to surrounding tissues. Secondly, it could be that low P stimulus promotes *SCR* transcription directly within these tissues. Two ways to test this could be to either use tissue specific *SCR* knockouts (i.e. antisense RNA) in the p*SCR*:SCR:GFP background and see whether this reduces or abolishes SCR:GFP signal in these tissues, or to photobleach SCR:GFP in the endodermis and see if this affects signal in surrounding tissues. If indeed it was found that SCR was moving between tissues, this would be an interesting finding as it has not previously been shown that SCR is capable of intercellular movement. Indeed a crucial part of its function is to restrict SHR movement in the endodermis (Cruz-Ramírez et al., 2012).

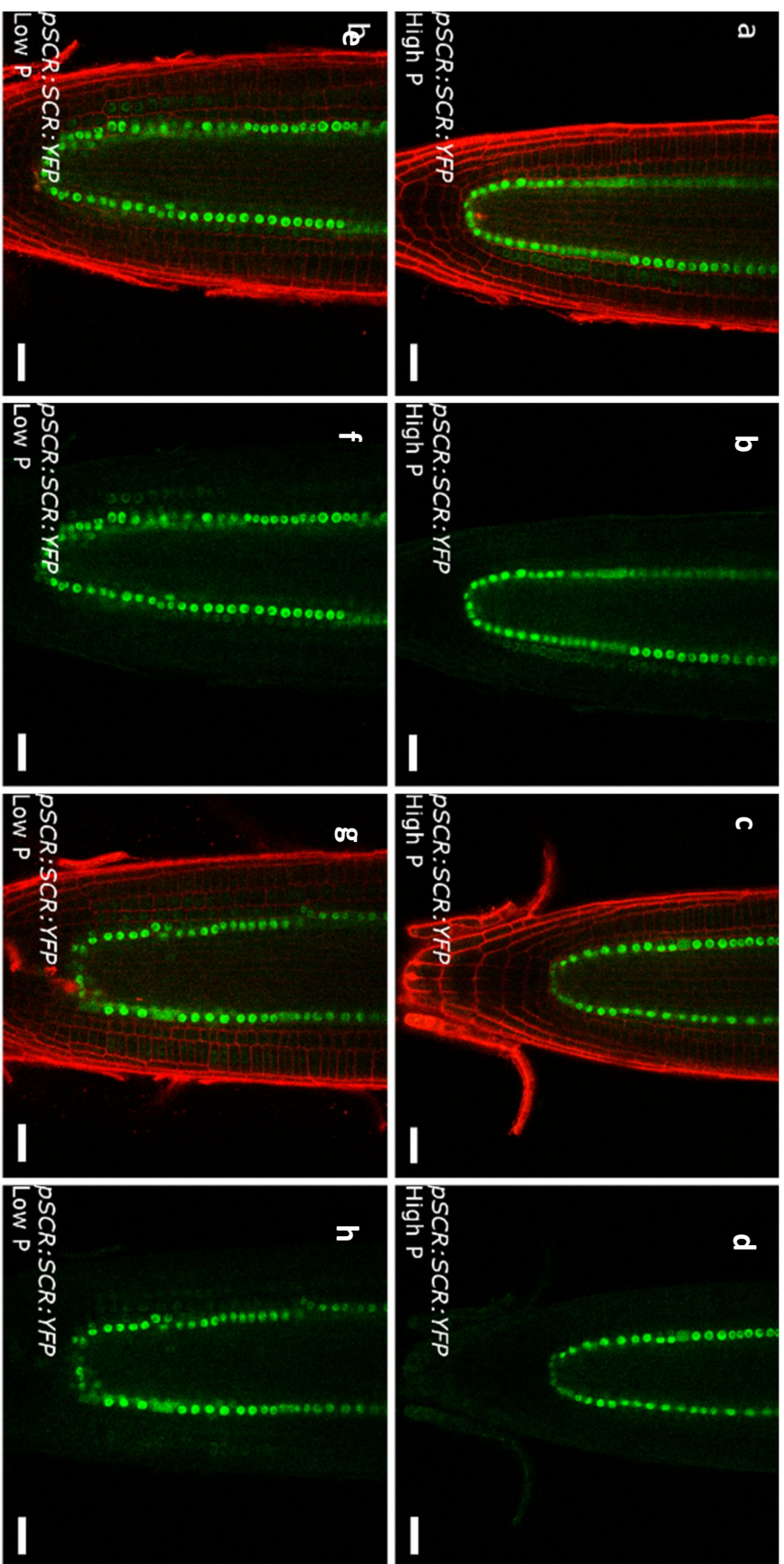


Figure 4.16 GFP fused to SCARECROW protein expressed under the SCARECROW promoter exhibits an extension to its expression domain under low P conditions

Confocal microscopy images showing root tips from pSCR:SCR:GFP transformant plants grown on high P (a) and low P (b). Green signal represents GFP fluorescence measured using the same excitation and detection settings for each treatment, red signal represents propidium iodide stained cell walls. Images show typical patterns of SCR:GFP localisation under high and low P; the extension of the SCR localisation domain under low P can be seen, with visible signal in the cortex. Scale bars= 25µM.

pSHR:SHR:YFP lines showed no visible difference between high and low P. In both instances, only a single layer of nuclear *SHR:YFP* was visible in the endodermis and QC. Cytoplasmic *SHR:GFP* was seen in the stele in both treatments (figure 4.18). Given the results from the *SCR:GFP* line, this provides an interesting result; if not *SHR:GFP* is seen outside of the endodermis then it must be that the *SCR* which is found outside of the endodermis under low P conditions is not associated with *SHR* and is not being transcriptionally induced by *SHR*.

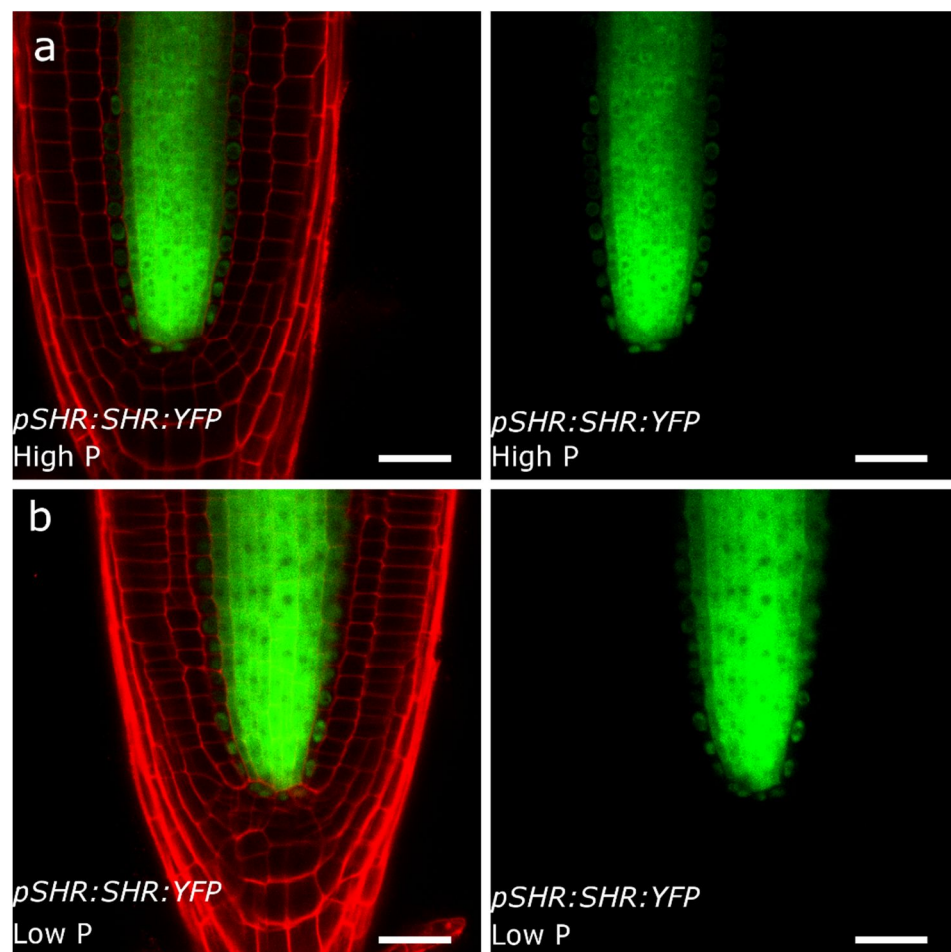


Figure 4.17 GFP fused to SHORT ROOT protein expressed under the SHORT ROOT promoter exhibits no change in localisation pattern or fluorescence intensity under low P conditions

Confocal microscopy images showing root tips from *pSHR:SHR:GFP* transformant plants grown on high P and low P. Green signal represents GFP fluorescence measured using the same excitation and detection settings for each treatment, red signal represents propidium iodide stained cell walls. Images show representative localisation patterns of *SHR:GFP* on low and high P; no notable change was seen in any low P treated roots. Scale bars= 25µm.

4.5 Expression of *BIRD* genes under a cortex specific promoter interferes with CCFN under high and low P

In the previous sections, *BIRD* gene mutant phenotypes and transcriptional expression levels were tested in response to low P to ask the question of whether these factors may be involved in regulating CCFN under low P conditions. These results have shown a likely involvement for *BIRD* genes in this process and hinted that the individual contribution of each gene may be complex. *jkd* mutant phenotype (higher than WT CCFN) was unaffected by phosphate in the media. This is not simple to interpret as it may be that both scenarios have reached a CCFN which is the “maximum” that can be accommodated by the root.

Alternatively the *jkd* mutant phenotype may represent a constitutive low P response in some capacity. It was found that *MGP*, *SCR*, and *SHR* were all differentially regulated at the mRNA level under low phosphate (figure 4.8). GFP reporter lines showed some changes in regulation at the promoter activity and protein abundance levels. For *JKD*, these were both in agreement with qPCR results whereby the promoter and translational fusions both showed down-regulation respectively. For *SCR* the translational fusion was in agreement with the qPCRs with an expanded expression domain. BIB protein level also exhibited a dramatic decrease under low P.

Therefore *BIRD* gene cDNAs were then expressed under a cortex specific promoter (Co2; Heidstra *et al.*, 2004) to decouple them from their native regulatory influences and disturb the pathway in a dominant manner. It was

hypothesised that by over-expressing *JKD* or *BIB*, it would be possible to suppress CCFN increase under low P, and expressing *MGP* or *NUC* might elicit a low P like response under low P. If these phenotypes were seen to interact with the low P response, then it could be suggested that they were playing a role in CCFN increase and this didn't occur via subversion of the *SHR/SCR/BIRD* pathway.

Plants were transformed with constructs encoding *BIB*, *MGP*, or *NUC* cDNA under control of the *Co2* promoter and fused at the carboxyl-terminus with GFP for confirmation of cortex specific expression. Plant transformation was performed using floral dip (materials and methods), selected using kanamycin, and expression confirmed by GFP fluorescence. Two independent transformants for the *NUC* construct and one for each *MGP* and *BIB* were recovered. These lines were grown under low or high P conditions for 12 days and then observed using confocal microscopy.

The results from this experiment show that expression of *MGP* under *Co2* promoter induces a constitutive high CCFN phenotype which phenocopies the WT low P response in this experiment. *Co2:MGP:GFP* roots exhibit an average CCFN of 10 on high P and 10.5 on low P; this differs to the WT response which is 9.1 on high P and 10.8 on low P suggesting a constitutive low P-like response for cortex specific *MGP* expression (figure 4.19). In contrast to this, the *Co2:NUC:GFP* construct did not have the same effect. The *Co2:NUC:GFP* lines had 9.3 CCFs (line 3.1) and 8.8 CCFs (line 4.1) on high P, and 11.2 and 11.1 on low P respectively, compared with the same WT as above (figure 4.19).

This experiment shows that *MGP* is capable of inducing proliferative periclinal divisions under high P conditions, but that this cannot be exacerbated by low P conditions. This confirms the previous reports of *MGP* acting to promote proliferative divisions in the cortex (Long et al., 2015; Welch et al., 2007).

Expression of *BIB* specifically in the cortex appeared to have the opposite effect to that of *MGP*. The results presented here show that a *Co2:BIB:GFP* line exhibits a CCFN of 9.3 on high P and 9.2 on low P, which suggests an insensitivity to low P. This finding is in keeping with previous work which has found that *BIB* behaves agonistically with *JKD* in the suppression of proliferative divisions in cortex cells (Long et al., 2015).

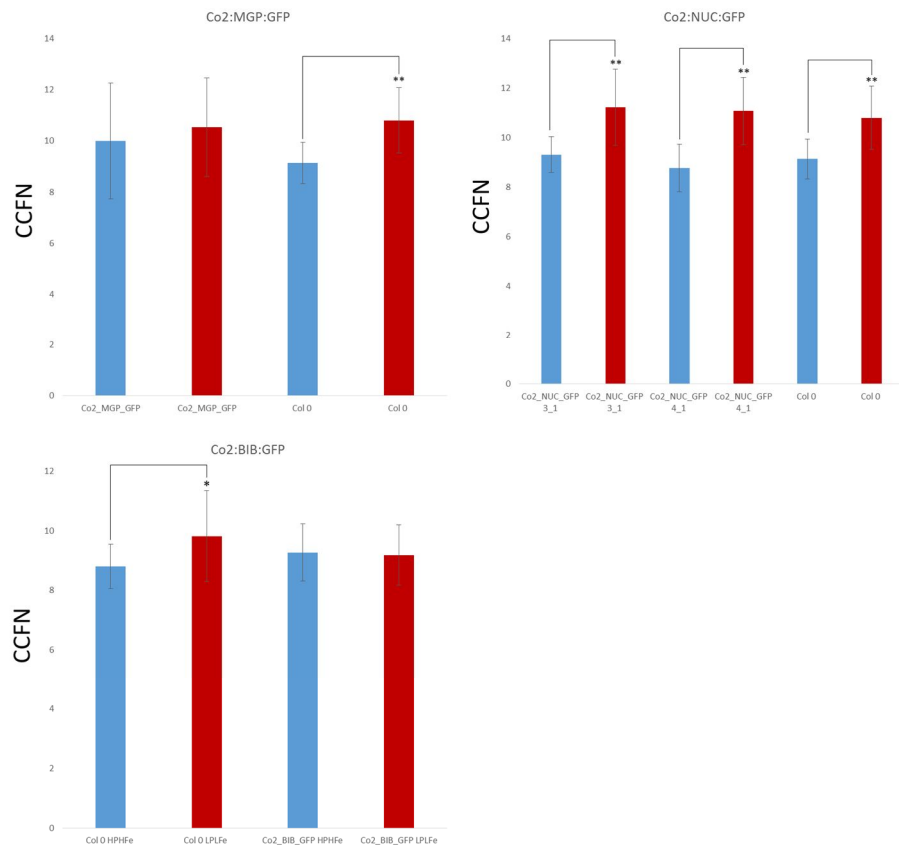


Figure 4.18 Expression of BIRD genes under the control of *Co2* promoter has an impact on CCFN under high and low P

Bar charts showing cortex cell file number for plants expressing *MGP* ($n=14$ for high P, 15 for low P) (a), *NUC* ($n=13$ [3_1 high P], 15 [3_1 low P], 16 [4_1 high P], 15 [4_1 low P]) (b), and *BIB* ($n=11$ for high P, 15 for low P) (c) under control of *Co2* promoter in the Col 0 background under low or high P conditions. Blue bars indicate average values under high P conditions and red bars the same values under low P conditions. *Co2* driven *MGP* expression causes a constitutive high CCFN phenotype on high or low P, which is not significantly affected by low P conditions. Two independent transgenic lines of plants expressing *NUC* under p*Co2* exhibit no effect on CCFN under low P conditions. p*Co2* driving *BIB* expression causes a defect in CCFN increase in response to low P. Differences between treatments are statistically significant to $p<0.05$ (*) or $p<0.001$ (**); black lines indicate which values were compared for statistically significant differences. Error bars represent standard deviation within treatments.

4.5 PIN2 protein undergoes a change in polar localisation under low P conditions

PIN2 transcript abundance was not altered under low P conditions according to qPCR results (figure 4.). Therefore it was proposed that the changes to PIN2 function which are required for CCFN increase under low P must occur post-transcriptionally. Alternatively, it may not in fact be required. To address this question, a *pPIN2:PIN2:GFP* reporter line was used to image PIN2:GFP localisation under low and high P conditions.

This experiment revealed that, as reported in previous literature, PIN2:GFP protein is localised to either rootward or shootward membranes of cortex and epidermal cells; this was maintained in both low and high P treated roots (figures 4.a and 4.b). There is also no clear difference in the signal intensity of GFP fluorescence between low and high P treated roots (compare figure 4.a and figure 4.b); this suggested that a strong change in PIN2 protein level was unlikely to be a large contributing factor to the CCFN response. Therefore the next step was to determine whether changes to the polar localisation of PIN2 in cortex, epidermis, both, or neither were occurring. Initially the software Cellset was used, which enables the definition of cell walls within fluorescence confocal images, which can then be used to map cell types and measure fluorescence intensities. Another function of Cellset is to determine which side of a cell wall a polar molecule resides on, therefore making a prediction of its polar localisation. However, although the predictions of Cellset were experimentally verified, many of these had low confidence values (numbers

below white bars in figure 4.d) and a large number were inconclusive (grey bars in figure 4.d). Additionally, in some images it appeared that signal from deeper cell walls was at times interfering with the predictions in the target domains. It was apparent that this could be done by eye, so therefore each of the images were examined by eye and a conclusion made as to which membrane PIN2:GFP appeared to be residing on. The main evidence used to do this was the presence of a 'tail'-like form to the localisation of PIN2:GFP domains which appears where the protein is present in the membrane as it curves around the corner of the inside of the cell wall (see red arrows, inset, figure 4.d). Using these tails, it was possible to determine which side of the cell the domain was, in cases where the tail was present. This did not, however, rule out the presence of a PIN2:GFP domain on the opposing membrane which may have lacked such a tail formation. Using this technique, along with judgement based on the spatial relationship between a PIN2:GFP domain and the PI stained cell wall, the polar localisation of each PIN2:GFP domain in the root was scored. Domains were scored as either shootward, rootward or unknown (i.e. could not be determined by eye; e.g. figures 4.a and 4.b: yellow bars indicate direction of localisation, membranes with no marker are unknown or with no GFP).

The data show that in the cortex, under high P conditions, 79.5% of PIN2:GFP domains are localised to the rootward membrane, 7.1% to the shootward membrane and 13.3 in an unknown direction. Under low P conditions, in the cortex, 75% of PIN2:GFP domains are localised to the rootward membrane, 13.8% to the shootward membrane, and 11.2% in an unknown direction. The increase in shootward facing PIN2:GFP domains from 7.1% to 13.8% after low

P treatment was found to be statistically significant ($p < 0.05$). In the epidermis, under high P conditions, 12.1% of PIN2:GFP domains were localised to the rootward membrane, 76.4% were localised to the shootward domain, and 11.5% in an unknown direction. Under low P conditions in the epidermis 10.2% PIN2:GFP domains were localised to the rootward membrane, 64.4% to the shootward membrane, and 25.4% in an unknown direction.

This finding is interesting as it suggests that a measurable, significant change has occurred in the subcellular localisation of PIN2:GFP in cortex tissue. Considering that this is the tissue where an increase in cell file number is observed then it makes sense that the cortex is where such a change would be seen. PIN2 has previously been shown to re-localise under changing environmental conditions in order to modify the flux of auxin through the root. PIN2-mediated auxin flux is especially prominent in its role in gravitropism (Rahman et al., 2010b), but is also observed during other responses such as avoidance of light near the soil surface (Laxmi, *et al.* 2008; Wan et al., 2012). Clearly PIN2 localisation is a dynamic and fluid process which is highly responsive to the surrounding conditions. The finding that PIN2 localisation is changed under low P conditions fits well in to the context of its fluidity and suggests that this process is part of a mechanism which is regulating auxin flow through the cortex, as it is doing in Laxmi et al., (2008); Rahman et al., (2010); Wan et al., (2012). Exactly how this change in PIN2 localisation affects auxin flux/accumulation is unclear and the use of an auxin reporter, such as DR5:VENUS, or DIIVENUS would be crucial in determining what auxin changes are associated with low P in the root tip.

4.6 Discussion

This chapter began with four main questions which were to be addressed, focussed on approaching the problem of what factors are involved in orchestrating the proliferation of cortex cell files under low P conditions. The first question asked was:

1. Are other components of the auxin transport/signalling pathway besides PIN2 required for the increase in CCFN which is observed under low P?
2. Are prominent components of the *SHR/SCR/BIRD* pathway required for this response?
3. Do any of these factors exhibit transcriptional changes in response to low P?
4. Do any of these components undergo subtle changes in expression at the pre- or post-translational level?

Within the auxin transport/signalling components which were assessed, the only auxin-related factor mutant which had a significant impact on the CCFN response to low P was *pin2*, which is insensitive to low P. All other mutants studied exhibited a wild type difference between low and high P treated roots, suggesting that individually, they did not play strong roles in the response. Two possible explanations for this finding arose; these were the possibility of redundancy between individual *PINs* and *ARFs* respectively and the possibility that other factors may be at play such as non-canonical auxin responses and/or repressor *ARFs*. The finding that only *pin2* exhibits low P insensitivity suggests that the auxin transport and response changes which occur must be specific to the PIN2 domain since this is the only essential biochemical in the CCFN response to low P so far identified. This then raises the question of which of these tissues is the most important for the CCFN increase. The fact that the divisions occur in the cortex could mean that the cortex is most likely to be the

site of the response, but it could be that PIN2 activity in both cortex and epidermis is required for the change in auxin flux.

2. To answer this question, mutants of *BIRD* genes, *jkd*, *mgp*, and *shr* were analysed for CCFN under high and low P in comparison to Col 0 wild type. This experiment yielded variable results; *jkd* mutant exhibited a constitutive high CCFN which was not exaggerated by low P treatment. *mgp* was hypothesised to exhibit a low P insensitive phenotype (i.e. wild type CCFN under low P) because its functional protein acts antagonistically with *JKD*, therefore if *JKD* is needed to suppress CCFN increase then perhaps *MGP* would be required to promote it (Long et al., 2015). However, the *mgp* mutant exhibited a wild type like response which suggested that the *BIRD* pathway does not play a prominent role in the CCFN increase response to low P. Similarly *shr*, which develops only a 'mutant' ground tissue layer which has been shown to express a mixed cortex/endodermis identity, exhibits a cell file number increase in this tissue in response to low P. Since SHR functional protein is involved in regulating SCR/BIRD complex activity, this result also went against the idea that this pathway plays a strong part in regulating CCFN increase in low P conditions. Similarly to the previous experiments, it was suggested that genetic/functional redundancy could be coming in to play here since there are a number of other IDD proteins active in the root (Coelho, et al., 2017). This raised the question of whether a more direct intervention in the pathway could interfere with the low P response.

3. Given that the results from mutant studies were variable and left open the possibility of redundancy, investigating the transcriptional activity of some of these factors was necessary to determine whether any of them undergo changes in expression level in response to low P. This question was addressed by the use of RT-qPCR performed using cDNA from root tips of low and high treated roots and revealed significant changes in expression of *SCR*, *SHR*, and *MGP*. This finding somewhat contradicted the mutant screen data; components of the *SHR/SCR/BIRD* pathway are differentially expressed under low P conditions, suggesting that they could be active in regulating CCFN response. This finding raised the question of which specific tissues these changes were occurring in, and whether they were also evident at the protein level.

In addition to the increase in expression of these components, it was also interesting that *PIN2* transcript level did not change significantly in response to low P. Considering that *PIN2* is so crucial to this response this result was somewhat unexpected but led to the conclusion that *PIN2* protein is probably regulated post-translationally under low P.

The results from the qPCR experiment showed that several genes (*SCR*, *SHR*, and *MGP*) are upregulated at the transcriptional level in response to low P. This came as an interesting result as it helped to uncover factors which may have a role in the CCFN increase response which could have been masked by genetic or functional redundancy in mutant screening. Therefore it was essential to determine the nature of these changes in expression at the tissue/cell specific level and to determine what, if any, affect this had on resulting protein levels.

To answer this question, promoter and protein reporter lines were used to assess the activity of *SHR*, *SCR*, *JKD*, *MGP*, *BIB*, *NUC*, *CYCD6*, and *PIN2* at the transcriptional and protein level.

These experiments revealed that there were changes in the expression and localisation of several of these factors: p*SCR*:*SCR*:GFP revealed that *SCR*:GFP appears to be expressed in new domains (or at least at higher levels therein) in the cortex and epidermis. This is concomitant with qPCR data which shows a four-fold increase in *SCR* transcript abundance. Other results were more contradictory, p*JKD*:*HIS2B*:YFP show increased expression in several cortex cell tiers whereas p*JKD*:*JKD*:YFP showed decreased level of protein in all 5 cell tiers measured. However, it is important to remember that these two lines were treated differently, with p*JKD*:*JKD*:YFP being grown on low or high P for 12 days, and p*JKD*:*HIS2B*:YFP being grown on high P for one week and then transferred to low or high P for one day. The latter experiment may not show the long term changes to the behaviour of *JKD* which may arise after prolonged low P exposure. The same can be said for the p*BIB*:*BIB*:YFP and p*BIB*:*HIS2B*:YFP reporters, whereby p*BIB*:*HIS2B*:YFP showed no expression in cortex under high P and the same pattern under low P but p*BIB*:*BIB*:YFP exhibiting presence of *BIB*:YFP in cortex under high P with complete abolition of signal under low P in most roots. While this shows that *BIB* protein is most likely strongly decreased under low P, it cannot be directly compared with the promoter data since both lines were grown under different regimes. It was also found that p*SCR*:*SCR*:GFP expression is seen in extra layers under low P conditions and even seen in the epidermis which is not observed in high P conditions. In order to generate a

clearer picture of these results and be able to compare the promoter and protein activities more accurately, it will be important to perform both experiments at the same time points. It would also be interesting to test both along a time-course to investigate how the response to low P changes over time.

The findings presented in section 4.4 reflect those from the qPCR results (figure 4.8) which show no changes in the level of transcript abundance of *CYCD6* under low P; the unchanging pattern of p*CYCD6*:GFP fits the previous data. It has previously been reported that *CYCD6* is a major downstream target of SHR/SCR/BIRD complexes, and that it is a reliable marker of cell divisions which are mediated by these factors (Sozzani et al., 2010). In particular, it is very clear in the *jkd* mutant that p*CYCD6*:GFP signal is strongly increased in the cortex along the length of the meristematic zone (Long et al., 2015; figure 4.12b). If downregulation of *JKD* really were a driving factor in this response, then it would be expected to see such an increase in *CYCD6* activity.

While these data reveal very informative clues about the involvement of these factors in the low P CCFN response, much of the protein reporter data does not uncover their behaviour in the short term (i.e 24hr) which precedes or surrounds the earliest proliferative divisions in the cortex.

The results in this chapter go some way to address the questions which were asked in the first instance. However following on from these findings, several more questions arise. Firstly, if PIN2 protein changes its localisation under low P conditions but its level of expression is unchanged then what is the role of

this? The question which can be asked here is: what effect does the change of PIN2 localisation have on auxin flux/accumulation in the cortex or surrounding tissue? Additionally since PIN2 is localised in both the cortex and the epidermis, what roles do these tissues specifically have on the accumulation of auxin in the cortex and surrounding tissues? Could a modulation of these activities lead to a change in auxin distribution under low P which is concomitant with the other changes we see?

To answer these questions, it will be necessary to take a two-pronged approach involving an experimental aspect and a mathematical modelling aspect. By using an existing model of PIN2 activity and auxin distribution and response dynamics (Band et al., 2012, 2014) it will be possible to simulate a series of scenarios based on the observations made here. This technique will help to generate predictions about PIN2 activity and auxin distribution which are founded in a tested model based on known system and pathways. These predictions can then be used to develop a hypothesis about what would be an expected outcome from the different scenarios of PIN2 localisation and auxin distribution *in vivo*.

Chapter 5: Testing the role of root tip auxin flux in the CCFN response to low P

5.1 Introduction

In chapter 4, the involvement of a number of possible candidate factors were tested for their involvement in the CCFN increase response to low P in *Arabidopsis* root tips.

This began with the testing of factors including a selection of root tip expressed PIN, AUX/LAX auxin transporters and activating ARFs by screening of mutants on low P for insensitive phenotypes. This experiment only revealed a putative role for PIN2, which was the only auxin related mutant tested which has a P insensitive phenotype in relation to CCFN. Reverse-transcription quantitative PCR showed that the level of *PIN2* transcript is not changed in response to low P (figure 4.8), which suggests that its involvement in the response is likely dependent on regulation at the post-translational level. However, observation of a PIN2:GFP protein fusion line showed that there is no obvious change in the level of PIN2 protein. This finding led to the conclusion that its contribution must be occurring as a result of change to protein function/activity, rather than abundance. Closer analysis of this marker line revealed that there was a small but significant shootward shift in the polar localisation of cortex-localised PIN2:GFP protein in response to low P. Collectively, these results suggest that PIN2 protein behaviour is modified differentially to affect the redistribution of auxin in such a way that increased proliferative divisions are initiated within the

early cortex. Whilst the previous chapter showed the requirement of PIN2 for regulating CCFN in response to low P, the data supporting the mechanism by which this effect is mediated remains unclear. This opens up the following questions, which will be addressed in this chapter:

1. *What expression domains of PIN2 are required for the CCFN response to low P?* This is important because it may indicate whether PIN2 is required to direct auxin towards/away from early cortex through the cortex itself, from the epidermis or if the response involves a modification to general polar auxin transport modulated by PIN2. To address this question, lines were developed which complement PIN2 protein in the *eir1* background using tissue specific promoters (Co2 and AUX1) to complement restore PIN2 in cortex and epidermis.
2. *Does blocking auxin response in PIN2 expression domains affect CCFN response to low P?* To address this question, new lines driving tissue specific expression of a stabilised AUX/IAA to impair auxin response in a subset of cells were developed.
3. *What is the consequence of tissue specific PIN2 expression on auxin gradient formation?* In order to predict what changes in polar auxin transport/auxin response are required to elicit an increase in CCFN under low P, an ordinary differential equation based mathematical modelling approach was used (Band et al., 2014). Using this methodology, these scenarios were simulated and compare tissue specific complementation and wild type with the *pin2* mutant to

decipher where auxin transport/accumulation is important to the low P response.

4. *What is the consequence of low P on auxin gradient formation in the root apex?* DII:VENUS, a fluorescent protein expressed ubiquitously which can be targeted for degradation by the TIR1/AFB/SCF complex in response to auxin (Brunoud *et al.* 2012), can be used to test changes in auxin response under low P.
5. *Do PIN2 and JKD act within a linear pathway to orchestrate cortex cell file number changes under low P conditions?* I hypothesised that since *jdk* mutant displays a higher-than-wild type CCFN in P replete conditions, and *pin2* suppresses CCF proliferation under low P, then *pin2* mutant would be able to rescue the *jdk* phenotype. Confirming this hypothesis would suggest that *PIN2* acts upstream of *JKD* in a pathway where *JKD* activity is modulated via auxin concentration in early cortex cells to control CCFN.

5.2 Expression of WT *PIN2* under cortex or epidermis specific promoters in the *pin2* background restores the WT phenotype

PIN2 activity in the root tip is required to increase CCFN in response to low P; it appears that this could be related to changes in *PIN2* localisation in the cortex

(figure 4.3 and figure 4.7). In order to test if *PIN2* function in one or both tissues is required, two tissue specific complementation lines were generated in the *eir1* background using the *Co2* and *AUX1* promoters to restore functional *PIN2* in cortex and epidermis respectively. Seeds were sterilised and sown on to either low P, low Fe (50 μ M, 5 μ M) or high P, high Fe (1mM, 50 μ M) media containing plates and grown for 12 days before being harvested, live-stained and imaged using confocal fluorescence microscopy. Cortex cell file numbers were then counted.

Three p*Co2*:*PIN2* Homozygous T₃ complementation lines (1.2, 3.7, and 4.2) in the *pin2* background were chosen for further analysis. These three lines showed wild type like phenotype exhibiting an average CCFN of between 8 and 9 when grown on high P media but have an average CCFN of between 10 and 12 when grown on low P. The differences between the values for CCFN of low and high P treated plants are all statistically significant at a confidence rate of $p < 0.001$ (figure 5.1a). A similar pattern is seen for the epidermal complementation line which uses a p*AUX1*:*PIN2* construct to complement *PIN2* in the epidermis in *pin2* background. Three independent lines of this construct were tested (1.2, 2.8, and 3.3), and all have an average CCFN of between 8 and 9 when grown on high P and between 10 and 11 when grown on low P. As with the cortex complementation line, the differences in response between low and high P treatments are statistically significant ($p < 0.001$) (figure 5.1b). These data from this experiment reconfirmed earlier findings and showed that *pin2* mutant lines exhibit a diminished CCFN increase response to low P, which is clearly observed in Col 0 wild type roots (figure 5.1 a and b).

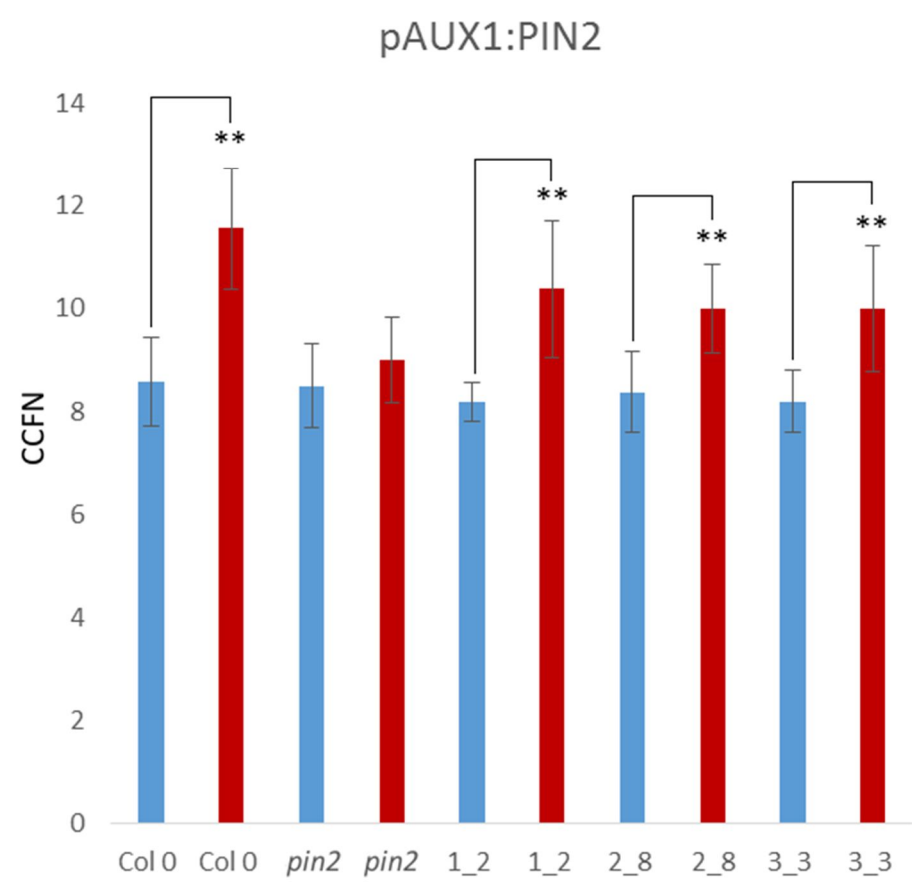
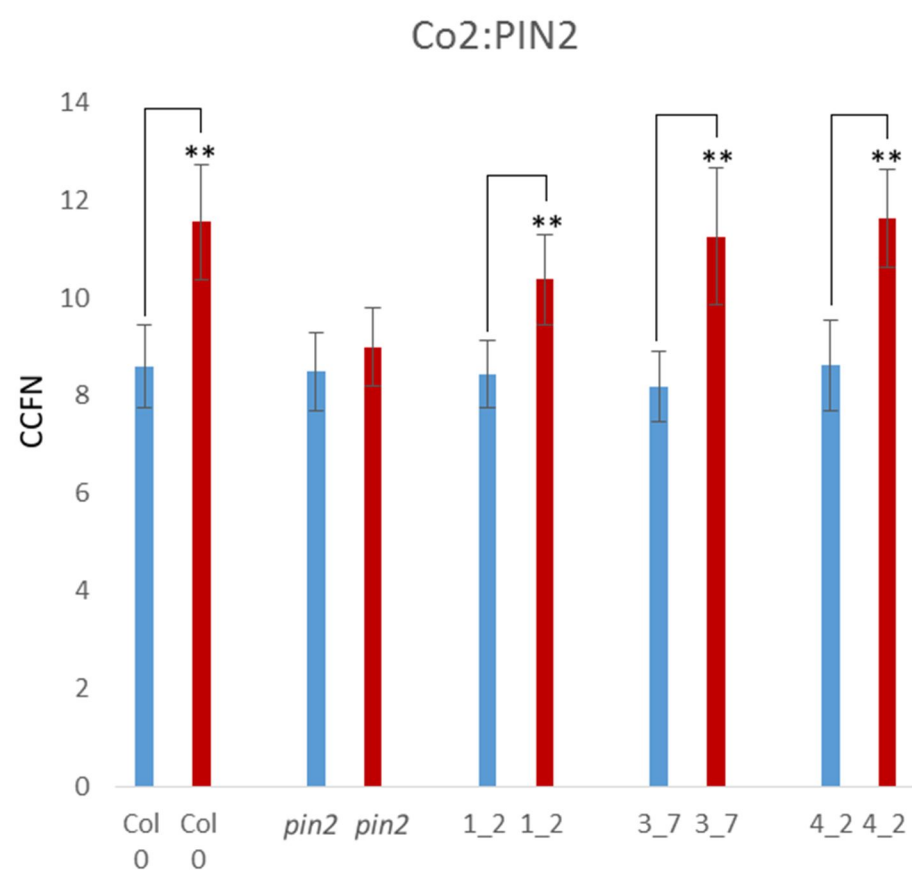


Figure 5.1 Functional *PIN2* expressed in cortex or epidermis is capable of rescuing low P induced cortex cell file number increase defect in *eir1* background

Bar charts showing cortex cell file numbers (CCFN) for Col 0 wild type, *eir1* mutant and three independent transgenic lines each of *PIN2* cDNA expressed in the *eir1* background under *Co2* (a) or *AUX1* (b) promoters. Blue bars indicate average values under high P conditions and red bars low P conditions. When expressed under either *Co2* or *AUX1* promoter, *PIN2* cDNA is able to rescue the insensitivity of *eir1* to low P in all three independent transformants for each construct. Differences are statistically significant to $p < 0.001$ (*); comparisons indicated with a black line connecting bars for compared sets of values. Errors bars represent standard deviation within a treatment.

To test if the AUX1:PIN2 and Co2:PIN2 constructs were being expressed as expected (i.e. in the AUX1 domain and cortex respectively) *in situ* immune localisation was used to detect PIN2 protein in root tips using anti PIN2 antibodies. Seeds of pAUX1:PIN2, pCo2:PIN2, *eir1*, and Col. 0 were sterilised and sown on to ½ MS salts with 1% sucrose and grown for 5 days. Tissue was then fixed using paraformaldehyde and stained according to the method used previously (Peret *et al.*, 2012). Roots were then mounted in 20% glycerol with propidium iodide and imaged using confocal fluorescence microscopy.

This experiment revealed that PIN2 antibody is specific to PIN2 as no signal was detected in *eir1* mutant background (Figure 5.2b). In contrast strong PIN2 signal was detected in epidermal and cortical cells of Col. 0 in agreement with Müller *et al.*, (1998); PIN2 domains can be seen to occupy rootward facing membranes in the cortex, and shootward facing membranes in the epidermis (Figure 5.2a; yellow arrows). The pAUX1:PIN2 line appears to exhibit no discernible PIN2 signal (Figure 5.2c), and pCo2:PIN2 shows some PIN2 localisation in the cortex proximal to the quiescent centre (Figure 5.2d; yellow arrows).

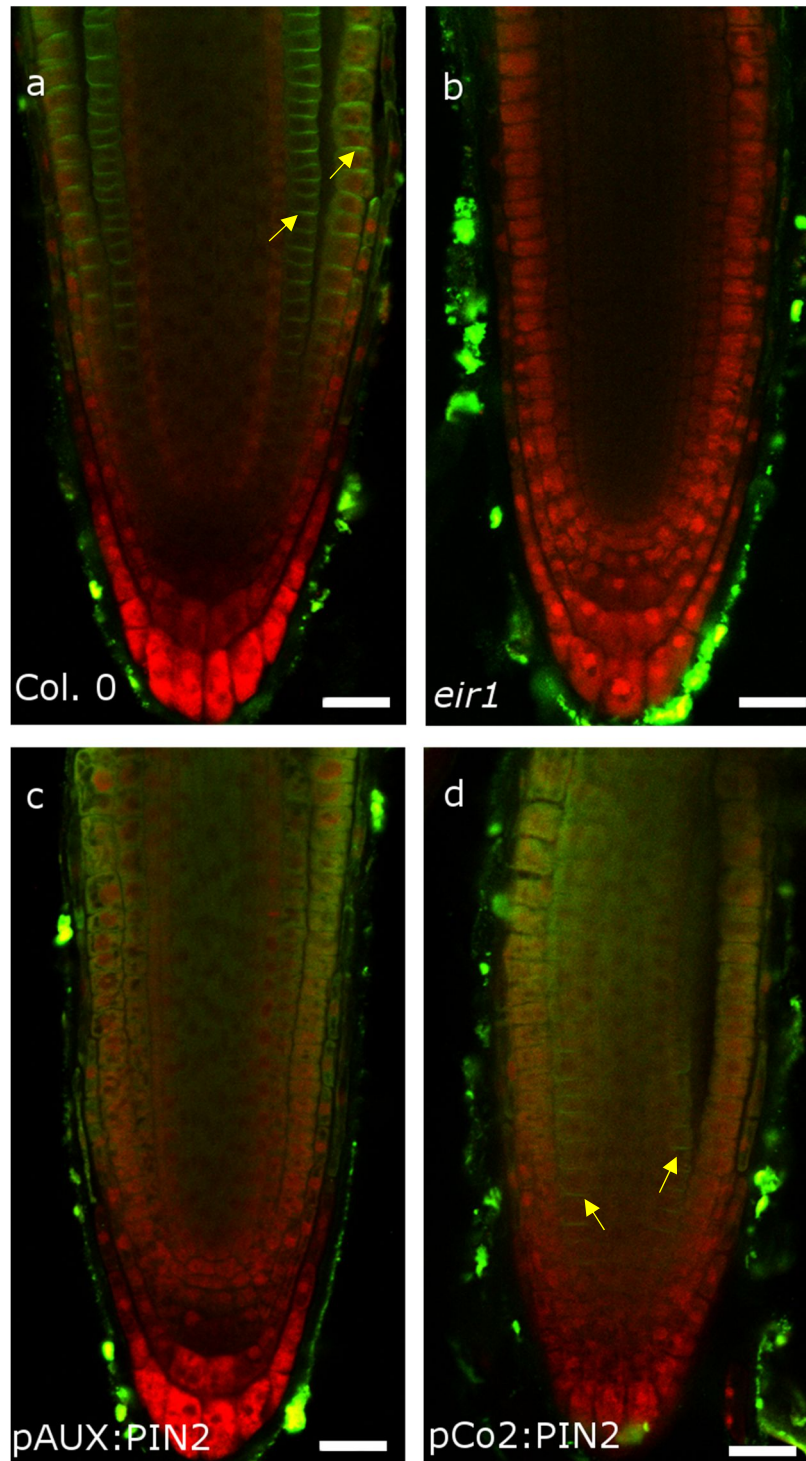


Figure 5.2 Immuno-localisation of PIN2 protein reveals that the Co2:PIN2 construct restores PIN2 protein in the cortex, but the pAUX1:PIN2 construct does not restore PIN2 protein in the AUX1 domain.

Confocal microscopy images showing immune-localisation of PIN2 protein in Col 0 (a), *eir1* mutant background (b), pAUX1:PIN2 in *eir1* background (c), and pCo2:PIN2 in *eir1* background (d). PIN2 domains can be seen clearly in epidermis and cortex in Col 0 (a; yellow arrows), and in the cortex of the pCo2:PIN2 line (d; yellow arrows) but is absent in *eir1* mutant and pAUX1:PIN2 line. Scale bars= 25μm. Green fluorescence represents secondary antibody tagged with Alexa fluor 488, red fluorescence represents propidium iodide.

5.3 Blocking auxin response does not affect CCFN under low P

To test whether canonical (i.e. TIR1 mediated) auxin response in the cortex or epidermis, the *axr3-1* gain of function allele of AUX/IAA17 will be expressed under tissue specific (Co2 and AUX1) promoters.

The *axr3-1* allele was originally discovered as a dominant, auxin insensitive mutant. Recent studies have revealed that this mutant is the result of a lesion in the DII domain of the AUX/IAA17 protein (Leyser et al., 1996; Ouellet et al., 2001). This results in an isoform of the protein which is not targeted for degradation by the TIR1/AFB/SCF ubiquitination complex, leading to an auxin insensitive phenotype whereby auxin cannot de-repress ARF proteins in complex with axr3 protein (Gray, *et al.* 2001). Since this mutant isoform has a strong auxin blocking effect, it can be used for tissue specific expression studies to probe the importance of auxin response under certain conditions or response scenarios. Therefore this approach to block auxin response in the cortex or epidermis was chosen in order to test whether an accumulation of auxin and increased auxin response is required in either/both of these tissues for increased CCFN under low P conditions.

In order to confirm whether these lines carried the transgene, PCR was used to amplify a fragment from genomic DNA which spanned the AUX or Co2 promoter in to the *axr3-1* sequence. This experiment revealed that both pCo2:axr3 1_1 and 9_2 carried the transgene and pAUX1:axr3 1_7 carried the transgene (although pAUX:AXR3 4_4 was not tested). Col. 0 negative control exhibited a negative result for both constructs, confirming successful

transformation in the *axr3* expression lines. This experiment indicates that the lines contained the constructs built to express the *axr3* gene, but does not confirm that they do actually express the gene. Future experiments could test for expression by using q-RT PCR to analyse the levels of *axr3* transcript. Additionally, an *axr3*:GFP fusion could be used to confirm correct localisation of the protein. Gravitropic experiments could also provide additional confidence that the transgenes are active.

Seeds were sterilised and sown on to either low or high P media and grown for 12 days before being stained with propidium iodide and observed using confocal microscopy. My results show that for lines with *axr3-1* driven under the *Co2* promoter, an average CCFN of 9.6 is seen on high P, and 9.1 seen on low for (line 1.1), or 9 on high P and 10.6 on low P (line 9.2; difference between low and high P statistically significant: $p < 0.05$). Wild type control for this experiment had an average CCFN of 9.1 on high P and 10.8 on low P (difference between low and high P statistically significant: $p < 0.001$) (figure 5.3a). These data show a differing result between the two independent transgenic lines used (1.1 and 9.2) where line 9.2 responds like wild type to low P with a statistically significant increase in CCFN, but line 1.1 does not exhibit an increase in CCFN suggesting low P insensitivity.

Expression of *axr3* under the *AUX1* promoter leads to a similar pattern of response to the expression of *axr3* under control of the *Co2* promoter. Line *pAUX1:axr3* 1.7 exhibits an average CCFN of 9.5 on high P and 9.2 on low P, which is not statistically significant. On the other hand, line 4.7 has an average

CCFN of 9.2 on high P and 11.7 on low P, which is statistically significantly different ($p < 0.001$). These are compared to the wildtype Col 0 control which has an average CCFN of 9 on high P and 11 on low P the difference between which is statistically significant ($p < 0.05$) (figure 5.3 b).

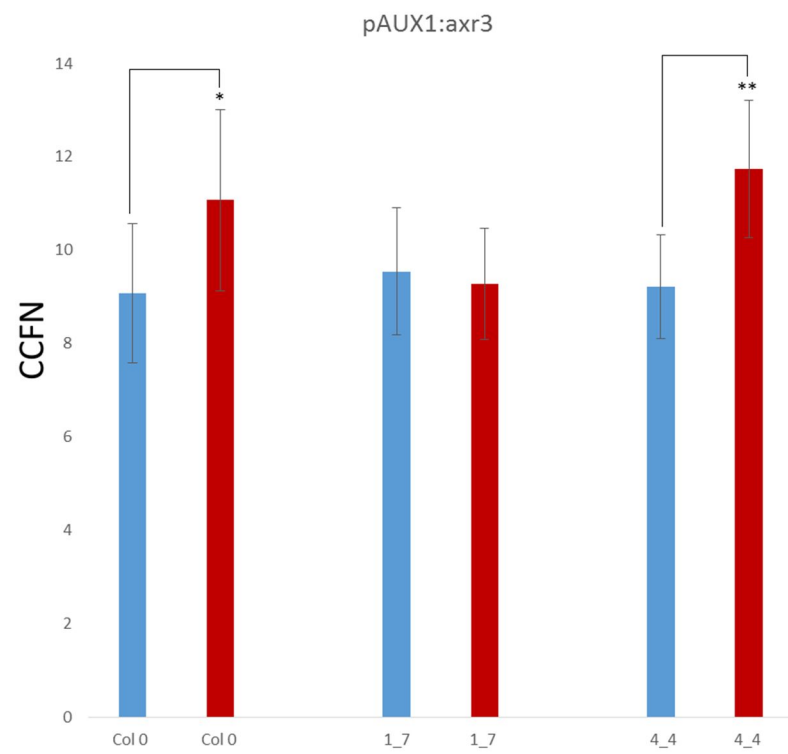
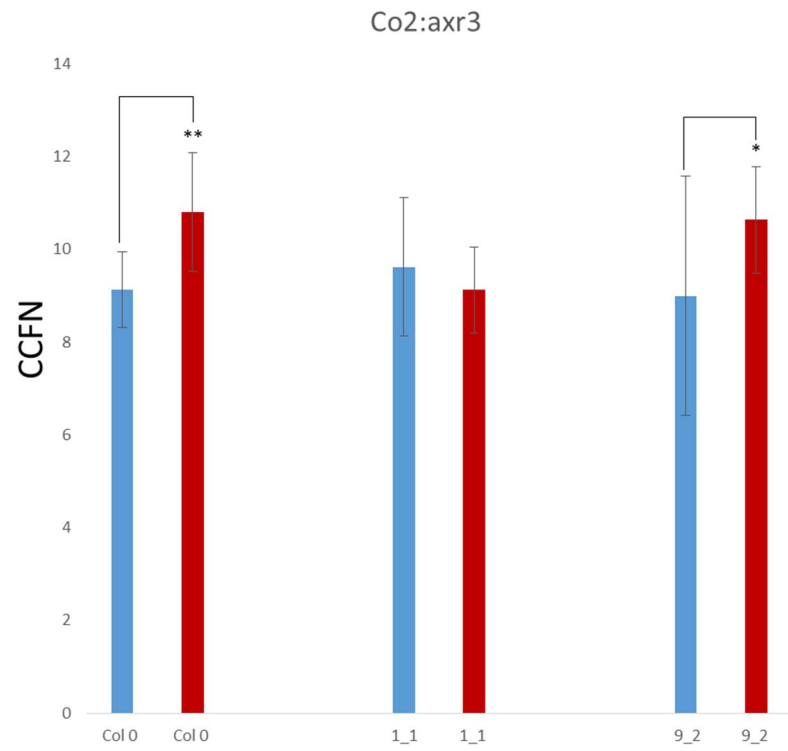


Figure 5.3 Expression of stabilised AUX/IAA protein *axr3* specifically in cortex or epidermis does not have a major effect on cortex cell file number under low P in Col 0 background

Bar charts showing cortex cell file numbers (CCFN) for Col 0 wild type and two independent transgenic lines each of *axr3* expressed under either *Co2* (a) or *AUX1* (b) promoter. Blue bars represent average CCFN for roots grown on high P media and red bars represent the same value for roots grown under low P conditions. Expression of *axr3* under control of *pCo2* (n= [line 1_1: 8 for high P, 9 for low P; line 9_2: 13 for high P, 11 for low P]) or *pAUX1* (n= [line 1_7: 15 for high P, 15 for low P; line 4_4 15 for high P, 15 for low P]) does not consistently impact upon CCFN increase under low P. For each construct, one independent line exhibits a defect in CCFN increase but the other does not express the same phenotype. (*) indicates statistical significance $p < 0.05$, (**) indicates statistical significance $p < 0.001$; compared values indicated by connecting black line. Error bars represent standard deviation within each treatment.

5.4 Mathematical modelling of auxin transport reveals that *pAUX1:PIN2*, and not *pCO2:PIN2* is capable of restoring wild type-like auxin distribution in the root tip

Using a modified version of a mathematical previously described by Band et al., (2014), it was possible to generate predictions of the effect which differential modulation of certain parts of the auxin transport pathway would have on auxin distribution. This model is built using experimental *in vivo* data on the subcellular localisation of PINs, AUX/LAXs, and ABCBs. By incorporating the effect of mutations in these transporters of DII VENUS distribution and their predicted auxin transport kinetics, a series of differential equations could be formulated to act as a model of auxin transport through the root. Applying the effects of these transporters as variables within these equations, they can be used to calculate the predicted steady state of auxin in each individual cell in a 2D map of the Arabidopsis root tip.

In this experiment, with the help of Dr Nathan Mellor, the parameters of the model were modified such that it would mimic the *pin2* mutant and the cortex and epidermis tissue specific PIN2 complementation lines described in section 5.2. The first outcome of this experiment was as would be expected: *pin2* mutation results in an accumulation of auxin at the root tip (stele, QC, columella, and lateral root cap) when compared with wild type transporter distributions. Additionally, as would be expected from the data presented in figure 5.1b, it was seen that steady state auxin accumulation in the root tip in

the *pAUX:PIN2* distribution was restored to something very similar to wild type (figure 5.4a and b). The model suggested a relative auxin level of around 1 in the meristematic epidermis and around 1 in the meristematic cortex in both wild type and predictions of *pAUX1:PIN2* in the *pin2* background (figure 5.4b). This can be seen in the heat map in figure 5.3b which indicates a pattern across the whole root tip which appears very similar to wild type distribution. Interestingly, however, the model where PIN2 activity was only present in the cortex predicted an auxin distribution pattern which was very similar to the *pin2* mutant. Figure 5.3b shows that the relative auxin levels are around 2 in the meristematic epidermis and 2 in the meristematic cortex overall. On average this is twice as high in these scenarios; however this is an even greater effect if only the first few cortex cells shootward from the QC are considered. Figure 5.4a shows that the values in the first 4-5 youngest cortex cells is three times higher than in the wild type in *pin2* and in *pCo2:PIN2* (in *pin2* background) models. Taken together, these results suggest that localisation of PIN2 in the epidermis is more instrumental in the reflux of auxin away from the root tip and out of meristematic cells. What exactly this means for the low P CCFN response is not entirely clear, however it does suggest that movement of auxin away from the meristematic tissue may be important. This data shows us that the ability of the plant to remove auxin from the meristematic cortex (and possibly other meristematic tissues) could enable the CCFN increase response to low P.

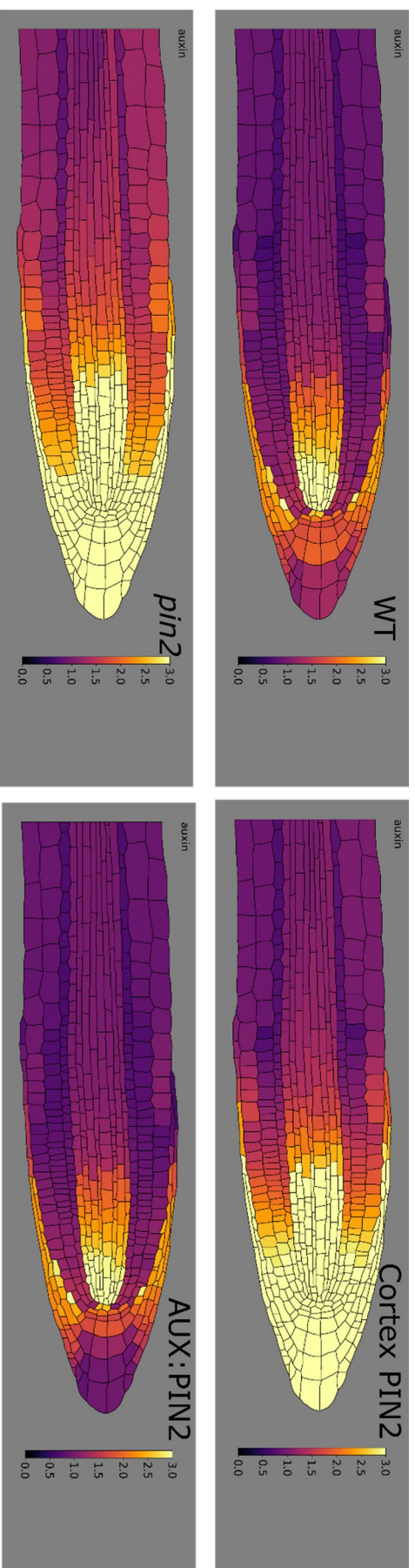
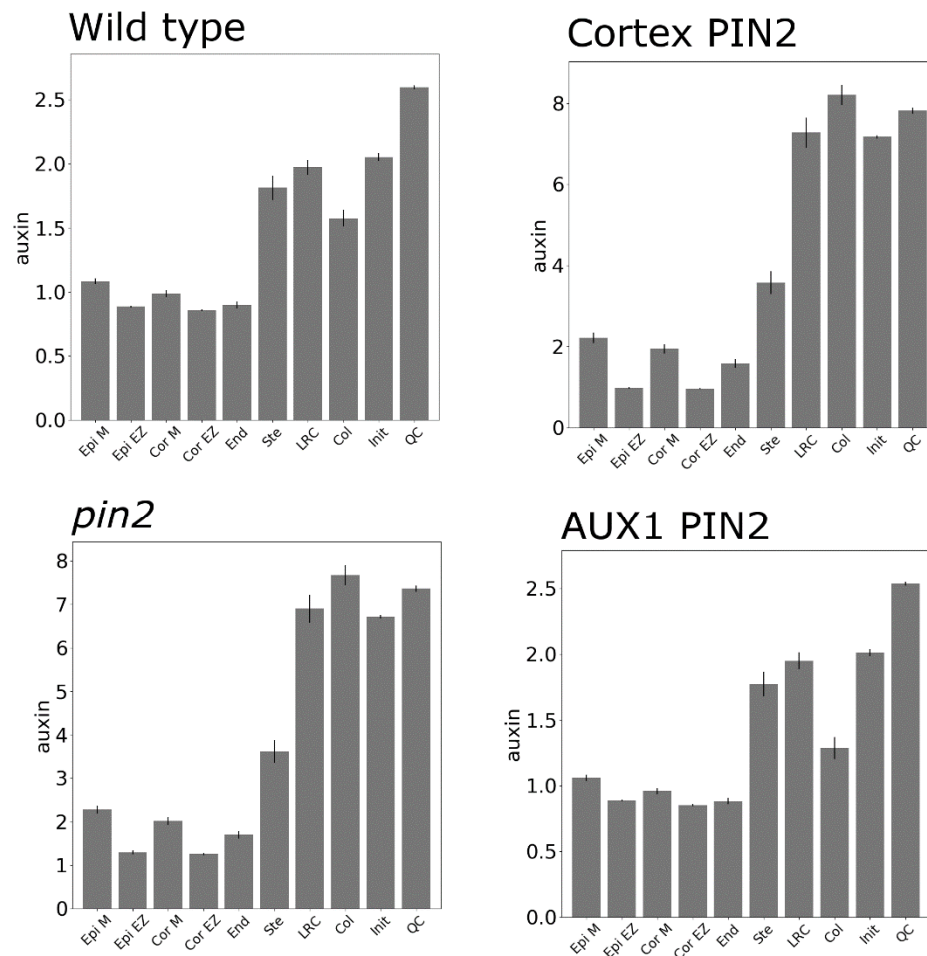


Figure 5.4 Mathematical modelling of root-tip auxin distribution suggests that PIN2 is required for decreasing auxin concentration in the cortex

(a) 2-dimensional schematics of root tip auxin steady states for models with wild type parameters, *pin2* parameters, *pin2* cortex:PIN2 parameters, and *pin2* AUX1:PIN2 parameters. The data predicts that the *pin2* mutation results in an accumulation of three or more times more auxin in the early cortex cells compared with wild type. According to the model, this effect can be rescued by the activity of PIN2 in the AUX1 localisation domains but not in the cortex alone. (b) Bar charts showing the relative average values of auxin concentration in different tissues under different model parameters. (Epi M= meristematic epidermis, Epi EZ= elongation zone epidermis, Cor M= meristematic cortex, Cor EZ= elongation zone cortex, End= endodermis, Ste= stele, LRC= lateral root cap, Col= columella, Init= initial cells, QC= quiescent centre). The data predicts a general increase in auxin in the root tip in the *pin2* and Cortex:PIN2 models. In some tissues this value is quadrupled (e.g. columella); in the meristematic cortex the value is approximately doubled.



5.5 DII:VENUS auxin reporter does not show a significant change in auxin response under low P conditions in the wild type or *pin2* background

Since it was established that PIN2 changes its localisation in response to low P, and the model in section 5.4 predicts that *PIN2* is required for the flux of auxin out of the root tip, the next step was to observe the distribution of auxin/auxin response in the root using an auxin reporter. DII:VENUS utilises the DII domain of AUX/IAA proteins, which is used to target proteins for proteolysis by TIR1/AFB/SCF in the presence of auxin, to create a VENUS (YFP) protein which

is degraded in response to auxin. This line was used to test whether there was a change in auxin response in the cortex under low P in Col background and *pin2* mutant *in vivo*.

Seeds were sterilised and sown on to high P (1mM P) containing media and grown for 7 days. Seedlings were then transferred to either high P (1mM P) or low P (50µM P) and grown for a further 24 hours. Seedlings were then stained with propidium iodide and imaged using confocal microscopy. Longitudinal sections were imaged using the same excitation/collection settings for all roots (figure 5.5a), and the fluorescence was measured using imagej software.

This experiment found that DII:VENUS in the wild type background exhibited an average corrected fluorescence measurement of 341165 for the first five cortex cells on high P; this value for low P was 274838 (Figure 5.5b). DII:VENUS in the *pin2* background on high P displayed an average corrected fluorescence value of 415744 in the first 5 cortex cells, whereas this value on low P was 315298 (Figure 5.5b). However, none of the differences between low and high P are statistically significant. Therefore no conclusions can be drawn from this experiment.

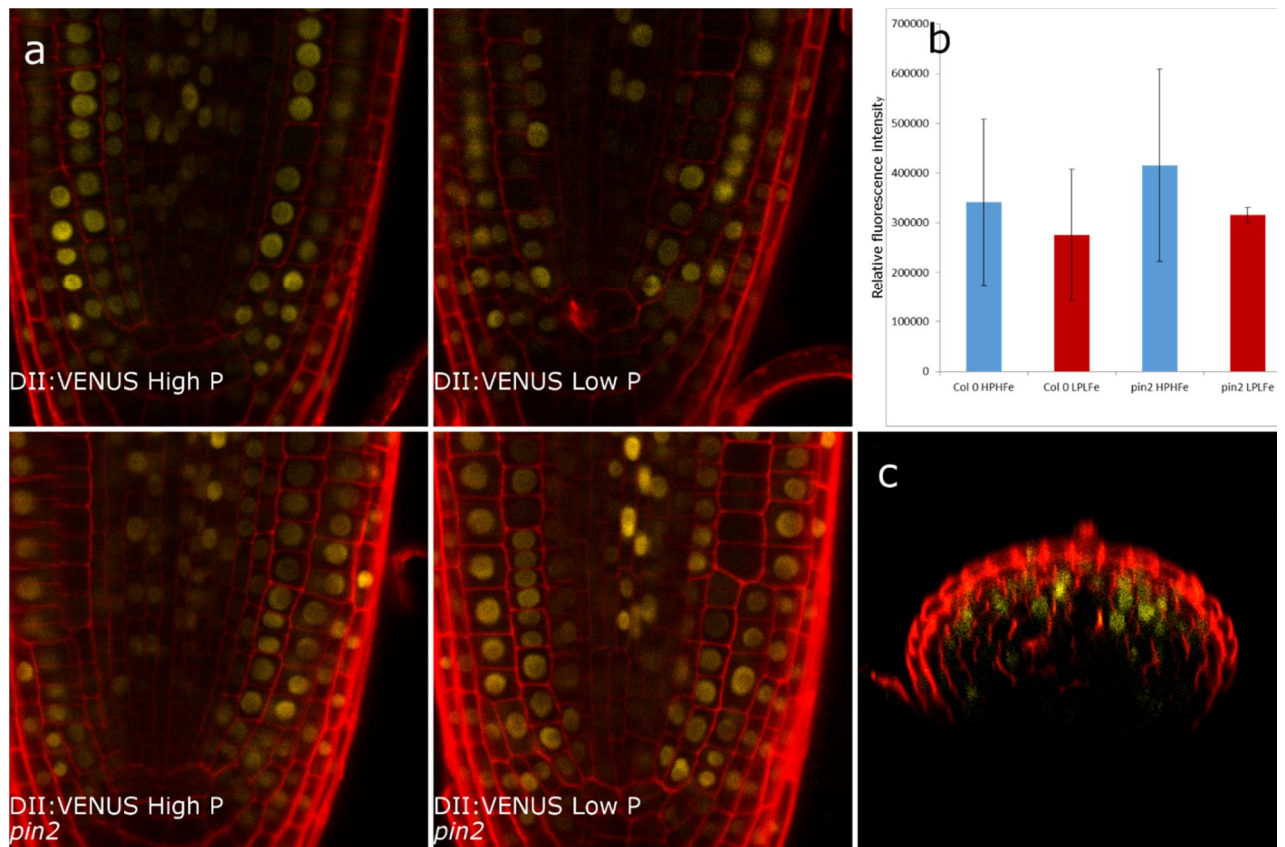


Figure 5.5 DII:VENUS shows no difference in auxin response under low P conditions or in *pin2* mutant

Images of DII:VENUS fluorescence under high (1mM) and low (50 μ M) P conditions in wild type and *pin2* backgrounds (a). No clear difference between any treatment or genetic background is visibly obvious. Average fluorescence values (y axis) for these lines was not statistically significant in any case (b), suggesting that a change in auxin response level was not seen. Attempts to generate three-dimensional images by stacking radial cross-sectional images from confocal microscopy resulted in images with significant optical aberration through and photo-bleaching (c). Yellow signal represents VENUS fluorescence, red signal represents propidium iodide fluorescence. Blue bars on chart: high P, red bars on chart: low P. Error bars represent standard deviation.

5.6 *pin2* mutation is capable of partially rescuing the *jkd* CCFN phenotype

Previous reports have demonstrated the relationship between the SHR pathway and auxin signalling. Cruz-Ramírez et al., (2012) found that auxin stimulates *CYCD6;1* in a SHR/SCR dependent manner, and Möller et al., (2017) describe how auxin establishes the SHR domain in vascular precursor tissue and *MGP/SCR* domains in ground tissue via ARF5 in the embryo. Therefore, since both auxin (via *PIN2*) and *SHR/BIRD* factors have both exhibited some involvement in low P related CCFN increase in my work, a logical next step was to probe for cross-talk between the two pathways in this response.

For this experiment I chose to focus on *JKD* since its mutant exhibits a high CCFN under P replete conditions. Because *pin2* has been shown to suppress the low P response, I wanted to know whether *pin2* could suppress the *jkd* phenotype. If this was the case then it would imply that *JKD* and other *SHR/SCR/BIRD* components lie downstream of *PIN2* mediated auxin redistribution in re-patterning cortex cell files under low P.

jkd and *pin2* mutants, both in the Columbia background, were crossed and the F₁ progeny propagated to then identify homozygous F₂ plants to obtain homozygous seed from. Once a homozygous line was identified, seeds were sterilised and sown on to plates containing replete P alongside *pin2*, *jkd*, and Col 0 as controls. After 12 days the roots were harvested, stained with PI and imaged using confocal microscopy. The data revealed that *jkd* mutant had statistically significantly more cortex cell files than either Col 0 or *pin2* (average

of 14 compared with 9.1 or 8.25, respectively; $p < 0.001$; figure 5.6). Interestingly, although *pin2 jkd* had significantly more cortex cell files than Col 0 and *pin2* (11.7), it had significantly less than the *jkd* mutant alone ($p < 0.001$; figure 5.6).

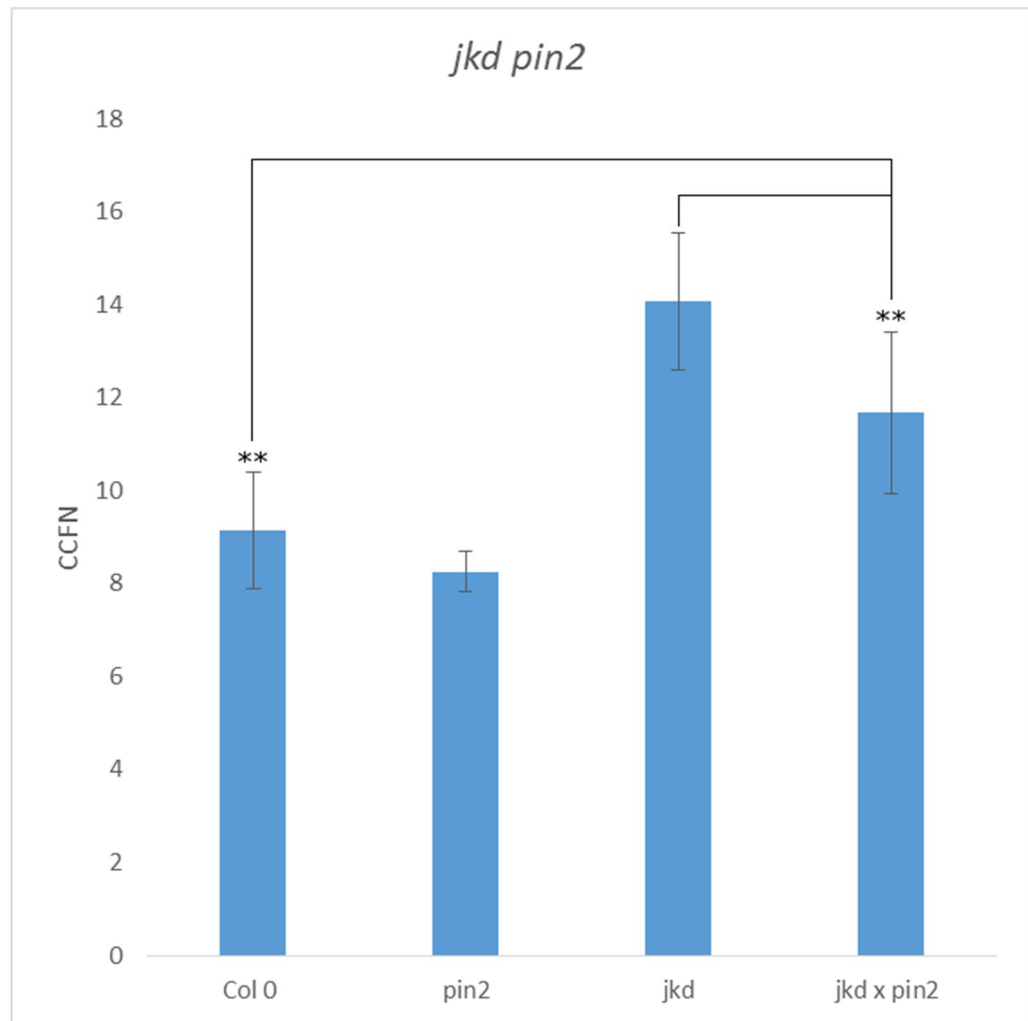


Figure 5.6 The *pin2* mutation is capable of partially rescuing the *jkd* CCFN phenotype

Bar chart showing values for cortex cell file number (y-axis) for Col 0 (n=16), *pin2* (n=16), *jkd* (n=15), and *pin2 jkd* (n=14) in P replete conditions. Error bars represent standard deviation. Black lines show relationships of statistically significant difference between values; **: $p < 0.001$.

5.7 Discussion

This chapter started with four questions based around hypotheses generated based on previous chapters. These questions were:

1. Does the CCFN response to low P require PIN2 activity in the cortex, epidermis or both?
2. Considering that *PIN2* is active in cortex and epidermis and may be affecting auxin response in cortex and/or epidermis, can CCFN in these tissues be affected by directly manipulating auxin response?
3. What is the predicted change in auxin accumulation/response under the scenarios of *pin2* mutation and tissue specific complementation using mathematical modelling?
4. Do *PIN2* and *JKD* act within a linear pathway to orchestrate cortex cell file number changes under low P conditions?

The first question was raised as it is a crucial step in understanding the role which *PIN2* has in mediating the CCFN increase seen in response to low P. To answer it, *PIN2* was expressed specifically either in the cortex or the *AUX1* domain (i.e. root cap and epidermis). This experiment yielded an unexpected answer to the question: that *PIN2* expression/activity in either of these domains is sufficient to drive changes to cortex patterning under low P. This answer was surprising as it was postulated that even though epidermal *PIN2* plays a role in mediating PAT, cortical *PIN2* would seem the logical most important for controlling auxin levels in the cortex. However it was concluded

that whatever auxin level change was occurring in the cortex under low P, it must be that *PIN2* and the regulation of its function in either tissue could produce an accumulation or reduction of auxin in cortex. It was also suggested that although *PIN2* in the cortex was the only localisation which underwent significant changes in response to low P, activity of *PIN2* across the root could be modified in a way not visible using the *PIN2*:GFP reporter. There are several possible explanations for this. It could be that the activity of *PIN2* in the epidermis has an impact on the auxin reflux modulation which results in additional periclinal divisions occurring in the cortex tissue. *PIN2* function/activity might be modified such that a change in the rate at which auxin is diverted away from the apical end of the cortex tissue and then shootward through the epidermis. This modulation may well be coordinated by *PIN2* in both cortex and epidermis in wild type plants, but could perhaps be managed by *PIN2* in just one. While *PIN2* polarity was seen to change significantly only in the cortex, this is not the only indicator of a change in the function of *PIN2*. There are other components which regulate *PIN2* activity and whose activity would not necessarily be obvious or measurable using the *PIN2*:GFP reporter. ATP-BINDING CASSETTE TYPE-B (ABCB) transporters have been shown to associate with PINs, including *PIN2* in their localisation domains at the plasma membrane. Blakeslee et al. (2007) showed how *PIN2* and ABCB1 proteins co-localise in the plasma membrane in the cortex and epidermis, and demonstrated that domains of *PIN2* and ABCB1 interacted in yeast-two-hybrid assays. This study and others have shown synergism between PIN and ABCB transporters; Blakeslee et al., (2007) show how the gravitropic defects of *pin2*

are enhanced in *abcb1,abcb19* background. Mravec et al., (2008) show how only *pin1, abcb1, abcb19* triple mutants had cotyledon development defects when *pin1* or *abcb1,abcb19* mutants did not. As well as potentially acting in concert with other auxin transport facilitators, PIN2 is subject to regulation by phosphorylation by a number of kinases. Some of this activity regulates the polar localisation and endocytotic recycling of PIN proteins; *PINOID (PID)* is required for gravitropic responses in roots by phospho-regulation of PIN2, one of whose mechanisms is control of PIN2 protein level via the M3 phosphorylation site (Ki et al., 2009). Other kinases have also been shown to regulate PIN2 membrane targeting and endocytosis, such as *D6 PROTEIN KINASES (D6PKs)*; Barbosa, et al., 2014) and *CDPK-RELATED KINASE 5 (CRK5)*; Rigó et al., 2013).

In addition to the existing understood pathways of phospho-regulation of PINs, PIN2 has diverged somewhat from other PINs in respect to its phosphorylation sites in the intracellular loop and their flanking regions (Barbosa & Schwechheimer, 2014; Weller et al., 2017). The importance of these differences and the role of some of the phosphorylation sites are not known, and could represent sites of action for regulatory modifications which are involved in modulating PIN2 activity. Due to the complexity of PIN2 function and its relationship with regulatory factors, and possibly other transporters, it is feasible that although no change in polarity of PIN2 in the epidermis was seen under low P, it could still be affecting auxin flux differently.

However, care must be taken to interpret these results as it was shown in Figure 5.2 that although the Co₂ driven expression of PIN2 was visible using immunolocalisation, the pAUX1:PIN2 line exhibited no discernible PIN2 expression in the root tip (Figure 5.2c). This is at odds with the phenotype of this line in that it is able to rescue the phenotype of the low P response in the *eir1* mutant background (Figure 5.1). One explanation for this could be that the AUX1 promoter is not as strong as the PIN2 promoter, although this is unlikely as the AUX1 promoter has previously been shown to express protein at a level detectable using immunolocalisation (Peret et al., 2012). Additionally, even if this were the case then it would seem unlikely that there would be protein abundance sufficient to function effectively enough to rescue the phenotype. These results could be further clarified by testing the gravitropic phenotype of the pAUX1:PIN2 line to test whether they rescue the agravitropic phenotype of *eir1*. If this is the case then it could be suggested that there is some PIN2 activity by the protein abundance is below the level which can be detected using immune-localisation. Additionally, developing new complementation lines using PIN2:GFP would enable much simpler confirmation of correct expression and targeting of the PIN2 protein.

Question two was addressed using the expression of a stabilised AUX/IAA (*axr3-1*) under the Co₂ or AUX1 promoter. By doing so, auxin response would be blocked in these specific tissues and the role of auxin response in low P induced CCFN increase could be probed. However, the two lines from each construct

both behaved differently, i.e. one blocked low P response, and one behaved like wild type. Since the results were contradictory a conclusion cannot be drawn without further work.

To address question three, a mathematical model using ordinary differential equations (ODEs) based on known parameters for auxin transporter localisations and auxin movement kinetics was used. This was queried by changing certain parameters to make predictions for auxin distribution under various circumstances. In order to determine what the role of *PIN2* might be under low P, the WT-mimicking model was compared with a *pin2* mutant model and models of my Co2 and AUX promoter driven *PIN2* complementation lines. The results from this experiment suggested that the consequence of *pin2* mutation is an accumulation of auxin at the root tip (including in the early cortex). Since this was rescued by *PIN2* in the *AUX1* expression domain (though not in the cortex), it was concluded that the ability of the root (via *PIN2*) to deplete auxin in the early cortex was responsible for this response. I.e. if auxin accumulation in the cortex occurs in *pin2 in planta* then it implies that this is blocking the low P response, suggesting that a low auxin signal is required.

Despite the apparent discrepancy between the model and the phenotype seen *in vivo*, it seems that the model teaches us that the most important function of *PIN2* during the low P response is to facilitate auxin reflux away from the root tip. This is made clear by the greater requirement of shootward *PIN2* in epidermal/root cap cells rather than in the cortex, and the accumulation of auxin in the meristematic cortex which is seen in the *pin2* mutant in the model.

This does seem in keeping with the findings presented in figure 4.19, whereby PIN2:GFP protein domains appear to shift to a more shootward orientation in the cortex, which would presumably be to direct auxin away from the early cortex.

Previous work which has investigated the action of auxin and downstream factors on cell division has suggested that in the meristem, auxin is required at the root tip for signalling patterning divisions. For example, Mähönen et al., (2014) show how auxin activates downstream *PLETHORA* genes which then play a role in regulating cell divisions within the meristem and division zones. They demonstrate that a gradient of *PLETHORA* along the meristem defines the different zones of the root tip (i.e. meristematic zone, MZ; differentiation zone, DZ; and elongation zone EZ). It seems that the auxin/*PLETHORA* interaction is an important determinant of cell divisions in early root tissues, and Mähönen et al., (2014) show that high auxin/*PLETHORA* at the root tip inhibits fast proliferative divisions and promotes slower patterning divisions. They suggest that reduced auxin/*PLETHORA* in the DZ results in fast anticlinal divisions, which followed by rapid cell expansion in the EZ results in elongation of the root. Such data shows that auxin response does not necessarily have a direct relationship to cell division/expansion but rather determines the orientation/rate of the division depending on the specific sensitivity of the identity of that cell.

The effect of auxin maxima in embryo patterning is well studied (Moeller *et al.* 2017, Smit and Weijers 2015), and such research has suggested that the action of auxin on cell division is dependent upon the identity and the position of the

cell. This differential activity allows auxin maxima to orchestrate divisions in different planes across groups of cells in the embryo, thus forming the architecture of the embryo. For example, Möller et al., (2017) show how although *ARF5* (*MP*, *MONOPTEROS*) is expressed quite broadly in the embryo, the concentration of auxin in to ground tissue precursor cells determines the orientation of the division plane. *ARF5* then becomes expressed specifically in vasculature and ground tissues, leaving these cells sensitive to tissue specific auxin-responsive downstream expression.

Möller et al., (2017) indicate that auxin has a strong role to play in patterning formative divisions in specific subsets of cells in the embryo, and for maintaining quiescence and stem cell identity in the root meristem. This means that specific auxin concentrations in these cells tune the downstream expression of developmental factors and that different cells have different downstream responses or sensitivity to auxin depending on their identity. Although the effects of auxin on embryo and root meristem patterning are well researched, the effect of auxin on cortex/ground tissue under certain environmental conditions is not well understood.

A series of experiments designed to test the hypothesis that low P results in auxin flux away from the cortex could be carried out. Firstly, the use of an auxin reporter to detect the levels of auxin/auxin response which is occurring in the cortex would be necessary to address the hypothesis of decreased auxin in the cortex under low P. This could be extended by crossing the *PIN2* complementation lines described earlier in section 5.3 with *DIIVENUS* in the

pin2 background which would help to understand how these constructs manipulate auxin flux to complement the *pin2* mutant response to low P. Such experiments could be complemented by the use of auxin biosynthesis mutants such as the *yuc* (*YUCCA*) mutants or *taa* (*TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS*) mutants. These mutants display numerous auxin related developmental/growth defects such as shade avoidance defects, floral development defects and root growth/development defects. Won et al., (2011) show how these enzymes coordinate the biosynthesis of IAA via the same pathway whereby TAAs convert tryptophan to indole-3-pyruvate (IPA) which is then converted to indole-3-acetic acid (IAA). Therefore, defects in this pathway caused by loss of multiple members of these families result in large changes to the levels of active IAA. An alternative to the use of these mutants could be the expression of auxin catabolising enzymes in the cortex. This could be done by using the *Co2* promoter to express an enzyme which converts IAA to an inactive form such as DAO1 (*DIOXYGENASE FOR AUXIN1*; Porco et al., 2016) which converts IAA to 2-oxoindole-3-acetic acid; or GH3 enzymes which conjugate IAA to amino acids (Staswick, et al. 2005). By manually reducing the auxin available to these cells, it would then be possible to discover whether such a scenario could confer sensitivity to low P, or CCFN increase under P replete conditions. These also have an advantage over the *axr3-1* expression experiments discussed above as they are not specific to one auxin signalling pathway (i.e. TIR1/AFB/SCF/AUX/IAA/ARF) and rather just mimic the reduction in IAA levels predicted under low P.

The role of cortex auxin level in this response could also be probed by the expression of *A. tumefaciens* indole-3-acetamide hydrolase (*iaaH*) specifically in cortex cells and growing seedlings on indole-3-acetamide (IAM) and low P (such as by Oka, *et al.*, 1999). This would increase IAA concentration in the cortex specifically and if it blocked CCFN increase under low P then it would be possible to conclude that reduced auxin is an important factor. It must be said however that this method is not totally selective since *Arabidopsis* has been shown to produce low levels of IAM (Pollmann, *et al.*, 2002).

Care must be taken when using such strategies as it has been shown that changes to the levels of certain auxin species can be made up for by alternative pathways (Porco *et al.*, 2016). A simpler, but less tissue specific, preliminary experiment to the latter approaches would be to grow seedlings on low and high P media with and without NAA; if raising overall auxin levels suppresses the low P response then this would support the first hypothesis.

In order to probe which downstream targets may be differentially regulated in response to a drop in auxin levels and low P, an RNA sequencing experiment could be performed. A construct for an enzyme which catabolises auxin (e.g. DAO1) would be driven under the *UAS* (upstream activation sequence) promoter and then crossed in to *pCO2:GAL4:GFP*. This cross, along with the *pCO2:GAL4:GFP* in its native background could be grown on replete and low P media and then their roots harvested for fluorescence activated cell sorting (FACS). Sorted cells (which would consist only of cortex cells) could be used to extract RNA from and then sequence all of the RNA molecules present. Using

bioinformatics analysis, factors which are up- or down-regulated in common between low P “WT”, and *UAS:DAO1* would reveal genes which are downregulated on low P in the cortex but also in response to low auxin. These genes would then be promising candidates to follow up as components in the low P-CCFN increase response pathway, and would also help to explain the relationship between PIN2, auxin and this response.

Question four asked whether there was an interaction between PIN2 and JKD in regulation cortex cell file number. Because PIN2 has a putative role in the CCFN response to low P, and it seemed that the *SHR/SCR/BIRD* pathway could be involved in the changes seen, it was hypothesised that the two pathways could be connected. Previous publications which showed the link between auxin, auxin transport, and establishing expression of *SHR/SCR/BIRD* related factors, it was suspected that *PIN2* might regulate *BIRD* genes as downstream factors during low P response. Since out of the *BIRD* genes, *jkd* has the strongest phenotype which could be related to the low P response seen in cortex, *jkd* was chosen as the most likely candidate to be regulated by *PIN2*. To test the relationship between these factors, *jkd* with *pin2* were crossed to see if *pin2* could rescue the *jkd* phenotype. In this experiment it was found that although *pin2* did not fully rescue the *jkd* phenotype, it was capable of suppressing it to a statistically significant degree. It was concluded that in the context of previous publications, and the fact that the two do seem to interact, it is most likely that *PIN2* acts upstream of *JKD*. However, it was not tested whether this was true under low P.

These results show that although *pin2* mutation does not completely rescue the *jkd* phenotype, it is capable of suppressing it. This suggests that PIN2 may be upstream of JKD activity, which is logical with regard to the work of Cruz-Ramírez et al., (2012) who suggest that auxin promotes *CYCD6;1* which is downstream of *SHR/SCR/BIRD* components. The requirement of auxin for the activity of this complex could mean that under low P, the modulation of auxin by PIN2 regulates the activity of *BIRD* genes such as *JKD*. The fact that the *jkd* phenotype is partially resistant to *pin2* suggests that the proliferation step itself is not dependent directly upon auxin (transport) itself but upon downstream factors, e.g. possibly *BIRD* genes. If this is the case then *PIN2* operate in the same pathway as *JKD* in the initial response. However, it is not possible to construct a model of this pathway without doing more work.

An important next step would be to perform the same experiment under high and low P conditions to determine whether *jkd* is capable of diminishing the *pin2* mutant response to low P. If this were the case, then it would corroborate that *JKD* is downstream of PIN2 since it would imply that a lack of regulation of JKD by PIN2 in the *pin2* mutant renders it incapable of orchestrating an increase in CCFN under low P. Another important piece of information would be to determine whether *JKD* (and other *BIRD* genes) are responsive to auxin in the post-embryonic root meristem. Möller et al., (2017) show that *MGP* is activated by auxin via ARF5 during embryo development and it would be very interesting to see if a similar pathway functions in the growing root tip. Such a relationship between auxin and *BIRD* genes would strongly suggest that *PIN2* modulates

CCFN under low P by altering auxin concentration in early cortex and thus regulating the activity of BIRD genes.

It could also be that PIN2 and JKD do not act directly within the same pathway but instead represent two parallel and complementary pathways which both play roles in patterning ground tissue. It could be that the effect of auxin modulation by *PIN2* does indeed regulate the downstream effects and expression of *BIRD* genes, but that it also affects other pathways which are involved in ground tissue patterning. BIRD genes similarly may be affected by auxin (via PIN2) under low P, but also regulated by other factors as well. For example, Paquette & Benfey (2005) show that GA is involved in the formation of middle cortex during ground tissue maturation and acts via *SHR* and *SCR*; Lee et al., (2016) make the same finding and also demonstrate that ABA is involved as well. Heo et al., (2011) showed that the *SCR*-related *SCARECROW-LIKE3* (*SCL3*) interacts with the *SHR/SCR* pathway and acts directly downstream of GA-DELLA mediated protein degradation to integrate endodermis-specific GA responses. Therefore it is clear that auxin is not the only plant hormone which has been shown to interact with the *SHR/SCR/BIRD* pathway, and the cross talk of all of these factors ultimately controls the downstream effects. Indeed, future experiments should investigate the roles of GA signalling components on CCFN increase under low P conditions in light of these previous reports.

6. General Discussion

This project aimed to address three broad issues regarding the increased cortex cell file number response to low phosphate in arabidopsis roots. These were:

1. How does the increase in cortex cell files in arabidopsis roots in response to low P manifest spatially, temporally, and in relation to P concentration?
2. What regulatory factors are involved in/required for this response?
3. How do these factors function and interact to orchestrate this response?

Chapter 1 characterised the response in terms of how rapidly the root responds in this way to low P, what concentration of P the root is sensitive to and where in the root meristem the change in patterning occurs. It was found that the roots were sensitive to a concentration of up to 400 μ M P and responded to a stress of 50 μ M P within 24 hours. Observations of the meristems of roots responding to low P and those grown on replete P suggested that the extra divisions occurred very early in the meristem. By analysing the difference in cell file numbers of the different tissues in the root between low and high P treated plants, it was inferred that since there was no significant change in endodermis cell file number between the two treatments, that the response must be occurring after the C/EID. These data provided a foundation and standard procedure for the work in the following chapters which addressed the question of what regulatory factors are responsible for controlling the response.

Previous work (Cederholm & Benfey, 2015; Ticconi et al., 2009) has shown that the low P response occurs within 24-48h but all of the studies to date have used concentrations of P in the range of 0-50 μ M which made it difficult to form a consistent understanding of the response. A critical concentration of P and time-scale of exposure which leads to the response were demonstrated. In addition, it was revealed where in the root meristem the response is initiated which is critical to developing an understanding of the factors which regulate it. Therefore, this study has advanced our knowledge regarding this response by giving a more detailed spatial and temporal understanding. This has allowed consideration of how individual components may control these processes which was done in the following sections.

This detailed understanding of the low P response enabled the effective assessment of the roles of potential underlying regulatory factors via the techniques of mutant screening, reverse-transcription quantitative PCR, and fluorescent reporter analysis. Interestingly, the first stage of this assessment revealed that although a strong role for *PIN2* was apparent (based on work by Zhang *et al.* 2003 and the present studies), none of the other *PINs*, *AUX/LAXs* or *ARFs* appeared to be required. It was concluded that with regard to the *PINs* this could be due to cross-activation of *PINs* resulting in functional complementation (such as reported by Blilou et al., 2005; and Vieten et al., 2005) or simply that only regulation of auxin flux in the cortex itself by *PIN2* was important for the response. Since no clear requirements for other *PINs* were revealed from this experiment, further investigation may be needed to

reveal a role for other *PIN*s or rule out their requirement. This would include the use of multiple mutants, the use of auxin transport inhibitors, and the testing of *pin4* mutants which was not performed in this project. The involvement of *PIN2* suggests that whilst the consorted action of *PIN*s may still be required, auxin reflux through the epidermis/cortex is crucial for this environmental response. Similarly, it was discovered that no activating *ARF*s were required; even the *arf6 arf8* double mutant did not reveal a role for these *ARF*s which are known to act redundantly. Therefore it was concluded that either there is further redundancy or that the transduction of the low P signal to cortex cells does not occur via these *ARF*s. Despite this set of experiments yielding the result that none of these factors has a strong role in the low P response. This finding has added to our knowledge because we now know that either these aspects of the pathway are not important, or a general auxin efflux/reflux response is required and this activity can be provided by multiple loci. Prior to this study, it was not known whether any other parts of the auxin signalling pathway were involved beside *PIN2*; this project has provided the information to guide the direction of future work by suggesting the use of higher order mutants to pursue this further.

Two future experiments in particular which would help to clarify this further are:

Due to the strong lethal phenotype of *mp/arf5* mutant, the role of *ARF5* could not be studied even though it is expressed in the relevant tissues and has been shown to control ground tissue patterning/initiation (and connect auxin with the SHR/SCR/BIRD pathway). To overcome this, the use of an inducible

dominant *bdl* (*bodenlos*) construct expressed in the cortex would be able to determine whether ARF5 is needed to transduce an auxin signal in response to low P (Herud, *et al.* 2016). It can be hypothesised that the *Co2:axr3-1* construct in chapter five is capable of blocking ARF5 activity (Ouellet, *et al.* 2001), but the use of a *bdl* mutant with a mutation in the DII domain would block ARF5 more specifically, as BDL specifically represses ARF5 in the embryo (Lau, *et al.* 2011), allowing the specific dissection of ARF5 function. The *Co2:axr3-1* construct also caused deleterious effects in shoot development/phyllotaxy and appeared to cause sterility or embryo lethality, so the true importance of the ARF-mediated auxin response may not have been reflected in this experiment. Similarly, this could also be overcome by the use of an inducible system expressed specifically in root cortex or a root cortex specific promoter alone- the important thing in future experiments would be to eliminate expression of the mutant *aux/iaa* in the inflorescence and embryo. Although many genes have been described as being specifically expressed in root cell types, the expression of these lines is rarely systematically tested in shoot/embryonic tissues; therefore careful selection of a promoter would be critical.

As *PIN2* remained the only auxin-related component with a clear role in the CCFN response to low P, its regulation was then tested using RT-qPCR and the use of the *pPIN2:PIN2:GFP* marker line.

RT-qPCR revealed no change in the expression of *PIN2* transcript under low P conditions compared with high P conditions. This pointed in the direction of post-translational regulation of *PIN2* being important. Therefore changes to

PIN2:GFP localisation in response to low P were then investigated in order to determine whether the polar localisation of PIN2 was changed to orchestrate the response. Indeed it was found that PIN2:GFP in the cortex exhibited a significant shift from rootward membrane localisation to shootward membrane localisation. This led to the conclusion that a likely mechanism of PIN2 function under low P was to re-direct the flux of auxin through the cortex in such a way as to coordinate new cell divisions leading to an increase in CCFN. This finding has added to the current understanding, as although *PIN2* was known to be required for the response (Zhang *et al.*, 2003), it was not known at all what its function in the response could be. Zhang, *et al.*, (2003) showed the requirement of *PIN2* in the response but they only used a small sample of 5 plants. Consequently their study lacked the depth necessary to show a significant change. This project has provided a greater understanding of what changes *PIN2* undergoes during the low P response. This work provides the foundation to understand in more detail how *PIN2* is able to regulate the response. For example, now that we know that *PIN2* polarity is changed under low P conditions, the next step might be to look for differential post-translational modifications to the *PIN2* protein. This approach also emphasises the ability of studying the responses of certain factors to plant stresses to shed light on new aspects of their regulation which may not be obvious under normal (i.e. P replete) conditions. This also enables the application of our understanding of basic plant biology to wider issues in terms of agriculture and the environment.

In addition to exploration of auxin related factors, chapter four tested the possibility of a role for the SHR/SCR/BIRD pathway. This started with the screening of *jkd*, *mgp* and *shr* mutants to identify whether any of these lines exhibited either low P sensitivity or insensitivity. *jkd* displayed the same high CCFN response as reported previously (Long et al., 2015; and Welch et al., 2007). However it was not hypersensitive to low P, suggesting that both low P roots and *jkd* are subject to a maximum cortex cell file number. The *mgp* mutant was not insensitive to low P treatment, which is not what would be expected if *JKD/MGP* was involved in the low P response considering that *mgp* has been shown to abolish the *jkd* phenotype (i.e. reduced CCFN; Long et al., 2015)). Finally, *shr* was found to exhibit an increase in the number of ground tissue cell files in response to low P. Even though the *shr* mutant exhibits a 'mutant' ground tissue layer (Helariutta et al., 2000), this tissue still displayed sensitivity to low P in the absence of *SHR*. Taken together, these results suggested that it is unlikely that the CCFN response to low P is regulated by the *SHR/SCR/BIRD* pathway.

Despite these results, RT-qPCR showed a significant increase in *SCR* and *MGP* and decrease in *SHR* transcript abundances, suggesting a change in regulation of these factors in response to low P. While the mutant studies indicate that none of these factors on their own are absolutely required for the low P response, qPCR suggested that regulatory changes in the BIRD/SHR/SCR network were indeed part of the low P response.

Following this, GFP reporter lines were used to measure the levels of transcription and protein abundance of *BIRD* genes, *SCR* and *SHR* under low P conditions.

These results revealed that *JKD* transcription was reduced in the first five cortex cells, *MGP* transcription was unchanged in response to low P and *BIB* transcription was not seen in cortex under high or low P conditions. This contradicted the qPCR results with *JKD* showing a change and *MGP* not showing a change, however it was considered that the exposure technique (i.e. 12 days growth on low P for qPCR rather than 1 day transfer to low P for promoter fusions) probably caused this discrepancy. Future experiments would begin by performing all of these experiments using exactly the same low P exposure technique.

Observation of translational reporters of *JKD*, *NUC*, *BIB*, *SCR*, and *SHR* suggested that these factors undergo changes in transcriptional and translational regulation under low P. *JKD* protein was strongly reduced under low P in all of the first five cortex cells, *NUC* showed very little change, *BIB* showed a strong reduction, *SCR* showed an extension of its expression domain to the cortex and epidermis, and *SHR* exhibited no obvious change in expression level or pattern.

In addition to mutant studies and expression analysis, I tested whether over-expression of *BIRD* genes in the cortex would affect the low P response. This experiment revealed that expression of *BIB* under the *Co2* promoter resulted in a low P insensitive phenotype, suggesting that *BIRD* proteins do impact on cortex patterning under low P.

Even though the results regarding the *BIRD* genes and *SHR/SCR* turned out to be fairly inconclusive, it can certainly be said based on the differential transcript abundance and the capacity of *pCo2:BIB:GFP* to suppress the low P response that the *BIRD/SHR/SCR* network is likely contributory to the response. It may not be that these regulators have a particularly strong influence on the response at an individual level, but further understanding the downstream targets of these could well reveal factors with more direct effects on the low P response. This project did not determine the exact relationship regarding how this network and low P responses interact, but having made a connection between the two could open a promising avenue for research. With all of the findings of chapters three and four, chapter five brought these findings together and tested the hypotheses of how all of the components identified earlier cooperate to coordinate the increase in CCFN. By restoring *PIN2* in the *pin2* background in cortex or epidermis respectively, it was revealed that *PIN2* in either of these tissues was capable of restoring the low P response. This suggests that *PIN2* is able to direct auxin flux in the required manner through either tissue. Expression of the dominant repressive *AUX/IAA* mutant *axr3-1* in cortex or epidermis revealed that blocking auxin response did not affect the low P response.

Next, to help understand what *PIN2* activity may be required for under low P, mathematical modelling was combined with what was learnt from the *PIN2* complementation assay. The *pin2* mutant model was compared with that of WT and both of the tissue specific complementation lines. This revealed that the *pin2* mutant exhibited an accumulation of auxin in the cortex which was

rescued by the pAUX1:PIN2 model (but not the pCo2:PIN2 model). It was concluded that this suggested that the capacity of *PIN2* to reduce auxin concentration in the cortex was required for the low P response. Since the *pin2* mutation causes auxin accumulation in the cortex, this implies that this is what blocks the low P response in *pin2*, suggesting that reduced auxin is the signal required to elicit CCFN increase under low P. This part of the project helped to develop the hypothesis that reduced auxin in the early cortex, modulated by *PIN2*, controls the CCFN response to low P.

This makes a very valuable addition to the field because it means that a simple question of whether reduced auxin response is seen under low P or not.

Following, gene expression analyses could be performed to test what may be downstream of this cortical change in auxin response and whether they are important for ground tissue patterning. Not only does this finding help to construct a hypothesis, but it also adds to our understanding of the model; why is it that *Co2:PIN2* in the *pin2* background can complement the low P-related phenotype but not the auxin distribution phenotype in the model?

This also emphasises how by applying the effects of external plant stresses to existing understanding can help to develop new ways of looking at these problems and to ask new questions. To address these questions would require a greater understanding of how reorganisation of PIN2 by low P affects auxin distribution. This could be investigated using alterations of PIN2 polarisation in mathematical models, to predict states of subcellular localisation which result in reduced auxin accumulation in the cortex.

Experiments could also be performed such as a screen of phosphorylation site

mutants in *PIN2* or using mutants of kinases which phosphorylate *PIN2* such as *PINOID* (Barbosa & Schwechheimer, 2014). If affecting the state of polarisation of *PIN2* in roots affects auxin distribution or low P response then it would provide an understanding of the changes in *PIN2* behaviour that are required.

Finally, chapter five investigated whether there is cross-talk between the factors which were identified in this project and possibly construct a model network. To test this, a *pin2 jkd* double mutant was generated to test whether the *pin2* mutation was capable of suppressing the *jkd* CCFN phenotype in the same way that it suppresses the low P CCFN response. The intermediate CCFN phenotype of the *pin2 jkd* double mutant makes it difficult to imply that *JKD* is directly upstream of *PIN2* in a linear pathway. Rather the phenotype suggests a situation whereby both of these factors are together involved in feeding in to a common regulatory network which ultimately is responsible for regulating this response. Feedback between the SHR/SCR/BIRD factors and auxin factors is known of, and it could be that both of these pathways work by feeding in to the same network in order to produce a strong low P response. However, it is likely that neither of these factors are entirely dependent on each other and that upstream and downstream components are still capable of mediating this response independently.

Drawing everything together, it can be concluded that this project has revealed much about the anatomy of the response and in which cells this originates. This background gives a good framework to develop and

understanding of the contribution of the factors which have been shown here to be differentially regulated under low P. The results from these experiments highlights the complexity of the network involved- no one factor so far seems to be crucial apart from *PIN2*. My work here demonstrates that the network around *PIN2* must feature a number of different factors. Further work would build on this, connecting together these components with the missing pieces of the puzzle and constructing a regulatory network. Work which would help to uncover how exactly these factors are contributing towards this response would be to study the expression of *PIN2* and other *PINs* in *jkd* and other *BIRD* mutants on low and high P. Such an experiment would reveal what, if any, role these transcription factors have in regulating auxin transport and whether this is dependent on low P. Additionally, transcriptomics and proteomics of roots grown on low and high P in *BIRD*, and especially *PIN2*, mutant backgrounds would help reveal what the downstream targets of these are. This is especially pertinent for *PIN2* since there must be downstream factors regulated by its manipulation of auxin flux which are absolutely crucial to the initiation of the CCFN response to low P. Identifying such factors would fill in the gaps and link the roles of auxin transport and ground tissues specification in root responses to low P. Not only would this work help in understanding the response to low P, but the modifications to this network caused by low P will broaden our understanding of the plasticity of plant development more broadly.

Bringing everything from my project together, I have provided new insights in to several areas

1. Cortex cells can respond to concentrations as high as 400 μ M P by increasing CCFN and this is independent of Fe
2. The response occurs in the first cortex cell after the CEI division
3. The response does not require individual *PINs*, *AUX/LAXs* besides *PIN2* or activating *ARFs* expressed in the root
4. The response is not fully dependent upon any component of the SHR/SCR/BIRD pathway
5. *PIN2* protein localisation changes under low P conditions
6. *PIN2* is most likely required in order to facilitate auxin flux out of early cortex cells
7. *PIN2* and *JKD* interact indirectly in coordinating CCFN in arabidopsis roots

The work presented in this thesis makes progress in explaining how arabidopsis roots respond to low P by increasing the number of cortex cell files in order to increase root hair density. This has answered several questions, but also asked many more. However, outside of the issues addressed directly in this thesis, the topic of ground tissue patterning and the factors which regulate it are relevant in a much broader context.

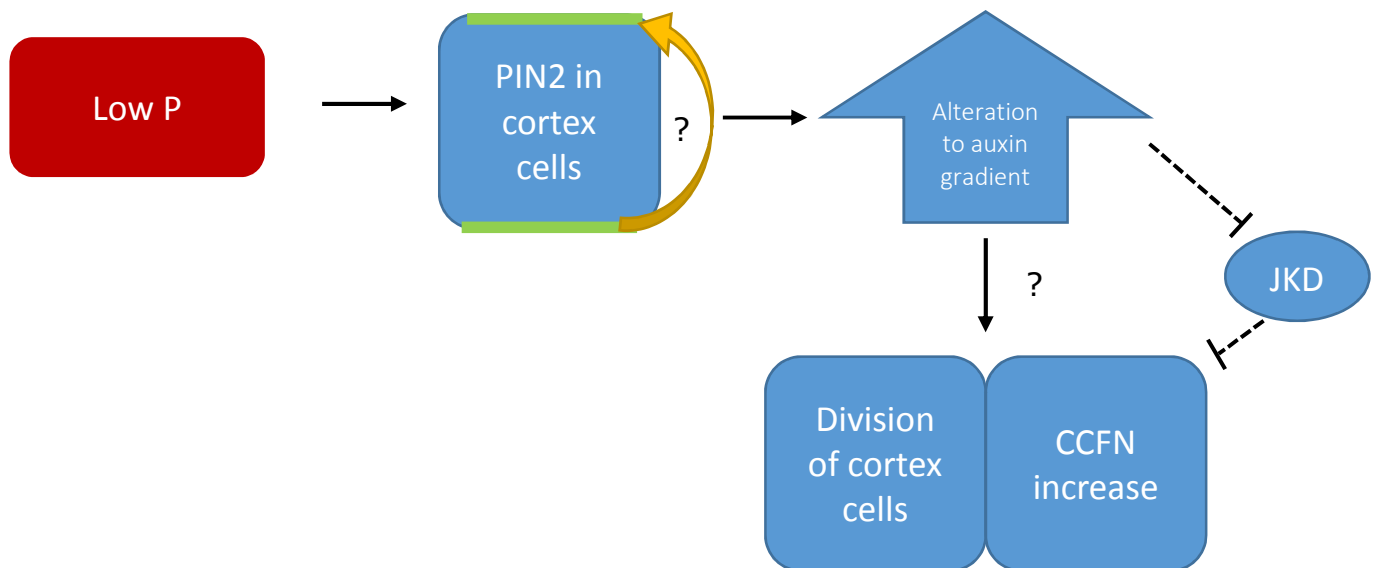


Figure 6.1 Schematic showing a hypothetical pathway via which low P results in division of cortex cell and an increase in CCFN

Low P leads to a shift in the polar localisation of PIN2 in cortex cells, whereby it becomes more localised to the shootward facing membrane. This facilitates an increase in shootward auxin flux (or decreased in rootward auxin flux) in the cortex cells. This then results in an increase in the number of periclinal divisions occurring in the first cortex cells, leading to an increase in cortex cell file number. These divisions maybe be partially regulated by auxin via the down-regulation of JKD or other BIRD genes.

Firstly, although this response is most logically interpreted as an adaptation to enhance P uptake, some confirmative work should be performed to study the agricultural relevance of this response. Some work has been done on the role of root hair in nutrient uptake, suggesting that higher root hair density can increase P uptake (Raghothama, 1999), and indeed Ma, *et al.* (2001) suggest that higher radial root hair density leads to increased P uptake potential (albeit via a mathematical modelling approach). However, what would be critical to this work would be to study how increasing CCFN influences P uptake. An experiment could be designed whereby an arabidopsis line with

increased CCFN (e.g. *pCo2:MGP*) or with increased radial root hair density (e.g. *wer*) is grown in P replete, P deficient (or both) soil/hydroponics alongside wild type controls. The tissue from these plants could then be harvested and their P content analysed (as in Alcock et al., (2017). This would give an indication of the magnitude of P uptake increase which is conferred by such changes to root anatomy.

Although this project has not produced the data to imply a direct role for the *BIRD* genes in CCFN increase under low P, the enhanced understanding of the response in addition to our knowledge of these factors which regulate ground tissue patterning could provide leads for genetic targets in crop improvement. For example the *jkd* mutant provides a strong candidate as a locus for manipulating crop root anatomy for enhanced P uptake. However, since *JKD* is required for root hair patterning on the epidermis as well as cortex cell patterning (Hassan, *et al.* 2010), an interesting project would be to assess the roles of downstream targets of *JKD* on CCFN. Moreno-Risueno et al., (2015) generated a rich dataset incorporating ChIP (Chromatin ImmunoPrecipitation) and transcriptomics from *BIRD* mutants which presents a high number of likely downstream targets of these factors. Using a dataset like this to dissect the downstream network of the SHR/SCR/BIRD complex could provide excellent candidate loci for understanding what factors are more directly responsible for modulating ground tissue patterning. This understanding could then be taken to crops in the form of focussed breeding efforts or transgenic technology to enhance P uptake via radial patterning in agricultural systems.

In addition to understanding how the downstream targets of these known ground tissue regulators affect these traits and low P responses in arabidopsis, it would be of great agronomic significance to transfer this understanding in to cultivated material. Pemberton *et al.* (2001) analysed the root hair patterning of 77 different species of eudicots. They found that the cortex cell file-dependent pattern of root hairs seen in arabidopsis (type 3) is seen in a diverse number of distantly related plant species across a number of orders. However, type 3 patterning is conserved within Brassicaceae and so is relevant to some plants of huge importance to mankind. A fascinating experiment would be to study the low P responses of other members of Brassicaceae (particularly those in *Brassica* such as *B. rapa*) and investigate whether these plants respond with increased CCFN. If so, investigating whether CCF patterning is regulated by homologues of the same genes in arabidopsis (i.e. *BIRDs*, *PIN2*) could open up leads for root anatomy as a mechanism for improving P uptake in vegetable and oilseed crops.

Brassica rapa is a diploid member of the *Brassica* genus and contributes the A genome to *B. napus* and *B. juncea* which are both amphitetraploids. As such, *B. rapa* has become the focus of genetic and biochemical research with the generation of T-DNA insertion mutants in to a turnip cultivar of *B. rapa* (X. Wang *et al.*, 2017) and the analysis of genetic diversity in a number of *B. rapa* accessions (X. Zhang *et al.*, 2016). Biochemical characterisation of factors in this species has been performed such as that of a lipase involved in pollen development (Ji *et al.*, 2017). Similarly, in *B. oleracea* (cabbage, broccoli)

studies in to the phylogeny and structure of gene families has been performed (Kakar et al., 2017). The opening up of these species as objects of study in advanced fields of genetic and biochemical research has the potential to provide a profound understanding the biology of food crops. An application of the understanding of traits such as CCFN and root hair density to these crops could have a powerful impact on P uptake and use.

A fruitful endeavour may be to examine the natural variation in cortex cell file amongst members of the *Brassica* genus. Eight cortex cell files is the basal number in *Arabidopsis thaliana* but this may not be the case for other *Arabidopsis* or *Brassica* species under P replete conditions. It would be interesting to uncover basal aspects of root cortex anatomy in other Brassicaceae and a search for natural variation within a genetically diverse population with genetic analysis could provide insight in to loci which control this trait. Following this, it could then be seen whether this number increases in response to low P. Identifying genetic variants in *Brassica* which result in increased CCFN under P replete conditions could provide breeding material for cultivars with increased P uptake in all conditions. Genome wide association mapping studies (GWAS) have been used to identify candidate loci controlling a number of important agronomic character in *Brassica napus* (oilseed rape) from branching traits (He et al., 2017) and plant height (N. Wang et al., 2016) to mineral nutrient uptake/content (Alcock et al., 2017). Indeed, quantitative trait loci have previously been identified which are associated with variation in root anatomical traits in maize (*Zea mays*) roots (Burton et al., 2015).

Applying the understanding of this trait in arabidopsis at the molecular and anatomical levels to the available tools for large scale genetic analysis could enable the integration of anatomical traits which enhance P uptake in to widely cultivated crops.

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Cellular Patterning of *Arabidopsis* Roots Under Low Phosphate Conditions

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Phosphorus is a crucial macronutrient for plants playing a critical role in many cellular signaling and energy cycling processes. In light of this, phosphorus acquisition efficiency is an important target trait for crop improvement, but it also provides an ecological adaptation for growth of plants in low nutrient environments. Increased root hair density has been shown to improve phosphorus uptake and plant health in a number of species. In several plant families, including Brassicaceae, root hair bearing cells are positioned on the epidermis according to their position in relation to cortex cells, with hair cells positioned in the cleft between two underlying cortex cells. Thus the number of cortex cells determines the number of epidermal cells in the root hair position. Previous research has associated phosphorus-limiting conditions with an increase in the number of cortex cell files in *Arabidopsis thaliana* roots, but they have not investigated the spatial or temporal domains in which these extra divisions occur or explored the consequences this has had on root hair formation. In this study, we use 3D reconstructions of root meristems to demonstrate that the radial anticlinal cell divisions seen under low phosphate are exclusive to the cortex. When grown on media containing replete levels of phosphorus, *A. thaliana* plants almost invariably show eight cortex cells; however when grown in phosphate limited conditions, seedlings develop up to 16 cortex cells (with 10–14 being the most typical). This results in a significant increase in the number of epidermal cells at hair forming positions. These radial anticlinal divisions occur within the initial cells and can be seen within 24 h of transfer of plants to low phosphorous conditions. We show that these changes in the underlying cortical cells feed into epidermal patterning by altering the regular spacing of root hairs.

Keywords: developmental biology, radial patterning, phosphate deficiency, root anatomy, *Arabidopsis*, root hair, cortex, light sheet microscopy

INTRODUCTION

Phosphorus (P) is a critical macronutrient and essential for plant growth. Unlike nitrogen or ammonium, which are soluble and diffuse through the soil, phosphate (Pi) is immobile within the soil as it often binds clay particles and forms insoluble precipitates (Brady and Weil, 2008). This means that the region around roots becomes quickly depleted of phosphate. Plants have developed several strategies for increasing phosphate acquisition, including changes to root architecture

(López-Bucio et al., 2002) and through root hairs (Bates and Lynch, 1996; Ma et al., 2003). Studies in both *Arabidopsis* (using the *root hair defective - rhd2* - mutant) and barley (using the *bald root barley - brb* - mutant) have demonstrated reduced growth in low Pi soils of hairless mutants (Bates and Lynch, 2001; Gahoonia and Nielsen, 2004).

Plants may exhibit different root hair adaptations to low P. For example, tomato, spinach, rape and *Arabidopsis* all exhibit an increase in root hair length under low Pi (Foehse and Jungk, 1983; Ma et al., 2001; Bhosale et al., 2018). However, these species also respond by increasing their root hair density in low Pi conditions. A recent study of root hair traits in 166 accessions of *Arabidopsis thaliana* showed that root hair density and length were not correlated, and that the genotypes that showed the greatest increase in root hair density under low Pi were mostly those that had fewer and shorter root hairs under Pi replete conditions (Stetter et al., 2015).

Arabidopsis epidermal cells can acquire one of two fates, they can form trichoblasts that go on to produce root hairs, or they can form atrichoblasts that cannot form root hairs. In wild-type plants, these two cell types form continuous files extending through the root meristem. The cell fate decision is controlled through positional information transmitted from the cortex; trichoblasts form in epidermal cells that overlie the cleft between to cortex cells, whilst atrichoblasts overlay just one cortical cell (Figure 1). In wild-type seedlings this results in a radial pattern in which files of trichoblasts are separated by one to three files of non-hair-bearing atrichoblasts (Dolan et al., 1993). The number of cells within each file is different, as trichoblasts are shorter than atrichoblasts (Dolan et al., 1993). Experimental analysis of master regulators of epidermal cell fate has shown that the differences in longitudinal cell length of trichoblasts is dependent upon the position of cells relative to the underlying cortex (Savage et al., 2013).

Pemberton et al. (2001) describe 3 types of epidermal patterning in angiosperms. They refer to the pattern in *Arabidopsis* of hair cells occurring in files separated by one to three files of non-hair cells as type 3. This type of patterning was found in all members of the Brassicaceae examined, as well as other families within the Brassicales and Caryophyllales. Most angiosperms displayed type 1 epidermal patterning, in which all epidermal cells can produce root hairs.

The molecular mechanism regulating epidermal cell fate is well understood in *Arabidopsis*. A transcriptional complex involving the transcription factors - WEREWOLF (WER), GLABRA 2 (GL2), ENHANCER OF GLABRA3 (EGL3), and TRANSPARENT TESTA GLABRA1 (TTGI) is required to specify atrichoblast identity (Grebe, 2012), and mutations in these genes result in plants with increased root hairs (Galway et al., 1994; Rerie et al., 1994; Masucci et al., 1996; Lee and Schiefelbein, 1999; Walker et al., 1999; Bernhardt, 2003). Whereas the transcription factors CAPRICE (CPC), TRIPTYCHON (TRY), and ENHANCER OF CAPRICE (ETC) promote trichoblast identity, and mutations in these genes result in few to no root hairs (Wada et al., 1997; Schellmann et al., 2002; Kirik et al., 2004). Together these components form a regulatory circuit, with CPC moving to trichoblasts where it competes with

WER to bind the GL3-EGL3-TTG1 complex (Bernhardt, 2003). JACKDAW (JKD) acts in a non-cell-autonomous manner from the underlying cortex cells to specify trichoblast vs. atrichoblast fate by regulating GL2, CPC, and WER expression via the LRR kinase SCRAMBLED (SCM) (Kwak and Schiefelbein, 2008; Hassan et al., 2010).

A plant such as *Arabidopsis* that exhibits type 3 root hair patterning could increase root hair density either in the longitudinal domain by a reduction in cell elongation, or in the radial domain by increasing the number of epidermal cells that differentiate as trichoblasts. Indeed, phosphate deficiency has been shown to result in shorter epidermal cells and greater root hair production in *Arabidopsis* (Sánchez-Calderón et al., 2005).

Under normal conditions, *Arabidopsis* roots almost always have exactly eight cortical cells. However, when grown on low Pi media, *Arabidopsis* plants show an increase in the number of cortical cells and this results in an increase in the number of cells at the trichoblast position (Ma et al., 2003; Zhang et al., 2003; Cederholm and Benfey, 2015). Mathematical simulations of epidermal cell fate in which different signal inputs are supplied from the cortex, support a model where a time-evolving signal specifies the recruitment of atrichoblasts from a default trichoblast pathway to control cell length and therefore delay atrichoblast cell fate specification (Savage et al., 2013). These simulations suggest that even under Pi deficient conditions, the core mechanisms regulating epidermal cell fate is functional although plants show increased cell length and form additional trichoblast files (Savage et al., 2013).

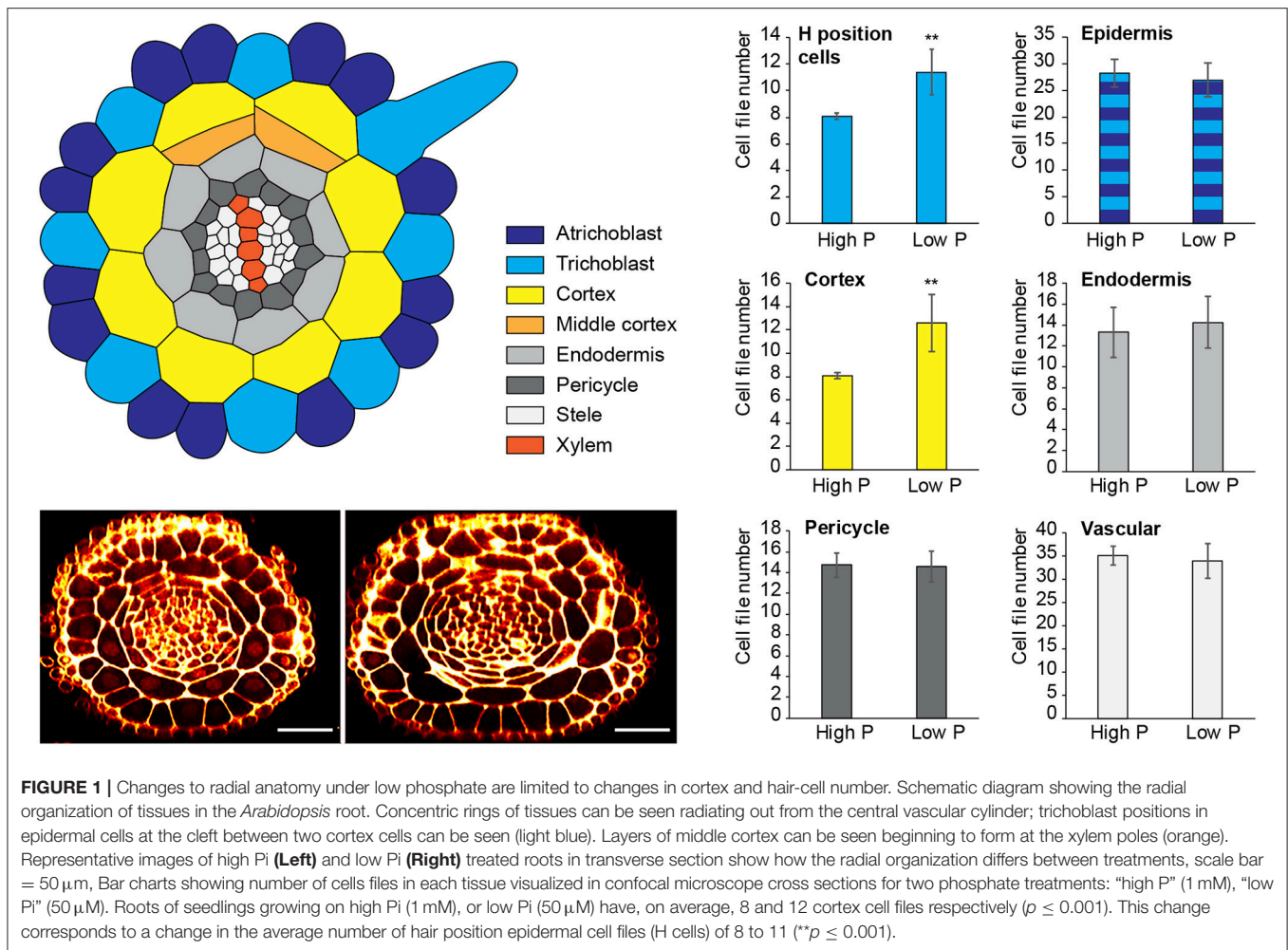
In this manuscript, we use a combination of different microscopy techniques to investigate anatomical changes in root radial patterning of *Arabidopsis* in response to low Pi. We show that increases in cortical cell file number occur dynamically within the meristem and respond rapidly to reductions in Pi. Furthermore, we show that these changes in the cortical tissues result in alterations in the final root hair patterning.

MATERIALS AND METHODS

Plant Material and Growth Conditions

All *Arabidopsis* experiments were performed using *A. thaliana* (Columbia ecotype) seeds. All seeds were sterilized with 5% sodium hypochlorite for 5 mins before being rinsed with 70% ethanol with 0.01% Triton X-100 once and several times with sterile water. Seeds were then sown on agar plates, sealed with micropore tape (3 M) and cold treated overnight to synchronize germination. Plants were grown under 12 h light, 12 h dark conditions at 21°C. To ensure consistency of our experiments in relation to the photoperiod, all plants were moved into the growth room within the first 2 h of the light period. Harvesting of roots for microscopy and transfer between different media was always done within the same 2 h period at the start of the light cycle.

Low and high P/low and high Fe media was made using a modified Hoaglands medium consisting of the concentrations of minerals listed in Table 1. 0.5g/L of 2-(*N*-morpholino)ethanesulfonic acid (MES) was added and the pH of the media was adjusted to 5.7 using potassium hydroxide.



Media was then added to bottles containing Sigma purified agar (catalog number A1296) 8 g/L and autoclaved before being used to pour plates.

Pseudo-Schiff Propidium Iodide Staining

Roots for PS-PI staining were harvested from the plate and fixed immediately in a 50% methanol 10% acetic acid aqueous solution and kept at 4°C overnight. Roots were then rinsed in sterile, deionised water three times before being incubated at room temperature in 1% periodic acid for up to 40 min. Tissue was rinsed 2–3 times with sterile, deionised water and then left in PS PI solution for 1–2 h [0.015 N hydrochloric acid (HCl), 100 mM sodium metabisulphite; 50 μ l/ml propidium iodide solution [1 mg/ml], freshly added each time]. Roots were again rinsed 2–3 times with water before being mounted on slides with 40% glycerol 30% chloral hydrate in water and left in the dark over night to clear.

Microscopy and Image Processing

Stained cleared roots were imaged using a Leica SP5 confocal microscope. A 488 nm laser was used to excite PI fluorescence

for imaging, PI fluorescence was collected using either a photomultiplier tube (PMT) or hybrid detector at between 600 and 800 nm.

For the generation of optical sections from PS-PI stained/cleared roots, Z-stacks of roots were generated using LAS-X Leica software then dissected in orthogonal view using ImageJ image analysis software. An example z stack is shown in Supplemental Movie 1. Cell counts were performed using a cell counter function in the same software. We use post-mitotic cell positions to infer previous cell divisions.

Light-sheet microscopy was performed using a Zeiss Lightsheet Z.1. A 514 nm laser was used to excite YFP as well as propidium iodide. Emission wavelengths were filtered using a bandpass filter BP 525–545 for YFP and BP 575–615 for propidium iodide. Root tips were excised from plants and stained in 5–10 μ l propidium iodide (PI)/ml in deionised H₂O and mounted in to a glass capillary (1 mm inner diameter) using low melting temperature agarose (Sigma-Aldrich) mixed with fluorescent beads from the PS-Speck Microscope Point Source Kit (ThermoFisher Scientific). Roots were imaged along six angles using the Multiview function in ZEN software. The ImageJ plugin Multiview-Reconstruction was used to assemble

TABLE 1 | Table showing concentrations of different nutrient components used in low Pi high Fe, high Pi high Fe, low Pi low Fe, and high Pi low Fe media preparations used for low and high P/low and high Fe treatments.

Nutrient	Low Pi high Fe	High Pi high Fe	Low Pi low Fe	High Pi low Fe
KNO ₃	3 mM	3 mM	3 mM	3 mM
Ca(NO ₃) ₂ 4H ₂ O	2 mM	2 mM	2 mM	2 mM
MgSO ₄ 7H ₂ O	0.5 mM	0.5 mM	0.5 mM	0.5 mM
(NH ₄) ₂ SO ₄	425 μM	0	425 μM	0
NH ₄ H ₂ PO ₄	50 μM	1 mM	50 μM	1 mM
H ₃ BO ₃	25 μM	25 μM	25 μM	25 μM
ZnNa EDTA	2 μM	2 μM	2 μM	2 μM
(NH ₄) ₆ Mo ₇ O ₂₄ 4H ₂ O	1 μM	1 μM	1 μM	1 μM
KCl	50 μM	50 μM	50 μM	50 μM
MnSO ₄ H ₂ O	2 μM	2 μM	2 μM	2 μM
CuSO ₄ 5H ₂ O	1 μM	1 μM	1 μM	1 μM
Fe-EDTA	50 μM	50 μM	5 μM	5 μM

multiview stacks in to a final stack using fluorescent beads as points of interest for registration (Preibisch et al., 2010, 2014).

Cell files of different cell types were counted by producing radial cross sections of roots from confocal Z-stacks assembled in ImageJ software, viewed in orthogonal views and counted using the cell counter plugin.

RESULTS

Changes in Radial Cell Proliferation in Response to Phosphate Are Limited to Cortex Cells

Although previous reports have shown that cortical cell proliferation is increased when *A. thaliana* plants are grown on low phosphate media (Ma et al., 2003; Cederholm and Benfey, 2015), it has been unclear whether the cortex is the only cell type to undergo additional radial anticlinal divisions to increase cell file number. In order to provide a greater understanding of precisely which cell lineages responded to low phosphate, we investigated this in higher resolution by generating 3D reconstructions of the root meristem based on a combination of fixation and labeling with pseudo-Schiff propidium iodide and generating z stacks using confocal microscopy (see Supplemental Movie 1 for an example). *Arabidopsis thaliana* seeds were sown on media containing 1 mM (high P), 50 μM (low P), or 1 μM (very low P) and grown for 12 days. We generated orthogonal projections through roots with which we could count cell file numbers of different cell types (stele, pericycle, cortex, and epidermis) within the meristem (Figure 1). At this age, *Arabidopsis* plants begin to mature, and we see the formation of some middle cortex cells. Previous studies have reported that this is the result of periclinal cell divisions within the endodermal layer (Baum et al., 2002; Lee et al., 2016). We have not included the middle cortex within these cell counts and only count cortex cell files that are in contact with the overlying epidermis.

We found no significant change in the number of stele, pericycle or endodermal cell files or in the total number of epidermal cell files (Figure 1), with the stele having between 30 and 35 cell files, and the pericycle, endodermis, and total epidermal cell files having 14–16, 12–14, and 26–28 cell files respectively. The only tissue that exhibited a change in cell file number was the cortex, where the number of cell files increased from 8 (1 mM P) to an average of 12 (50 μM P) or 11 (1 μM P).

These results show that the radial cell proliferation was restricted to the cortex, and differ from those shown previously by Cederholm and Benfey (2015), who also observed differences in the number of endodermal as well as cortical cells. This could be due to differences in the age of plants analyzed, as this study used 12 day old plants, whereas that by Cederholm and Benfey (2015) used approximately 6 day old plants or may be due to differences in the composition of the media.

The Root Cortex Response to Low Phosphate Is Concentration Dependent

Different studies by a variety of groups investigating low Pi stress in *Arabidopsis* seedlings have used a variety of Pi concentrations to mimic a stress scenario. We therefore considered it important to determine the specific concentration of Pi that will trigger cortical cell proliferation, to facilitate comparisons with the plethora of data already existing for architectural and anatomical changes.

To find the lowest concentration of phosphate, at which a response in cortical cell file number (CCFN) was seen, plants were grown under a concentration range from 0 M to 1 mM Pi. All concentrations between 0 and 400 μM Pi elicited an increase in CCFN. Within this range there was little difference in the number of cell files, with 0 M, 50, 100, 200, and 400 μM all showing ~10–11 cortex cell files (Figure 2). Root length and lateral root density was also measured for these plants, and it was observed that plants grown on <50 μM phosphate typically exhibited more severe stress symptoms than those grown on higher concentrations, including dramatic inhibition of growth, anthocyanin accumulation and chlorosis. After 9 days root lengths were 69 ± 9 mm on high Pi and 26 ± 1 mm on low Pi. These general plant phenotypes were similar to those previously reported for plants grown in phosphate deficient conditions, such as Bates and Lynch (1996) and Trull et al. (1997). These results show that the changes in CCFN occur at Pi concentrations that do not severely affect plant growth, suggesting that this is an adaptive response rather than simply a disorganization of the meristem in response to phosphate starvation.

It has been shown previously that iron concentration in media affects root architectural responses to low phosphate (Hirsch et al., 2006; Svistoonoff et al., 2007; Ward et al., 2008; Dong et al., 2017; Gutiérrez-Alanís et al., 2017) as under low Pi conditions roots can accumulate high concentrations of free iron in the root meristem. This accumulation seems to be particularly high in the QC and surrounding initials and has been suggested to have a toxic effect that can lead to meristem disorganization and mislocalisation of key patterning regulators such as the transcription factors SHR and SCR (Ticconi et al., 2004, 2009). Whilst there

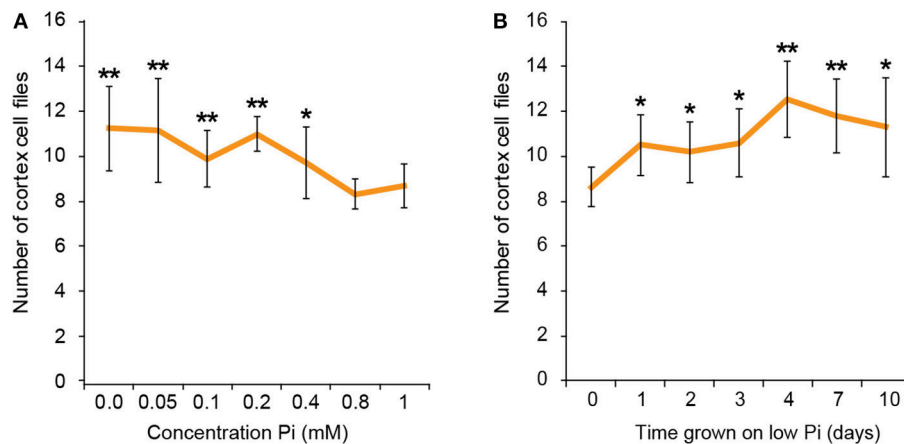


FIGURE 2 | Response to low phosphate is concentration and time dependent. Cortex cell file numbers increase when roots are exposed to medium containing 400 μM or lower. At lower concentrations the number of cortex cell files is as high as 13, but at 800 μM –1 mM the number of cortex cell files remains at 8 **(A)**. Error bars represent standard deviation. Number of plants used in cortex cell file counts 0 M: $n = 8$; 50 μM : $n = 12$; 100 μM : $n = 11$; 200 μM : $n = 10$; 400 μM : $n = 12$; 800 μM : $n = 11$; 1 mM: $n = 11$. **Statistically significant difference to 1 mM Pi ($p \leq 0.005$); * $p \leq 0.05$ **(A)**. Cortex cell file number increases to an average of 10 after 24 h exposure to low Pi, increasing to an average of 12 after 4 days. **(B)** Error bars represent standard error. Numbers of plants used in cell file counts 0 days: $n = 11$; 1 day: $n = 10$; 2 days: $n = 11$; 3 days: $n = 10$; 4 days: $n = 9$; 7 days: $n = 10$; 10 days: $n = 10$. **Statistically significant difference to 1 mM Pi ($p \leq 0.005$); * $p \leq 0.05$.

is increasing evidence that iron toxicity may be responsible for some of the extreme responses observed for *Arabidopsis* plants grown on agar plates under phosphate limiting conditions (such as a severe reduction in primary root length). More recent studies have mitigated this iron toxicity effect when using media with $<1 \mu\text{M}$ Pi by reducing iron levels concomitantly with Pi reduction.

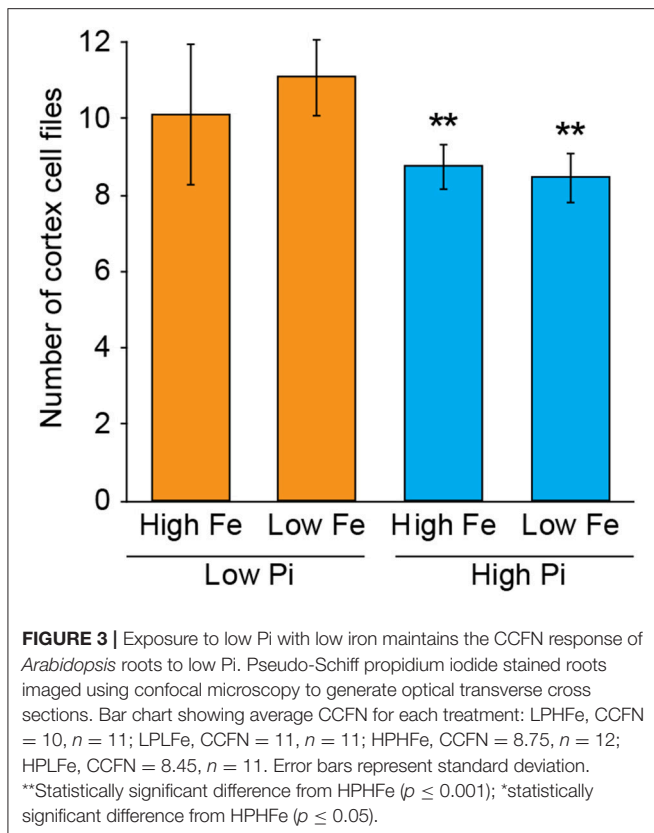
To test whether the response in CCFN to reduced phosphate in the media was primarily associated with a lack of Pi or an excess of iron, we set up experiments with *A. thaliana* sown on to agar plates containing media with high Pi high Fe (1 mM $\text{NH}_4\text{H}_2\text{PO}_4$, 50 μM Fe-EDTA), high Pi low Fe (1 mM $\text{NH}_4\text{H}_2\text{PO}_4$, 5 μM Fe-EDTA), low Pi high Fe (50 μM $\text{NH}_4\text{H}_2\text{PO}_4$, 50 μM Fe-EDTA), and low Pi low Fe (50 μM $\text{NH}_4\text{H}_2\text{PO}_4$, 5 μM Fe-EDTA). We counted CCFN in all these lines, and observed that when plants are exposed to low Pi, but also 10-fold less iron (5 μM Fe-EDTA) an increase in CCFN was still present. On this media, roots have a CCFN of 11; a result that is higher than on low Pi high Fe **(Figure 3)**. This data supports the hypothesis that alterations in CCFN seen in Pi limiting conditions are primarily a response to low Pi and not due to an increase in Fe and/or Fe toxicity in the root.

Changes to Radial Cortex Anatomy Occur After 24 H Exposure to Low Phosphate Just Above the Cortex Endodermal Initial

Previous studies have shown that cortex proliferation in response to low Pi in *Arabidopsis* roots is visible within the root tip. However none of this work provided an explanation as to where exactly in the root meristem the proliferative divisions take place. In order to address this gap in the knowledge, we assembled 3-dimensional image stacks using confocal microscopy on chloral hydrate cleared pseudo-Schiff propidium iodide stained root tips

grown on low or high P. By assembling these image stacks in ImageJ we determined the number of cortex cell files at specific cell tiers behind the quiescent center. Counting the number of cortex cell files at each tier revealed a trend for decreasing CCFN with distance from the QC in low Pi treated roots **(Figure 4)**. Under high Pi conditions, CCFN was consistently 8 at each of the 10 tiers distal to the QC **(Figure 4)**. However under low Pi the average number of CCFs at tier one is around 12, which then decreases to 11 at tier 10 **(Figure 4)**. In order to reveal a clear view on the cortex cells only, we isolated the cortex cells from the rest of the cell layers by manually drawing a black mask in Adobe After Effects. We unrolled this layer (using ImageJ's function RadialReslice) to produce a longitudinal image of the cortical cell lineages **(Figure 5 and Supplemental Movie 2)**. Whilst 8 continuous cell files can be traced from just above the quiescent center until the start of the elongation zone for plants grown on high Pi, when grown on low Pi, we observe multiple events in which CCFN is increased in relatively small numbers of cells. From these images it was concluded that the proliferative divisions occur within the meristem, and as we saw many independent events altering CCFN in a relatively small longitudinal domain. We speculate that this is likely to be a dynamic processes perhaps responding to local levels of Pi.

We next asked if the changes in CCFN observed on low phosphate were a dynamic event capable of adapting rapidly to a low phosphate environment. We reasoned that if this cortical re-patterning is to confer an adaptive advantage to, then it should occur quickly, for example in the case of a root grows though a patch of soil low in P. To address the dynamics of this response, seedlings were grown on plates containing replete levels of Pi (1 mM). Plants were then transferred to 50 μM Pi containing medium after 4, 7, 10, 11, 12, and 13 days. Twenty four hours after the final transfer, when seedlings were 14 days old, roots were harvested from all the 50 μM Pi plates, alongside control



plants from 1 mM Pi plates and roots imaged using pseudo-Schiff staining coupled with confocal microscopy. Strikingly, we observed a CCFN response to low Pi within 1 day of transferring plants to low Pi (Figure 4). Within this timeframe we saw a statistically significant increase in average CCFN from 8.6 to 10.5.

After this time point, the average CCFN remains between 9.5 and 12.5 with a small amount of variation depending on the transfer stage (Figure 3). These findings are broadly in line with other changes in plant response to low Pi as they occur much slower than changes in gene response. For example, changes in the expression of transcription factors such as WRKY75 occur within 3 h of transfer to low phosphate media (Devaiah et al., 2007).

Proliferation of Cortical Cells Alters Epidermal Patterning

Previously, it had been hypothesized that altering cortical cell file number would provide a mechanism through which root hair density can be increased in the radial dimension. We examined positions of epidermal cells laying in the H (trichoblast) position (i.e., spanning a junction between two cortical cells) and the N position (i.e., overlaying just one cortical cell). We found that the number of epidermal cells in the H position increased from 8 (1 mM P) to 11 (50 μ M P) under Pi limiting conditions (Figures 1, 4B). These results support previous observations by other researchers (Ma et al., 2001).

In order to confirm that this change in positional information for a selected subset of epidermal cells was sufficient to alter cell fate specification in these cells, we used light sheet microscopy to fully reconstruct 3D representations of mature tissues to evaluate the radial distribution of both expression of the root hair marker COBRA-like *COBL9* (Roudier et al., 2002) and the presence of the root hairs themselves in context of the underlying cortical cell geometry. We used light sheet microscopy because it enables a large volume to be imaged rapidly, it can be used with living samples and it does not require any clearing protocol which could change the cellular structural integrity. When plants were grown on high phosphate we observed a pattern as previously reported with files of trichoblasts over clefts between cortical cells, with these files being separated by 1–3 files of atrichoblasts (Figures 6–8 and Supplemental Movies 3–5). We only occasionally saw deviations from this stereotypical pattern, with for example, occasional ectopic expression of *COBL9* in epidermal cells overlaying a single cortex cell. When plants were grown under low phosphate we saw a breakdown of this regular spacing and frequently found four non-typical root hair patterning scenarios. The first scenario was that we saw adjacent files of trichoblasts as a result of two neighboring epidermal cells overlaying two adjacent cortex cell clefts (Figure 5). In reconstructions of roots grown on low Pi, we not only observed root hair forming cells forming at positions overlaying a cleft between two cortical cells, but in all reconstructions, we saw several examples where epidermal cells both expressing *COBL9* and forming root hairs were found over a single cortical cell (Figures 6–8 and Supplemental Movies 3–5), which we denoted as scenario 2. We also observed several instances in which epidermal cells lying in the cleft between two cortex cells neither express *COBL9* nor form root hairs (scenario 3). Finally, the fourth scenario we noted was that of root hairs emerging from a file of epidermal cells overlaying cleft between more than two cortical cells. For roots grown on high or low Pi we observe examples in which cells expressing *COBL9* either fail to form root hairs or only produce very short root hairs, although this is likely to come in part from an asymmetry in the availability of water imposed by the agar plate (Bao et al., 2014). Using a conventional microscopy approach we measured the root hair length on the side of the root facing away from the agar plate and observed an increase in root hair length for plants grown on low Pi (High Pi, $153 \pm 73 \mu$ m; Low Pi, $270 \pm 128 \mu$ m). Collectively, these results suggest that rather than simply increasing trichoblast number, alterations in the cortical cell patterning file number have significant effects in which the regular root hair patterning mechanism of the mature root becomes unstable and a more chaotic pattern emerges (Figures 6–8).

DISCUSSION

Arabidopsis thaliana roots have previously been shown to respond to low phosphate by producing extra cortical cells (Ma et al., 2001; Cederholm and Benfey, 2015). However, although this phenomenon had been reported, it had not been explored in great detail. For example, it was unclear if the cortex was the

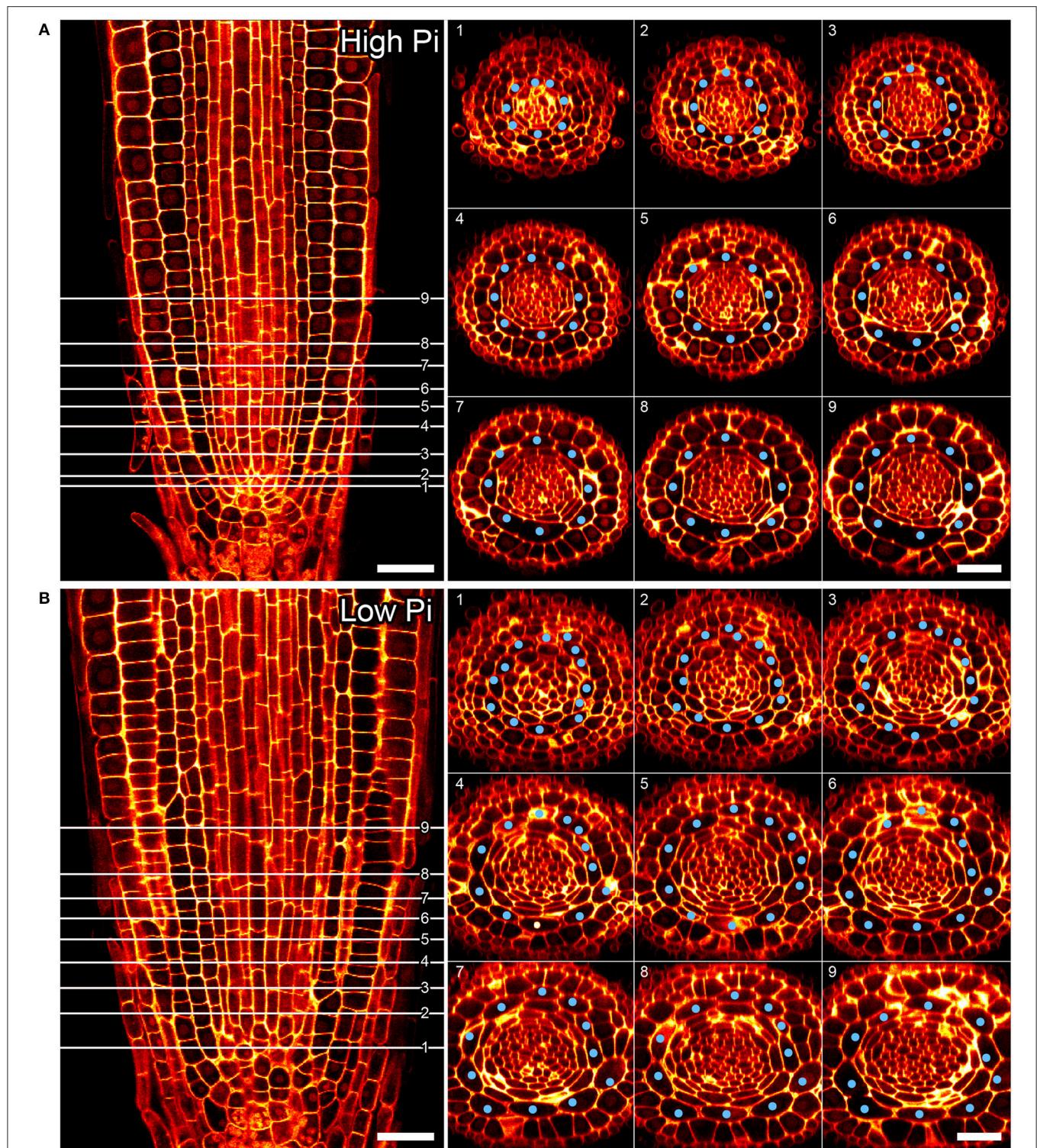


FIGURE 4 | Cortical cell proliferation occurs close to the root apical meristem. Reconstructed confocal Z-stacks of imaged pseudo-Schiff propidium iodide stained roots show that cortex cell file (blue spots) numbers are highest in proximity to the growing tip/cortex endodermis initial (CEI) cells in roots grown on 50 μM phosphate (**B**). Plant roots grown on 1 mM phosphate containing medium exhibit 8 cortex cell files from 1 to 10 cell tiers (white lines) behind the cortex endodermal initial cell (**A**). Cell file counts high P: $n = 17$; low Pi $n = 30$, differences between low and high Pi statistically significant $p \leq 0.001$. Scale bar = 25 μm .

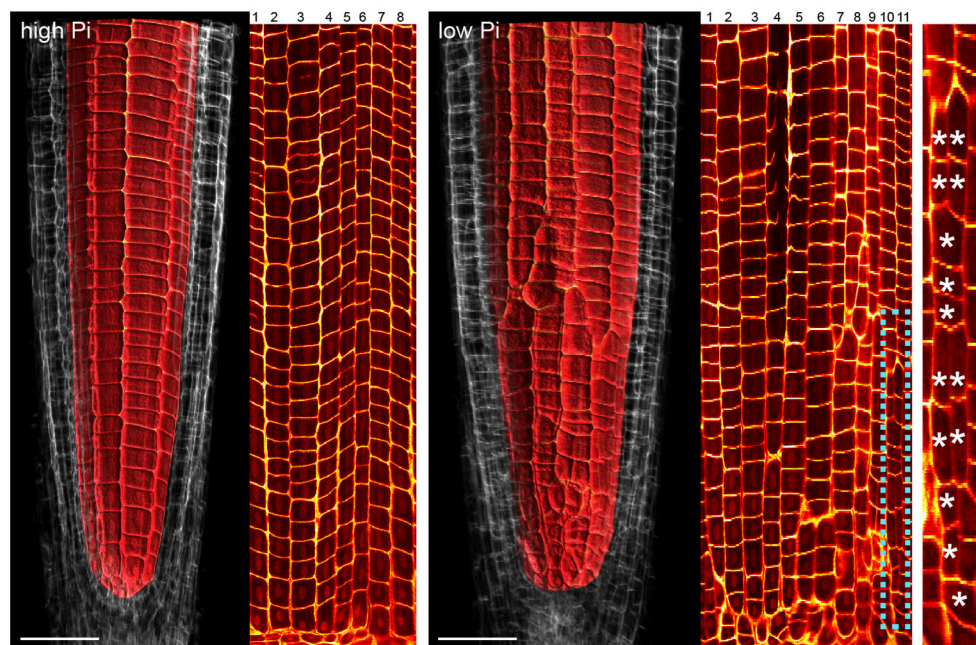


FIGURE 5 | Cell lineage analyses reveal multiple cell division events leading to increases in radial anticlinal cortical cell file number. Unrolled images showing cell lineages in the cortex of *Arabidopsis* roots under high Pi (**Left**) and low Pi (**Right**). Using Adobe After Effects we digitally dissected the membranes between cortex cells. In ImageJ. We unrolled this data set in order to visualize the cortical cell lineages within the root meristem. The dissected cell layers unrolled are shown in Supplemental Movie 2. Whilst 8 continuous cell files can be traced from just above the quiescent center until the start of the elongation zone for plants grown on high Pi, when grown on low Pi, we observe multiple events in which CCFN is increased in relatively small numbers of cells. These events are marked with asterisks. Scale bars = 50 μ m.

only cell type to show increased cell file number under low Pi, it was unknown which cortical cells underwent extra cell divisions, the exact timing of this response was unknown and it was unclear whether this was a specific response to low Pi or a result of iron toxicity. Furthermore, although changes in CCFN were reported, these were not correlated experimentally with alterations in root hair density or patterning.

To address these issues we exploited two methods for generating 3D reconstructions of the root meristem for plants grown under high and low Pi conditions. The first involved fixing roots and using a modified pseudo Schiff reagent to stain cell walls with propidium iodide. These stained roots were then visualized on a confocal microscope. This technique allowed us to image around 20–30 roots for each sample in a medium throughput manner. This confocal based approach allowed us to build upon the original data produced by Ma et al. (2003) that used just 6 roots. The second approach was based upon analysis using light sheet microscopy of roots stained with propidium iodide. In order to fully resolve the entire volume of the mature root we captured Z-stacks along six different angles coupled with the bead-based multiview reconstruction alignment (Preibisch et al., 2010). Four roots grown on low Pi and four roots grown on high Pi were analyzed, as well as a further 2 roots on high Pi and 3 roots on low Pi expressing the *COBL9* marker. This has allowed us to observe that trichoblasts can form in positions overlaying a single cortical cell, in plants grown on low Pi; a phenomenon that has not previously been described.

Collectively our data builds upon previous work showing files of extra cortical cells throughout the meristem (Cederholm

and Benfey, 2015) by demonstrating that several rounds of radial anticlinal divisions occur close to the root apex. Our experiments transferring plants from Pi replete to Pi limiting conditions show that anatomical changes occur quickly, with the first changes occurring within 24 h of transfer. These results are in keeping with those required of an adaptive response evolved to maximize Pi acquisition from soil. For a root to maximize Pi capture from a heterogeneous soil environment, the developmental program controlling root hair patterning must be able to adapt quickly to changes in the local environment at a range of concentrations. We observed that changes in CCFN occurred at Pi concentrations between 0 and 400 μ M, with the strongest effect seen at 50 μ M. This was surprising as this concentration is far above what is seen in most soils (Raghothama, 1999), but this could be explained as the availability of Pi on plates is likely to be very different from that in the soil. Recently the relevance of traditional gel-based systems for investigating the effects of phosphate limitation on root growth has been challenged, and a new system has been developed in which Pi is adsorbed onto Al_2O_3 particles (Hanlon et al., 2018). This has the advantage of delivering buffered levels of Pi to roots at concentrations closer to the Pi levels of natural soils. Although there are changes to *Arabidopsis* root architecture and anatomy when roots are grown on buffered vs. non-buffered medium, increases in CCFN on low Pi medium have been observed with both systems (Hanlon et al., 2018). This supports existing data showing that changes in CCFN are a direct response to low Pi, however it also indicates that alterations in the responses seen at different concentrations of Pi in this study cannot be

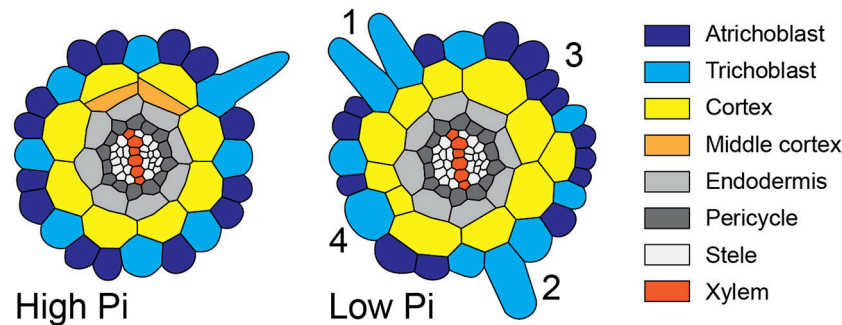


FIGURE 8 | Schematic diagram showing atypical root hair patterning scenarios found under low Pi conditions. Schematic representations of root radial cross sections under high Pi (**Left**) and low Pi (**Right**) conditions showing changes in tissue patterning including four atypical root hair patterning scenarios. These scenarios are: (1) the formation of 2 adjacent hair cells files forming over two adjacent cortical cells clefts, (2) the formation of a hair cell in a non-hair-cell position, (3) no root hairs forming from an H-cell file, and (4) one H cell file forming over two adjacent cortical cell clefts.

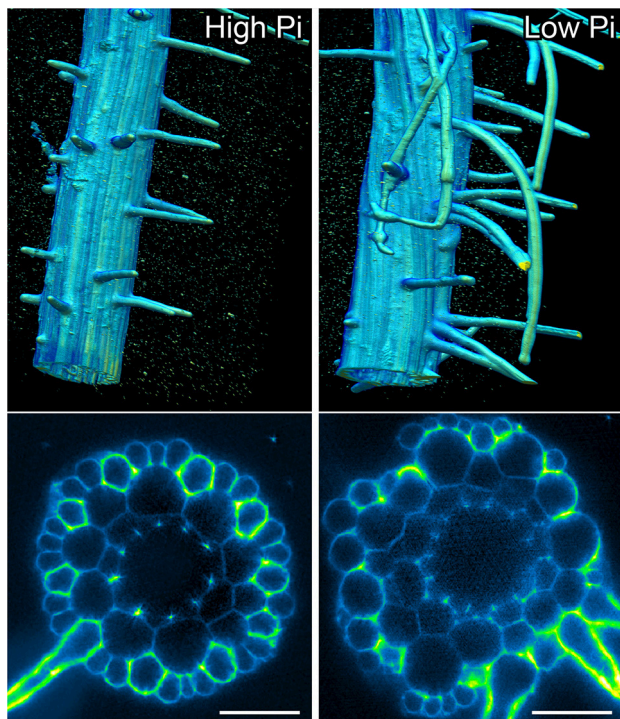


FIGURE 6 | Light sheet imaging reveals atypical root hair patterning scenarios under low Pi. Anatomy of a mature part of *Arabidopsis* under high Pi (**Left**) and low Pi conditions (**Right**). Multi-view recording along six directions captured using Light Sheet Fluorescence Microscopy. A 3D reconstruction (Arivis Vision 4D software) of the fused image stack is shown in the upper panel and a single slice cross section is presented in the lower panel. Roots were stained with propidium iodide. Scale bars = 50 μm .

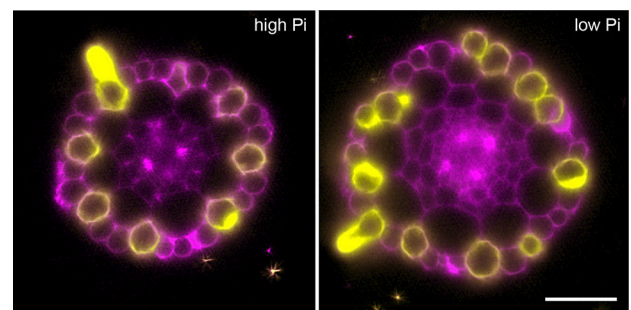


FIGURE 7 | Expression of the COBL9 marker shows atypical specification of trichoblast identity under low Pi. Anatomy of a mature part of *Arabidopsis* under high Pi (**Left**) and low Pi conditions (**Right**). Multi-view recording along six directions captured using Light Sheet Fluorescence Microscopy to visualize the pCOBL9::GFP gene (yellow) and propidium iodide (magenta). Scale bars = 50 μm .

interpreted literally and do not represent levels found within soils.

Whilst our results were generally in keeping with those published previously (Ma et al., 2003; Cederholm and Benfey, 2015), they differed in a few minor aspects. Cederholm and

Benfey (2015) reported changes in the number of endodermal cells. However in our assay the numbers of vascular cells, pericycle cells, endodermal cells and total epidermal cells did not differ significantly between low and high Pi conditions. We also observed changes in cortical cell file number on transfer to Pi limiting conditions earlier than has been reported by other authors. Cederholm and Benfey (2015) report changes in cortical cell file number at 2 days post-transfer, Ticconi et al. (2009) report changes in ground tissue in the Pi hypersensitive phosphate deficient root 2 (*pdr2*) mutant after 2 days of transfer; whilst we observed the first changes occurring after only 24 h.

One limitation for performing studies limiting phosphate on agar plates, is that under low Pi conditions, roots can accumulate high concentrations of free iron in the root meristem (Hirsch et al., 2006; Svistoonoff et al., 2007; Ward et al., 2008; Dong et al., 2017; Gutiérrez-Alanís et al., 2017). This can have a toxic effect leading to meristem disorganization and ultimately termination. Our data demonstrates that the increased cortical cell file number, is direct a result of low Pi rather than extra iron.

Although increased CCFN has been proposed to offer an advantage to improving Pi acquisition under low nutrient conditions, it is only one of several traits employed by *Arabidopsis* to increase root hair density, as decreased epidermal cell size impacts root hair density in the longitudinal dimension and increased root hair length can increase uptake of Pi from the soil (Foehse and Jungk, 1983; Ma et al., 2001). However, when we followed the radial distribution of root hairs and compared it with the underlying pattern imposed by the cortex, we observed alterations in the regular spacing of root hair cell rather than a general increase. We frequently observed multiple files of root hairs forming adjacently (scenario 1), we observed root hairs forming in positions where the epidermal cells overlay just one cortical cell (scenario two), epidermal cells overlaying a cleft between two cortical cells producing no root hairs (scenario three), and root hairs forming from single epidermal cell files overlying two adjacent cortical cell clefts (scenario four). These scenarios are summarized schematically in **Figure 8**. However, we did not see a significant increase in the radial density of root hairs. Our results challenge the assumption that the trait of increased CCFN has evolved in species that exhibit type 3 root hair patterning as a mechanism specifically to increase root hair density, although it is possible that different effects could be observed at different concentrations of Pi.

It is important to develop understanding of the mechanisms through which roots respond to low nutrient environments in order to develop new lines that can utilize nutrients existing in the soil more efficiently. In this paper, we perform a detailed anatomical study of how radial anatomy is altered under low phosphorus in *Arabidopsis* and the effect that modulating cortical cell file number has on controlling density of root hairs in the radial dimension. This study will inform future programmes that may seek to modulate root hair density in plants similar

to *Arabidopsis* as it challenges the utility of focusing on CCFN. It opens up interesting questions regarding whether increased cortical cell file number offers an alternative adaptive advantage to growth on low Pi, and provides an intriguing example for future studies about how patterning events in one tissue can affect patterning in others.

AUTHOR CONTRIBUTIONS

AB, GJ, and DvW designed experiments. GJ, SC, and DvW performed experiments and analyzed results. AB, GJ, and DvW wrote the manuscript with input from LB, AF, and IK.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fpls.2018.00735/full#supplementary-material>

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