EXPRESSION DOMAIN ANALYSIS OF FOUR MEMBERS OF THE PLASMODESMATA-LOCALIZED PROTEIN FAMILY IN *ARABIDOPSIS*

by

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ABSTRACT

There are two modes of cell-to-cell communication between plant cells: the apoplastic and symplastic pathways. The apopolastic pathway involves endocytosis and exocytosis, whereas, the symplastic pathway involves movement through plasmodesmata (PD). PD are channels that directly connect the cytoplasms of neighboring cells and allow for the symplastic trafficking of proteins, RNA and other small molecules. PD, over the years, have been implicated in plant growth, development, survival and innate immune responses. Protein analysis of cell wall extractions identified a family of proteins called *Arabidopsis* PD-localized protein (PDLP), named for their association with PD. There are 8 type-I membrane receptor-like proteins in the PDLP family. PDLP1 and PDLP5 have been shown to regulate plasmodesmal aperture. Here, the expression domains and patterns of four PDLP members, *PDLP4, 6, 7* and *8* were investigated using the yellow fluorescent protein and/or β-glucoronidase reporter systems. The results of this study distinct tissue and cell specific expression patterns of these members. *PDLP4* promoter was active in primary and lateral roots, both in the mature endodermal cells with developed casparian strips and the lateral root cap. *PDLP6* promoter was active in the shoot apical meristem. It was also transiently induced in the primary root vasculature and root tip. *PDLP7* promoter was active in the companion cells of cotyledons and young true leaves. As well as in the primary root, specifically phloem pole pericycle, companion cells and the columella root cap. Similar to *PDLP7*, *PDLP8* promoter was active in the vasculature of cotyledons and roots, including the companion cells and

the phloem pole pericycle. The expression patterns of *PDLP4* and *PDLP7* were quite exciting given that no information is known about the role of PD in that tissue. Thus, analysis of the *PDLP4* and *PDLP7* genes was carried out using auxin treatments of the *PDLP4pro:GUS* and *PDLP7pro:GUS* lines and phenotyping the transgenic knockout lines for each gene. Observation and quantification of primary root growth of individual mutants revealed significant differences between the growth of *pdlp7-1* lines and WT plants. Gravitropic assays coupled with histochemical assays revealed a role for *PDLP4* and *PDLP7* in lateral root emergence. While confocal imaging of mutants revealed abnormal root cap cell morphology and subsequently hindered sloughing of root cap cells, similar in phenotype to the *SOMBRERO, BEARSKIN 1 and BEARSKIN 2 (SMB, BRN1* and *BRN2)* knockouts. SMB, BRN1 and BRN2 are NAC domain transcription factors involved in cell wall modifications. Based on expression patterns, auxin responsiveness, and knockout line phenotypes, I propose a mechanism for lateral root propagation in phloem pole pericycle cells in *pdlp7-1* lines. Additionally, a role for *PDLP7* and *PDLP8* in floral transitioning. And lastly, a method to study symplastic movement of the transcription factor WUSCHEL by regulating PD aperture within the shoot apical meristem using *PDLP6*.

Chapter 1

LITERATURE REVIEW

1.1 Introduction

Plant cells have distinct cell walls that set them aside from their eukaryotic counterparts. Cell walls act as physical semi-permeable barriers and as structural components for the cell. They protect against water pressure and keep the cells from lysing in a hypotonic solution; this makes plant cells turgid. Turgidity is vital not only for respiration but also in transpiration and maintenance of cellular trafficking. Over the years, eukaryotes have developed many strategies for symplastic communication. In plant cells, cell-to-cell communication is made possible by nano-channels called plasmodesmata (PD) (Singular: plasmodesma). PD traverse across cell walls of adjacent cells. When first discovered it was believed that PD acted as gaps in cell walls that assisted signaling by passive diffusion of smaller molecules. It was later discovered that it was possible for soluble molecules as well as membrane-bound molecules to be actively transported through PD. There are four known modes of transport via PD. The first being through the cytoplasmic sleeve, the second by entering the lumen of the desmotubule, the third defined by lateral diffusion in the plasma membrane (PM) and lastly by lateral diffusion in the membrane of the desmotubule (Wu and Gallagher, 2012).

Plasmodesmal aperture is tightly regulated by several known and unknown mechanisms. The basal resting size exclusion limit (SEL) of PD is between 0.8-1.2 kDa, acting as a filter for the cell, but under certain conditions PD can be opened and then closed again, to accommodate proteins over 20 kDa. PD are capable of changing their permeability in response to biotic and abiotic stressors such as wounding or infection.

PD can also be permanently closed and degraded (Lucas et. al., 2004). This is seen in guard cells. It has been evident through several studies that PD have the ability to mediate movement of proteins, RNA molecules, viruses and viral particles from cell to cell. More specifically, PD are a necessary pathway in the transport of non-cell-autonomous proteins (NCAPs). NCAPs are proteins translated in one cell that actually function in another. These proteins must be exported out of the cell to their destination. Therefore, playing a crucial role in plant survival, growth, development and innate immune response. This literature review will focus on the mechanisms of PD regulation, the role of symplastic trafficking in stem cell niches and *Arabidopsis* growth and development.

1.2 Plasmodesma Structure and Modification

PD connect the cytoplasm of neighboring cells by crossing their cell walls. PM lines the PD channel, while an appressed endoplasmic reticulum (AER) runs through the pore. The space in between the plasmalemma and desmotubule is called the cytoplasmic sleeve (CS). Within the CS, several PD targeted proteins are located at the PM of PD. PD also have a cell wall component composed of the cell walls of adjacent cells (Figure 1.1).

Several cytoskeleton-associated proteins have been found to regulate, associate with, or simply accumulate at PD. Actin is one such key component of PD structure (White et. al., 1994). Actin was found to perfectly align along the neck of the channel. It is hypothesized that this perfect alignment suggests that actin is involved in relaxing and contracting PD. The actin cytoskeleton was also found to be involved in regulating PD SEL. Cell to cell transfer of viral movement proteins (MP) exploit actin

depolymerization (Chen et. al., 2010). Myosin, tropomyosin and microtubules also contribute to the cytoskeleton of PD. Myosin, known to be a motor protein, is the most prevailing actin binding protein at PD (Chen et. al., 2010). Evidence suggests that myosin at PD is required for the localization and targeting of other proteins to PD. Microtubules at PD are necessary for its structure, aperture and transport of cargo to PD. Lastly, callose, a (1,3)-β-glucan is found to accumulate at the PD neck region between the cell wall and membrane, acting as a gate keeper opening and closing PD (Levy et. Al., 2007).

There are two kinds of PD, primary and secondary (Faulkner et. al., 2008). Primary PD are formed during cell division and secondary PD are forged into existing cell walls, independent of cell division. Secondary PD biogenesis is not yet comprehensively understood- but it is thought to be similar to the formation of primary PD. During primary PD formation, part of the ER is trapped at the phragmoplast (scaffold for cell plate assembly) during cytokinesis (Ehlers et. al., 2001). When originally formed, primary PD are very simple. However, with time can become branched and modified. One mechanism of plasmodesmal branching is like that of primary PD biogenesis. As the cell wall thickens the unbranched PD need to stretch to ensure they traverse across both cell walls. Golgi vesicles containing cell wall material, trapped ER and cytoplasmic material then fuse with the PM, often creating a X or Y shaped intermediate (Faulkner et. al., 2008). The shape of the branched PD is determined by the shape of the original entrapped ER. In contrast to branching PD, adjacent PD may also fuse together producing an H shaped structure. One strand of appressed ER may also be divided by vesicles depositing cell wall material, this is called

twinning PD (Kragler et. al., 1998). PD distribution and frequency may also be changed throughout the lifetime of the plant, most distinctively seen in vascular bundles (Botha et. Al., 1988).

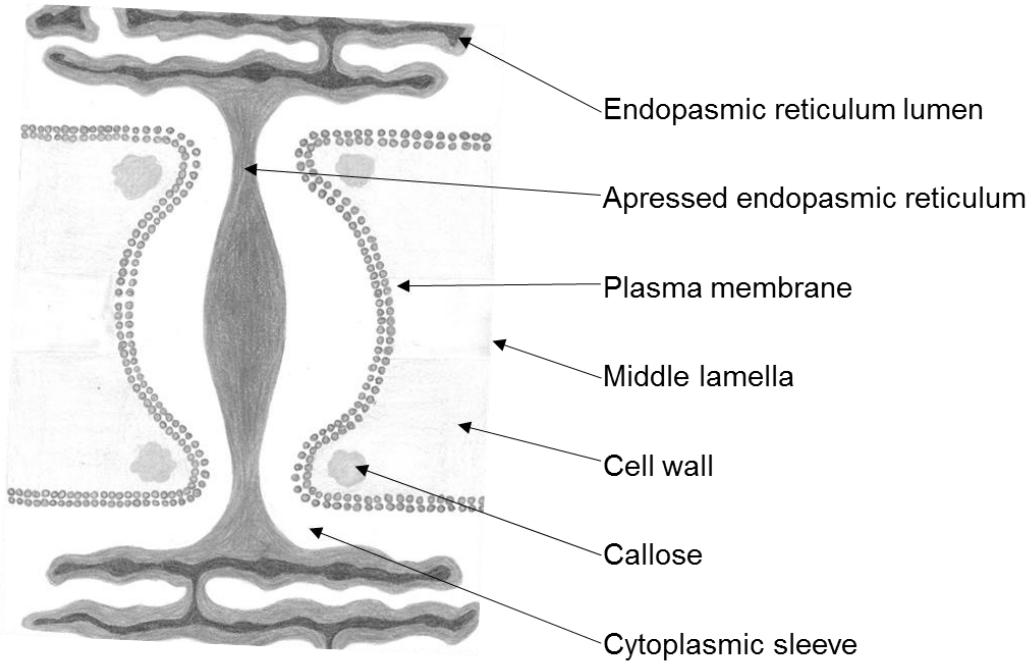

Figure 1.1 Illustrative representation of a simple unbranched plasmodesma. ER lumen, endoplasmic reticulum lumen. AER, appressed endoplasmic reticulum. PM, plasma membrane.

1.3 Players Involved in the Regulation of Plasmodesmal Aperture

1.3.1 Plasmodesmata-Localized Proteins Limits PD Permeability

Plasmodesmata-localized proteins (PDLPs) are a family of proteins that are found to localize at PD (Thomas et. al., 2008). This family of eight proteins is categorized as type I membrane receptor-like proteins. A 2008 survey of membrane bound proteins of highly purified *Arabidopsis* cell walls revealed the later labeled protein PDLP1. Sequence analysis predicted that this protein had a N-terminal signal sequence, transmembrane domain (TMD) and two domains of unknown function (DUF domains)

and a short c-terminal tail. Sequencing data was also used to identify all homologs of the first found protein, PDLP1. Fluorescently tagged PDLP1showed that PDLP1 localized at PD, when expressed under its own promoter, via confocal microscopy. Both the TMD and signal peptide sequence are required for PD targeting (Thomas et. al., 2008).

Transgenic lines overexpressing PDLP1 demonstrated a dramatic decrease in free Green Fluorescent Protein (GFP) movement, cell-to-cell, implying PDLP1 was involved in closing PD aperture and molecular symplastic trafficking. However, the exact mechanism is yet unknown.

A recent study from our laboratory showed that the hormone salicylic acid (SA) acts as a signaling molecule in a defense pathway that induces PDLP5, another PDLP family member, to close PD in response to pathogen invasion. SA upregulates PDLP5 and surprisingly PDLP5 overexpression feedback regulates SA (Wang et. al., 2013). PDLP5, sublocalizes at the central region of PD. The original proposed mechanism suggested that pathogen invasion upregulates levels of EDS1 (enhanced disease susceptibility protein 1- found in *Arabidopsis*), which in turn upregulates SA levels, somehow activating transcription of PDLP5, leading to PD callose accumulation. The proposed mechanism has a missing link referred to as X, the component downstream of PDLP5 expression. X is believed to be one or more members of the CalS family. The study suggested PDLP5, is an essential molecular link that ties together the immune signal generated by SA with the PD closure response in *Arabidopsis thaliana* (Wang et. al., 2013).

A knock down mutant, *pdlp5-1*, was formed from a T-DNA insertion in the first intron of PDLP5 was studied to resolve changes in morphological phenotype. It was found that the mutant plants were similar to wild type plants in growth and development. However, the overexpression line, *PDLPOE,* negatively impacted the growth of *Arabidopsis*, indiscriminate of the life stage of the plant (Lee et. al., 2011). *Pdlp5-1* inhibits PD closure, increasing permeability of molecules. In comparison, *PDLP5OE* leads to a decrease in PD assisted transport. When quantifying PD callose levels via alanine blue staining and confocal microscopy, Callose levels were decreased in pdlp5-1 mutants and increased in PDLP5OE transgenic lines. The exact mechanism of how PDLP5 regulates PD callose levels or which CalS are being utilized is still unknown. However, a recent study from Cui et. al. (2016) revealed that PDLP5 works with CalS family members CalS1 and 8 to close PD in response to abiotic stressors such as oxidative stress. Further studies of all the PDLPs will reveal the functions to each PDLP isoforms.

1.3.2 Expression of Callose Synthases/Glucan Synthases Result in Decreased Plasmodesmal Size Exclusion Limit

Callose controls PD permeability during both abiotic and biotic stresses. Deposition of callose has been shown to close PD. Callose turnover at PD is tightly regulated Callose synthases (CalS), also known as Glucan Synthases (GSL). 12 CalS family members have been identified in *Arabidopsis*. It is hypothesized that each may be tissue-specific or active in response to different physiological conditions and stresses. Only 8 of the 12 CalS have been characterized. The functionality of CalS2, 4, and 6 remain a mystery. CalS1 and 10 are involved in cytokinesis and cell plate formation. CalS 5, 9, 10, 11 and 12 play a role in microgametogenesis and pollen formation, while

CalS1 and 8 play roles in abiotic stress response, as mentioned in Chapter 1.3.1. CalS3 is PD: root- stele specific. CalS7 mediates sieve plate and phloem transport. CalS12 is activated in response to wounding and pathogen infection. There is functional redundancy among many of the CalS members. Out of the 8 known CalS, 5 present a relationship with PD, thus far: CalS1, 3, 7, 8 and 10. Vatén et. al. (2011) showed gain of function CalS3 mutants resulted in an accumulation of PD callose, limiting PD permeability. CalS3 was first identified because of sequencing data of three different allelic gain of-function mutations that are collectively refer to as *cals3-d*. These mutants were identified from a genetic screen for altered vascular patterning. Moreover, movement of the transcription factor SHORT-ROOT and microRNA165, which are necessary for vascular patterning in roots, proved to be PD dependent. Thus, CalS3 mediated PD closure was linked to root development. Reverse transcriptase- polymerase chain reaction (RT-PCR) analysis and in situ localization studies of CalS3 transcription levels and reporter lines CalS3: GFP/CalS3: GUS showed that CalS3 is expressed in the stele and in the root meristem during plant development. More specifically, immunogold labeling showed that CalS3 was located in the vicinity of PD. Sieve pores of the sieve plate and sieve areas are developed from PD. This process is yet again poorly understood, and the role of callose is ambiguous. It is a well-known fact that callose is present in sieve plates. Wounding a plant can cause it to rapidly accumulate callose at sieve pores (Xie et. Al., 2008).

Four CalS7 mutants, collectively labeled cals7, found using T-DNA insertions provided insight to CalS7 functionality (Xie et. al., 2011). Homozygous mutants failed to produce cals7 transcripts all together, representing true knockouts. RT-PCR

expression patterns of wildtype (WT) plants revealed high levels of expression in the phloem cells of vascular tissue, implying a possible role in phloem callose synthesis. In CalS7 mutants, aniline blue stained callose deposition was not seen in phloem cells or at sieve plates. Sieve plates were visualized using a propidium iodine stain and confocal microscopy. Indicating CalS7 played a distinct role in phloem localized callose synthesis and sieve plate formation. Mutant plants even displayed distorted sieve pore structures.

CalS10 mutants show clustered stomata, indicating a role in patterning during plant development. CalS10 mutants also led to decreased levels of PD localized callose, resulting in deregulation of symplastic movement. During cytokinesis callose accumulates in PD at the cell plate, regulating cell-to-cell signaling. Some homozygous CalS10 mutants, established by random T-DNA insertion screens, turned out to be seedling lethal and display multiple phenotypes such as axial dissymmetry and dwarfism. RT-PCR expression analysis of CalS10 revealed transcript in all organs and various different parts of each organ. Expression was relatively higher in dividing cells. Further studying revealed incomplete cell walls in mutants. Normally aniline blue staining of callose is done to outline cell walls, in this case it was used to quantify callose levels and identify the role of CalS10 in cytokinesis (Chen et. al., 2009).

1.3.3 Plasmodesmata Callose Binding Proteins Upregulation Results in Over Accumulation of Callose at PD

PD callose binding protein 1 (PDCB1) was first discovered through proteomic studies of cell wall components isolated from *Arabidopsis*. PDCB1 was shown via confocal microscopy to localize at PD and bind callose. Immunogold studies showed sublocalization specifically at the neck regions of PD. PDCB1 is a glycosylphosphatidylinositol (GPI) anchored protein facing the extracellular domain.

Overexpression of PDCB1 resulted in an over accumulation of callose PD, implying a role in regulating PD via callose. Over accumulation of callose at PD, leads to decreased PD aperture, therefore indicating PDCB1's involvement in symplastic trafficking via PD (Simpson et. al., 2009).

1.3.4 Expression of Plasmodesmata β-1,3-Glucanases Result in Increased Plasmodesmal Size Exclusion Limit

Glucanases breakdown sugars. *Arabidopsis* β-1,3-glucanase_Pd-associated protein (AtBG_Pap), also known as PdBG, a GPI lipid-anchored protein, was the first β-glucanase discovered to regulate PD aperture. To isolate AtBG_Pap, proteins in PD rich tissue in *Arabidopsis* were fractionated using SDS-gel electrophoresis. All proteins on the gel were proteolysed using trypsin. Resolving peptides were then analyzed using mass spectrometry. Software called Sequest compared the results to simulated proteolysed proteins in the non-redundant National Center for Biotechnology Informatic (NR-NCBI) database. Further computational analysis of AtBG_Pap indicated two transmembrane regions and a signal peptide sequence, while the other proteins did not (Levy et. al. 2007). These findings became grounds for further exploration.

Several studies were done to confirm the protein's association with PD post wounding. Functional studies of an AtBG_Ppap mutant, in *Arabidopsis*, established by random T-DNA insertions via agrobacteria, which failed to transcribe AtBG_Pap, demonstrated reduced cell-to-cell movement of GFP via a fluorescent light microscope and a confocal laser scanning microscope. The mutant plant also resulted in higher accumulation of aniline blue stained callose at PD, in comparison to WT plants. The

transgenic AtBG_Pap mutant in *Arabidopsis* was obtained by the *Arabidopsis* Biological Resource Center in Ohio.

The AtBG_Pap:GFP fusion protein, in *Nicotiana Tabacum*, was shown to co-localize at PD along with aniline blue stained callose. The fusion protein in tobacco was expressed using a pCambia vector and transformation via agrobacteria. These studies indicated AtBG_Pap opens PD by callose degradation in response to wounding. Furthermore, two other BGs labeled Plasmodesmal located β-1,3-glucanase 1 (PdBG1) and Plasmodesmal located β-1,3-glucanase 2 (PdBG2) were recently identified for their role in lateral root development. It was discovered that the two PdBG's controlled PD opening by regulating callose levels in xylem pole pericycle (XPP). PdBG1-PdBG2 double mutants instigated clustering of lateral roots, signifying that symplastic connectivity is crucial for lateral root positioning and spatial patterning (Knox et. al., 2014).

To arrive at these results, studies on the relationship between symplastic connectivity and lateral root development were conducted. Transgenic lines expressing reporter GFP were visualized at different stages of root development. GFP movement was decreased in stage II and III primordia and completely excluded from stages IV-V primordia. Results indicated that PD closure at those stages was crucial for proper development. Symplastic transport is controlled by callose at PD neck regions. Immunofluorescent detection of callose levels at the various stages of lateral root formation further supported the idea that callose assisted PD opening in the early stages was vital for proper development. The callose degrading enzymes associated with this regulation, PdBG1 was found by using transcriptome data sets to screen for genes

involved in lateral root initiation that might also be expressed in XPP. GUS staining revealed that the PdBG1: GUS transgenic fusion protein was localizing at PD. Phylogenetic analysis and expression profile data suggested a gene, later called *PdBG2*, may have functional redundancy to PdBG1. Microarray data uncovered high expression of PdBG2 in XPP.

When studied, using gene trapping for mutagenesis purposes, the expression patterns overlapped with that of PdBG1. To determine PdBG1 and PdBG2 role in lateral root growth, PdBG1, PdGB2 and PdBG1-PdBG2 double mutants were studied. Single mutants and WT plants showed no significant differences. However, the double mutant accumulated approximately 3 times more callose than the WT roots. Callose levels were quantified using aniline blue fluorescence intensity (Benitez-Alfonso et. al., 2013). Collectively these studies propose that symplastic connectivity during development of lateral root primordia is coordinated by callose regulated PD aperture via the two redundant enzymes: PdBG1 and PdBG2.

1.4 *Arabidopsis* Plant Growth and Development

1.4.1 Shoot

The *Arabidopsis* shoot develops into all non-root organs and tissues. This consists of the cotyledons, true leaves, stem, and reproductive organs such as the flower. Phenotypic studies done on early flowering mutants revealed morphological phenotypes not just of the flowers, but the leaves and bolts as well. Sterile Apetala (SAP) a gene regulating flower and ovule development is characterized by morphological abnormalities in sepals and petals as well as its sterilizing effect on the plant (Byzova et.

Al., 1999). Proper shoot development is vital for reproduction and subsequently species survival.

1.4.2 Root

The *Arabidopsis* root consists of several cell layers. The inner most layers are known as the stele which comprise the pericycle layer and vascular tissues such as phloem, xylem and their precursors and companion cells. Directly outside of the stele is the endodermal layer, between the endodermal and epidermal layer lies the cortex. Cells in the pericycle give rise to lateral roots. The vascular tissues are involved in the transport of nutrients and water and possibly even the hormone auxin. The endodermis combined with a special cell wall modification called the casparian strip function as an impermeable selectable barrier to keep nutrients and water from diffusing out of the stele and deciding what gets in. The cortex is known to play a role in abiotic stress, directional cell growth and development in the root. The epidermal layer can be modified to produce elongated cells known as root hairs which function in water uptake and absorption in the soil. The root also consists of a meristematic zone (root apical meristem) and a zone of high cell turnover known as the root cap. The root apical meristem, made up of a quiescent center and stem cells, is responsible for root growth. The root cap comprising a lateral root cap (LRC) and columella root cap (CRC) is involved in regulating stem cell fate and root gravitropic response. The root cap is made of cells that are constantly being killed, replaced and sloughed off. The rationale behind this is still unclear (Durand et. Al., 2009). A visualization of root trip anatomy adapted from Yvone Jailais is shown below.

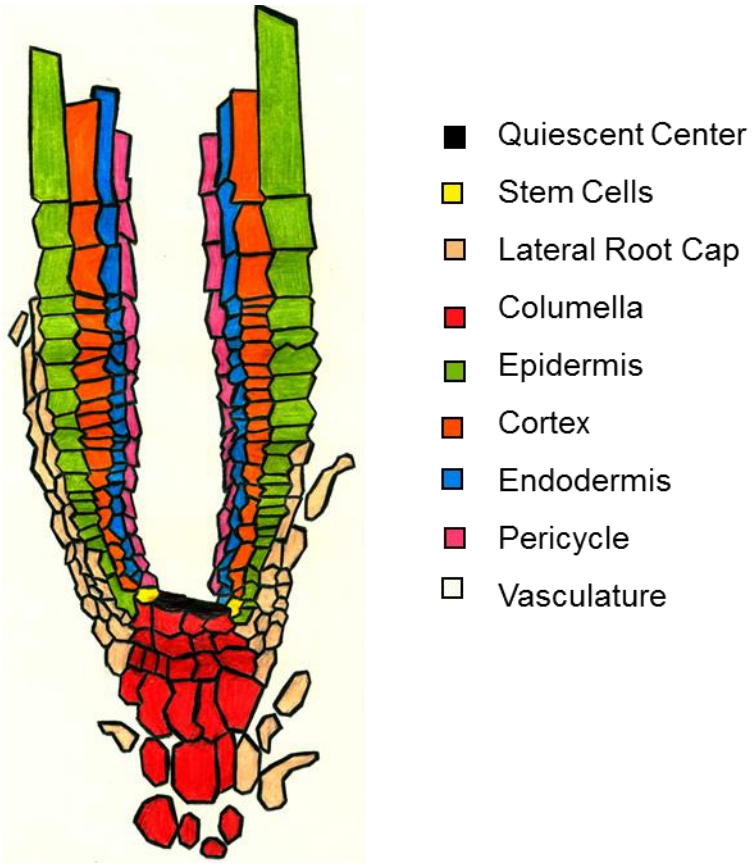

Figure 1.2 Representation of root tip anatomy depicting the positioning of the provasculature (that eventually forms into phloem and xylem), pericycle, endodermis, cortex, epidermis, QC, LRC and CRC. Note the stem cells are not distinguished. Based on illustrations made by Yvon Jaillais.

1.4.2.1 Casparian Strip Formation and Function

The endodermis in the roots acts like a selectivity filter for water and nutrient transport, vital for growth and development. This specificity is made possible by the formation of the lignin-based casparian strips. The casparian strip is a ring-like cell wall modification present in all mature endodermal cells. Casparian strips are analogous to animal tight junctions. Casparian strip formation is orchestrated by the *MYB36* transcription factor. Its formation is initiated by localization of Casparian strip domain proteins (CASPs) in the cell membrane where the strip will eventually form. Localized

CASPs recruit Peroxidase 64 which helps assemble the lignin polymerization machinery. Mutant screenings conducted to find endodermal mutants revealed proteins SCHENGEN1 and 3 (SGN1 and 3). SGN1 localizes to the endodermis in a specifically polar manner and stimulates the central positioning of the casparian strip. In SGN3 knockout, the casparian strip is missing completely. Seedlings without a casparian strip present with altered water transport, latent potassium deficiency, hypersensitivity to low potassium and other elemental homeostasis defects (Geldner et. Al., 2016).

1.4.2.2 Lateral Root Propagation and Emergence

The lateral root primordial (LRP) originates from pericycle stem cells which are located deep within the primary root tissues. LRP emerge through the overlying root tissues by the induction of auxin-dependent hydraulic changes and cell separation in the adjacent cells. Like-auxin 3 (LAX3) plays a vital role in the concentration of this signal in the cells overlying the lateral root primordial. Delimiting of LAX3 expression to 2 adjacent cell files which overly new lateral root primordial is critical in ensuring auxin regulated separation of cells occurs only along their shared walls.

There are three steps involved in lateral root emergence: 1) initiation 2) endodermal crossing 3) cortical/epidermal crossing. Auxin stored in the CRC, is transported to lateral root founder cell (LRFC). LRFCs, found in the pericycle layer of the root, undergo asymmetrical anticlinical divisions initiated by auxin signaling. These newly divided cells become stage I primordia. Auxin then flows out of the LRFC into the surrounding endodermal cells. The local casparian strip is degraded and the endodermal PM of the overlying cells fuses. The LRP know becomes dome shaped and protrudes past the endodermal layer. Defects in cell wall remodeling enzyme and auxin

exporters lead to halted or delayed LR emergence. The cortical and epidermal cells overlying the LRP are not modified but instead pushed apart to allow the LR to emerge. Auxin is moved from the endodermal and into the subsequent layers for proper emergence (Overvoorde et. Al., 2010).

1.4.2.3 Roots Respond to Gravistimulation by Auxin and Starch Accumulation in the Root Cap

Gravitropism is a turning of the growth movement by a plant while responding to gravity. This is a general feature in every higher plant. Charles Darwin was among the very first scientist to discover that roots usually show positive geotropism and the stems show negative geotropism. This means that the roots normally grow following the direction of the force of gravity while the shoots grow in the opposite direction. Gravity profoundly influences plant development and growth. Plants respond to the changes in the orientation using the gravitropic responses. The hormone Auxin regulates the root growth by the targeting the Aux/IAA repressor proteins for the degradation (Gray et. Al., 2010).

Genetic ablation of the root cap in *Arabidopsis* revealed that it was the major gravity sensing organ, consisting of the LRC and CRC. The first part of the plant that touches the environment in the soil is the root cap. Studies also show that it is in fact the starch and auxin present in the root cap that is responsible for the gravitropic response. In *Arabidopsis*, auxin is initially expressed in the shoot and is transported through the stele via PIN1 and AUX1 (efflux carrier proteins) and accumulates at CRC. From the CRC, PIN3 and PIN7 transport auxin into the LRC. The CRC is also home to starch granules known as statoliths (specialized amyloplasts). Auxin and statoliths work

together to respond to gravistimuli. It is hypothesized that changes in starch sedimentation and auxin distribution creates cellular signals that trigger the changes in root growth. However, the exact mechanisms remain a mystery (Band et. Al., 2012).

1.4.3 A Combination of Symplastic and Apoplastic Signals Regulate Stem Cell Maintenance

Plant pluripotent cells are found in microenvironments that are known as meristems. The two basic meristems, root apical meristem, which will be discussed later, and the shoot apical meristem (SAM) are the ones that are responsible for a plant's longitudinal growth and they are situated at the tip of the root and the shoot respectively. Furthermore, plants have the ability to develop a secondary meristem, known as the cambium, which allows the plants to grow radially. Since stem cells are enclosed in rigid cell wall structures, they are not mobile, the growth of plants is mainly possible by cellular expansion and division. In shoot and root meristems, there are groups of cells that are actively dividing mitotically, thus, creating fields of misplaced cells that adopt different functions as they pass through the various functional domains. In recent years, a more refined picture of regulation of the activity of the meristematic regions has begun emerging. It involves interplay between phytohormonal signals, transcriptional networks of regulation and chromatin remodeling factors. PD are thought to be aiding these signals in symplastic trafficking. During the various stages of cell differentiation, dynamic control of the permeability of PD allows the formation of symplastic domains. The functional domains thus allow only specific programs of development to occur in the restricted regions.

Two distinct pathways contribute to stem-cell maintenance in the SAM. Bilateral and radial pattering information is combined in order to position the stem cell niche and the vast amplifying cell population which surrounds the niche. The major transcription Factors which are required for stem-cell maintenance are homeodomain transcription factors SHOOT MERISTEMLESS (STM) and WUSCHEL (WUS) (Endrizzi et. Al., 1996).

The 3 layers of stem cells are maintained by 2 mechanisms in *Arabidopsis*. In a scanning electron micrograph of heart stage (early) embryos, stem cells marked with CLAVATA3 (CLV3), a receptor-like kinase, show an underlying organizing center, which expresses WUS. In the WUS pathway, the expression of WUS in stem cell organizing center maintains the overlying cells. Initiation of WUS expression seems to depend on continuous positional information, while a new organizing center can be reestablished after laser ablation of those cells. There are two known activities that limit the domain of WUS expression at an early embryonic stage: polar auxin transport systems which include PIN1, an auxin efflux carrier, and also the activity of HANABA TARANU GATA factor. Special positional cues, however, have not been reported for the expression of WUS) (Endrizzi et. Al., 1996).

The STM pathway for stem-cell maintenance is strictly defined by the STM home domain transcription factor, which suppresses differentiation of cells in the stem-cell area and also in the surrounding transit amplifying cells. The CUP-SHAPED COTYLEDON (CUC) factors are among the many positional cues for STM expression initiation, and these are members belonging to the NAC-domain family. In the embryonic stage, these factors are limited to the lateral subdomain via the polar-auxin transport regulator. Polar

auxin transport thus seems to restrict stem-cell promoting activity of the CUC and the STM transcription factors to some lateral domains at the top half of early embryos. One more positional cue for the expression of STM may be generated or developed from provascular tissues by ZWILE (ZLL), a protein that is required for the appropriate and proper establishment of the STM expression. ZLL is analogous to the *D. melanogaster* PIWI that is also implicated in non-cell autonomy in stem cell maintenance, and both encode a RNA-induced silencing complex (RISC) substituent. Suggesting that the role of the RISC complex in generation of small RNAs are very crucial in maintenance of stem cell niches. The function of WUS appears to be limited to the promotion of stem cell pool maintenance, whereas, STM is also active in the stem cell daughters to assist in defining the size of transit amplifying cells' population and maintain embryonic leaf boundaries. Despite their varied roles, both STM and WUS are needed to keep the stem cells undifferentiated.

A rather important question is, if the maintenance of stem cells by STM or WUS involve the control and regulation of different or the same target genes. The repression of differentiation of cells and the maintenance of cell division potential are vital functions of stem cells and comparison of the targets of both factors should show if there is some overlap in the effector genes. STM inhibits activities of promoter factors for differentiation, an example being the Myb factor ASI which is similar to the animal stem cell regulators. This inhibition involves both the STM and the redundant homologs of STM. It is unclear, however, if the regulatory interactions of ASI and STM occur in the STM domain or only at the boundary of transit amplifying cell population. Some evidence has also shown regulation of the cell cycle since both WUS and STM regulate

the *Arabidopsis* response regulators abbreviate as *AAR* genes, involved in response to plant hormone cytokinins. The cytokinins can induce cell division and can hastily control the expression quantities of D cyclins that are involved in the cell cycle progression.

A combination of apical- and radial-basal-pattering input allows for positioning of stem-cell niches in roots. The directional transport of small indolic plant growth regulator, auxin, via polarly localized and placed trans-membrane proteins of the PIN family contribute to auxin accumulation in stem cell niche area. Auxins determine expression of double-AP2-domain transcription factors PLT2 and PLT1, which stands for PLETHORA. These provide transcriptional input for specification of stem cell during and even after embryogenesis (Galinha et. Al., 2007).

In addition, provascular expression of GRAS-family proteins initiates movement of SHORTROOT (SHR) protein to the surrounding cell layer. The regulated protein movement leads to the nuclear activity of the SHR protein and its target, SCARECROW (SCR), single-layered and passes through PLT1 and 2 expression domains. Overlap between highest level of PLT1 and 2 expressions plus the SCR and SHR protein expression domain defines the organizer identity. Also, SCR expression outside of the organizer contributes to size of transit-amplifying cell population in non-cell autonomous manner. The separate functions of PLT genes in the organizer-cell specification, transit-amplifying and stem cell maintenance divisions haven't been reported yet.

Arabidopsis polar auxin transport facilitators PIN2 and PIN1 determine the direction of auxin flow by the polar membrane localization. The SHOOTROOT gene is expressed in central tissues, but the SHR protein is not able to be transported efficiently into nucleus. The SHR protein moves to peripheral cells by PD. After this, it

accumulates in nucleus and activates SCARECROW (SRC). The SHR and SCR in combination with PLT specify stem cell niche at the cellular resolution. The stem cells then produce daughter cells outwards (Paquette et. Al., 2005).

Though PLT2 and PLT1 together with SCR and SHR are all very crucial for stem-cell maintenance, these two-control different enhancer trap markers for organizing cells and loss of each group affects the maintenance of stem cell pool at varying rates. This difference indicates that the SHR-SCR and PLT pathways don't fully cover the same set of target genes. It is going to be informative to compare comprehensively all the targets of four transcription factors in stem-cell niche to gather insights into the underlying mechanisms of the root stem-cell specification.

Within the root apical meristem structure there is a group of four cells termed the quiescent center (QC). The QC controls root stem cell (also referred to as initials) fate of the surrounding stem cells. It is through that the QC cells send out signals to the neighboring stem cells to signal cell division and differentiation. The QC was shown to express WOX5, a WUS homolog, that promotes columella stem cell (CSC) fate. WOX5 expression relies on SCR, which is also expressed in the QC. WOX5 and SCR can act redundantly to maintain stem cell integrity of the cortex initials. REPRESSOR OF WUSCHEL 1 (ROW1) was found to bind to the promoter region of WOX5 to repress its expression. Meanwhile, the CRC secretes peptide CLAVATA3/EMBRYO SURROUNDING REGION 40 (CLE40) to promote CSC differentiation into CC. Overexpression of WOX5 results in accumulation of CSC while overexpression of results in more layers of CC. It was found that secreted CLE40 interacts with ACR4/CLV1 complex to limit WOX5 expression. Both ACR4 and CLV1 localize to PD in the root

apical meristem (Zhang et. Al., 2015). Even though CLE40 moves apoplastically, it is hypothesized that an "unknown stemness" factor is trafficked through PD and interacting with ACR4/CLV1 complexes.

The transcription factor SHR is also expressed in the steele, and symplastically trafficked into the endodermis, endodermis/cortex initials or the QC. In the endodermis, SHR activate miRNA165, which itself is then trafficked into the steele. This movement through PD is regulated by CalS3. miRNA165 suppresses PHB, which is necessary for vascular formation and radial patterning. Indicating PD-dependent movement locally affects developmental decisions. There is still a lot of work to be done in exploring PD's role in stem cell maintenance and cell differentiation.

1.5 Project Hypothesis and Aims

The goals of this project are to analyze the expression patterns of four PDLP family members, *PDLP4*, 6, 7 and 8, and relate their expression pattern to tissue specific control of PD and subsequently their consequence on *Arabidopsis* growth and development. My hypothesis is that the distinct expression patterns of *PDLP4*, 6, 7, and 8 will allow for tissue specific regulation of PD, similarly to PDLP5. My first aim is to determine the spatiotemporal localization of PDLP homologs using the GUS reporter system. My subaim pertaining to aim I is to use the GUS reporter system to deduce responsiveness of the PDLP promoters to Auxin hormone. My second aim is to determine cell specific expression of PDLP homologs using a fluorescent reporter. Lastly, I aim to elucidate the functionality of PDLP homologs using knockout line phenotyping.

Chapter 2

MATERIAL AND METHODS

2.1 Plant Materials

Arabidopsis Columbia ecotype (Col-0) seeds were received from the Blake Meyers lab at the University of Delaware. T-DNA insertion knockout line seeds *pdlp4-1* and *pdlp7-1* were obtained from the Arabidopsis Biological Resource Center (ABRC). The remaining transgenic lines used to study promoter activity and protein localization were made as described on page X using the pMB vector. *Nicotiana Benthamiana* seeds were a gift from Fraunhofer USA Center for Molecular Biotechnology.

2.2 Plant Growth Conditions

Arabidopsis seeds that were sown directly into soil, which was treated with Gnatrol Biological Larvicide (Valent Biosciences Corporation), were grown in a Conviron walk-in growth chamber (GR Series, Controlled Environments Inc.) at 22°C in 60% humidity under 16/8-hour light/dark daily cycle. Seeds that were used for phenotyping, imaging, screening and staining were initially sterilized by washing in 3% bleach for 5 minutes and then rinsed 3 times with autoclaved water before being placed in 4°C for 48 hours. They were subsequently plated on 0.5x Murashige and Skoog (MS) salt in 1% agar plates and grown vertically in a reach-in growth chamber (Percival Scientific) at 22°C under continuous light conditions for a maximum of two weeks.

Benthamiana plants were obtained as seedlings from Xu Wang in the Lee lab at the University of Delaware and grown in Conviron walk-in growth chamber (GR Series, Controlled Environments Inc.) at 22.7°C in 70% humidity under 16/8-hour light/dark daily cycle.

2.3 Image Acquisition

Confocal images of seedlings were taken by Zeiss LSM 710 confocal microscope. Plant samples were placed into NUNC Lab-Tek®II Chamber (#1.5 German Coverglass System, Cat# 154453), covered with coverglass and flattened. The YFP tag was imaged using a C-Apochromat water-immersion 40x/1.2 objective, a 20X objective and a 10X objective with the 514 nm Argon laser, BP 505-550 nm. Transmitted light images were taken using a DIC 40X/1.4 objective water lens, a 20X objective air lens and a 10X objective air lens on either the Zeiss Axioplan 2 or Axiovert 200 microscope systems.

2.3.1 Propidium Iodide Staining

1 mg/mL solution of propidium iodide was diluted by adding 100 µl of PI stock solution into 900 µl of water. Seedlings were then submerged in the diluted solution for 10 minutes in the dark. For immediate imaging, no was necessary.

2.4 Establishing Transgenic Lines

2.4.1 List of Transgenic Lines

Table 2.1 List of transgenic lines and their backgrounds

Construct	Background
pMB:PDLP4pro:er-YFP	WT
pMB:PDLP6pro:er-YFP	WT
pMB:PDLP7pro:er-YFP	WT
pMB:PDLP8pro:er-YFP	WT
pMB:PDLP4pro:GUS	WT
pMB:PDLP6pro:GUS	WT
pMB:PDLP7pro:GUS	WT
pMB:PDLP8pro:GUS	WT

2.4.2 Construct Design and DNA Cloning

In creating the YFP reporter lines, the promoters for *PDLP4*,6,7 and 8 were amplified from the Wildtype *Arabidopsis* Col-0 ecotype genomic DNA, each with a forward and reverse primer as listen in Table 2. The promoters were then sublconed into an existing plasmid, pMB35S-ER-cYFP (shown below) using the AscI and NotI restriction enzyme sites. In creating the GUS reporter lines, the GUS gene was subcloned into the YFP clones for each promoter via the XhoI and XbaI restriction sites.

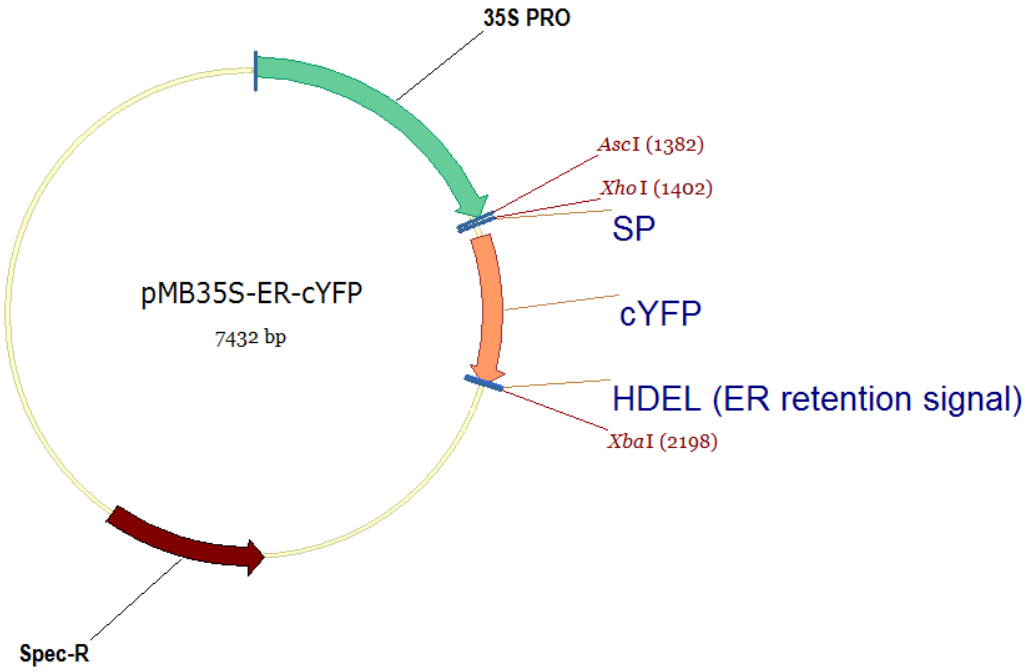

Figure 2.1 The pMB35S-ER-cYFP plasmid map representing the location of the 35S promoter sequence, signal peptide sequence, citrin YFP sequence, the ER retention signal sequence and the AscI, XhoI, XbaI and NotI restriction enzyme sites.

Table 2.2 Primers for promoter cloning

Name	Sequence
PDLP4pro_Fw	GAA GAT CTG CGG CCG CGA TTA TAC GAT CAG TTG TTG AGA GGA
PDLP4pro_Rev	CCG CTC GAG GCG CGC CAG TCA AAA CTA CGA GGG AAG AGA G
PDLP6pro_Fw_BgN	GAA GAT CTG CGG CCG CGC TAT CAC AAA AAG ATT TTG TAC G
PDLP6pro_Rev_AsX	CCG CTC GAG GCG CGC CGA CTT TCG ACG ATT GCT TCT TTT TG
PDLP7pro_Fw_BgN	GAA GAT CTG CGG CCG CTA GTG TAG AAT TCG CCA CTG AAG C
PDLP7pro_Rev_AsX	CCG CTC GAG GCG CGC CTA GTG GAG AAG GAG AGA AAG ACA ATA GC
PDLP8pro_Fw_BgN	GAA GAT CTG CGG CCG CAT CTC TTA TGA TCA ATC TAA TTA GC
PDLP8pro_Rev_AsX	CCG CTC GAG GCG CGC CAT TTT CTT GTT CTT TGA GAT TGT TTT CAA AGG

2.4.2.1 Plant Genomic DNA Extraction

Wildtype *Arabidopsis* Col-0 ecotype genomic DNA was used as template for amplifying the promoter regions of each gene. Genomic DNA was extracted from 1 week old leaves. Tissue was frozen in liquid nitrogen and then ground in 1.5mL microcentrifuge tubes with pestles. Then 400µL of extraction buffer containing 200mM Tris-HCl, pH=8.0, 250mM NaCl, 25mM EDTA and 0.5% SDS was added and tubes were put in a 65℃-water bath for 15 minutes. 155 µl of alkaline lysis solution II containing 5M Potassium Acetate, glacial acetic acid and nano-pure water were mixed in. The homogenate was then centrifuged for 5min at 16,000×g and the supernatant was transferred into new tubes. 500µl of 100% isopropanol was added and the tube was inverted gently several times to mix, followed by incubation at room temperature for 10

min. The mixture was then centrifuged at 16,000×g for 10min. After discarding the supernatant, the pellet was washed with 70% ethanol before being dried in a fume hood for 10min. The pellet was then resuspended in 50μL of 10mM Tris-HCl, pH=8.0.

2.4.3 Bacterial Cell Culture, Transformation and Plasmid Extraction

Bacteria was cultured in LB media containing 1% tryptone, 0.5% yeast extract, 1% NaCl, pH=7 and shaken at 200-250 rpm, or on LB plates containing the same ingredient as the media and 1.5% agar. When concentrations of corresponding antibiotics were as follows, the concentrations were: 100 μg/mL spectinomycin, 50 μg/mL gentamycin and 50 μg/mL rifampicin. *Escherichia coli* DH10 strain was cultured at 37°C, and *Agrobacterium tumefaciems* GV3101 strain (+pSOUP) was cultured at 28°C. For electroporation, 10-30 ng of plasmid was added into 40 μL electro competent cells. The mixture was electro-shocked at 1.8 kV and 200 μL SOC media composed of 20g/L Tryptone, 5 g/L Yeast Extract, 4.8 g/L $MgSO_4$, 3.603 g/L dextrose, 0.5g/L NaCl 0.186 g/L KCl was added immediately. The *Agrobacteria* were recovered at 28°C by shaking for 2 hours. All of the culture was spread using glass beads on LB plate and incubated at 28°C for two days. The DH10 were recovered at 37°C by shaking for 1 hour then spread on LB plates and incubated at 28°C for two days. For plasmid extraction, transformed DH10 was cultured in LB broth overnight and 1 mL culture was centrifuged at 16000×g for 1min. The bacterial pellet was resuspended in 100 μL ice cold resuspension solution containing 50 mM glucose, 25 mM Tris base, 10 mM EDTA and 0.1 mg/mL RNaseA. Bacteria were lysed in 200 μL solution containing 0.2 M NaOH and 1% SDS, and neutralized by 150 μL solution containing 3 M KAc and 5 M HAc. The lysate was centrifuged at 16000×g for 10 min, and 400 μL supernatant was mixed

with 1 mL ice-cold 100% ethanol and then stored at -20°C for at least 2 hours before being centrifuged at 16000×g for 10 min. Pellet was washed with 70% ethanol and dried in hood, and 50 µL 10 mM Tris buffer (pH=8) was used to dissolve the DNA.

2.4.4 Tobacco Infiltration

Nicotiana Benthamiana was infiltrated with agrobacteria for transient gene expression to ensure the target genes/fusions were being properly expressed. *Benthamiana* were grown to 3 weeks old under conditions described in Chapter 2.2. A single colony of *Agrobacterium* containing the target plasmid was inoculated in 2 mL LB media with appropriate antibiotics and grown overnight at 28°C. A*grobacteria* was spun down and re-suspended to OD600=0.4 in resuspension solution consisting of 1 mL 2-(*N*-morpholino) ethanesulfonic acid (MES) buffer pH 5.76, 1 mL 0.5M MgCl, 50 µl acetosyringone and 47.95 mL H2O. The infiltration was performed with a 5 ml syringe (loaded with resuspension buffer and *agrobacteria*) by simply pressing the syringe (no needle- on the underside of the 4^{th} and 5^{th} true leaves) and exerting a counter-pressure with a finger on the other side. Once a spreading "wet" area in the leaf was observed, plants were covered under a dome for recovery overnight. Gene expression via either GUS staining or fluorescence depending on the inserted plasmid was then observed 48 hours post infiltration.

2.4.5 Floral Dipping: *Agrobacteria* Transformation of *Arabidopsis thaliana*

Arabidopsis were transformed using *agrobacteria* via floral dipping as described in Clough and Bent, 1998. Plants used in transformation were grown until they bolted (~3 weeks) and then the primary shoot was cut off to allow for enhanced growth of multiple secondary bolts. Once several side shoots have flowered, plants are ready to be

dipped. You must prepare the *agrobacteria* that contains your gene of interest by growing a 200mL culture in LB broth at 28℃ with the appropriate antibiotics. Spin down the culture and resuspend to OD600=0.8 in 5% sucrose solution (no less than 200mL). Then Silwet L-77 to a concentration of 0.01% was added and mixed well. The above shoots of each plant were then dipped in the *agrobacteria*/sucrose/silwet solution for 30 seconds each. Each plant was then laid on its side and covered under a dome for 24 hours to maintain humidity, hide from light and recover. After the plants had senesced, seeds were collected (~8-10 weeks). Transformed seeds were then picked using plates with Basta or Kanamycin as needed.

2.5 GUS Staining

The β-glucuronidase (GUS) histochemical staining assay was preformed per Weigel and Glazebrook (2002). Staining solution consisted of 100mM NaPO4, pH=7, 10mM EDTA, 500 mM potassium ferricyanide, 500 mM potassium ferrocyanide, 1 mM 5-Bromo-4-chloro-3-indoxyl-beta-D-glucuronide cyclohexylammonium salt (X-Gluc, Gold Bio, Cat# G1281C1), 0.1% Triton X-100. Plant tissues were submerged in this GUS solution and vacuum infiltrated using a house vacuum for 5 minutes at room temperature and then incubated at 37℃ for 1 hour. Post staining, tissues were cleared of chlorophyll and fixed by submerging them in 90% acetone at -20℃ for 24 hours. Images of plant tissue were taken using a Zeiss AxioCam camera and processed via the Zeiss AxioVision software.

2.6 Hormone Treatment of Seedlings

Sterilized seeds were plated 0.5x Murashige and Skoog (MS) salt in 1% agar plates and grown vertically in a reach-in growth chamber (Percival Scientific) at 22°C

under continuous light conditions for 5 days. They were then transferred on to 0.5X MS salt 1% agar 100µM 1-Naphthaleneacetic acid (NAA) plates for 12 hours or MS salt 1% agar 100µM salicylic acid (SA) plates. Seedlings were then stained with GUS and imaged. Images of plant tissue were taken using a Zeiss AxioCam camera and processed via the Zeiss AxioVision software.

2.7 Gravitropic Studies

Gravitropic studies done to phenotype the knockout lines were conducted as described in Peret et. Al., 2012. Initially you grow seedlings vertically on plates to 4 days old, under conditions described in Chapter 2.2. At that point, the plates were rotated 90 degrees and grown vertically for an additional 48 hours. Seedlings were then submerged in 70% ethanol for clearing chlorophyll and to halt subsequent cell divisions. Lateral root progression was then quantified at the root bend in groups by stage of LRP. Data was recorded and analyzed.

Chapter 3

RESULTS

3.1 PDLP Family Members PDLP4,6,7 and 8 Have Distinct Expression Domains

I employed two approaches to analyze the expression patterns of the four PDLP family members in this study. One approach was using the GUS reporter system to determine the spatiotemporal expression pattern of each member. Promoter regions were identified by amplifying the region upstream of the target gene's start codon right up until stop codon of the previous gene. Target gene's promoters drove the expression of GUS, a cell autonomous enzyme that is only very rarely expressed in plants. Cleavage of 5-bromo-4-chloro-3-indolyl β-D-glucuronic acid (X-gluc), a β(1,3) glucan, catalyzed by GUS results in primary product, 5-bromo-chloro-3-inodyl, which is colorless and soluble. This primary product is then oxidized and dimerized, as part of the visualization process of the histochemical assay, to form the bright blue insoluble final product (indigo). The second approach was to analyze the expression patterns at the cellular resolution using a citrin-YFP transcriptional fusion that contained an ER retention signal. These results are summarized in Figure 3.1. *PDLP4* promoter activity was seen in the primary and secondary roots and their LRCs. *PDLP6* promoter activity was seen in the SAM and transient activity in the primary root and its tip. *PDLP7* promoter activity was seen in the cotyledons, true leaves primary root and its CRC. *PDLP8* promoter activity was seen in the cotyledons and primary root. The siliques and flowers at different stages for all the GUS lines (*PDLP4pro:GUS, PDLP6pro:GUS, PDLP7pro:GUS* and *PDLP8pro:GUS*) were also vacuum infiltrated. However, no staining occurred indicating the promoter was not active in these regions.

	Cotyledons	Hypocotyl	Primary root	Root cap	Lateral root/ LRP	Leaves	Stem	Flower	Siliques	SAM	Vasculature
PDLP4			✓	✓	✓						
PDLP6			✓	✓						✓	
PDLP7	✓	✓	✓	✓	✓	✓					✓
PDLP8	✓	✓	✓			✓					✓

Figure 3.1 Schematic overview of *PDLP4*,6,7 and 8 promoter expressions in different organs found throughout the *Arabidopsis* life cycle.

3.1.1 PDLP4 is Expressed in the Endodermis, Lateral Root Cap and Endodermal Cells Overlying Lateral Root Primordia

Figure 3.2 shows the results of GUS staining in transgenic lines expressing GUS under the *PDLP4* promoter. An image of the entire 5-day-old seedling (Figure 3.2A) revealed consistent staining in the upper half of the root and sporadic staining in the lower half of the root. Figure 3.2B shows staining begins in the root/hypocotyl junction but is omitted from the hypocotyl. Figure 3.2C suggests a pattern consistent with endodermal staining in the upper root and in endodermal lateral root primordia (LRP)-overlying cells in the lower root (Figure 3.3D).

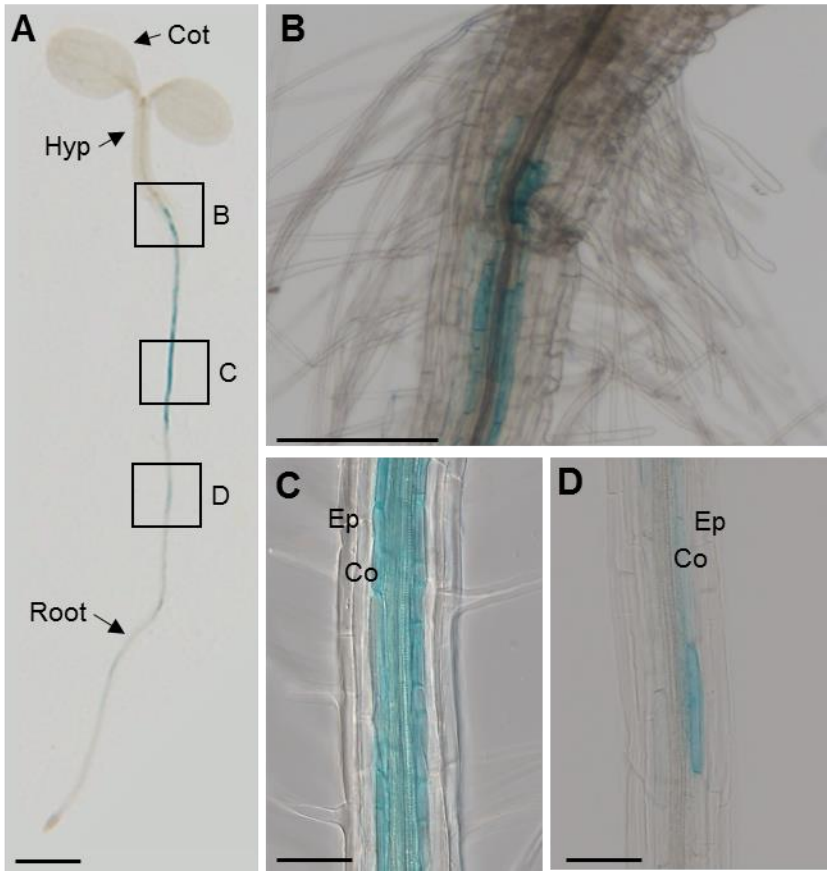

Figure 3.2 *PDLP4pro:GUS* expression pattern of a 5-day-old seedling, shown via GUS stain. (A) Lower magnification image showing whole seedling. Cot; Cotyledon, Hyp; Hypocotyl. Size bar, 2mm. (B) *PDLP4* expression pattern at the hypocotyl/root junction. Size bar, 100µm. (C, D) Endodermal expression pattern of *PDLP4pro:GUS* in the primary root. Ep, epidermis, Co; cortex. Size bars, 50µm.

Examination of transgenic seedlings expressing the erYFP protein under the *PDLP4* promoter confirm that the promoter is active in the endodermal layer (Figure 3.3A, 3.3A'). Furthermore, less intense signal was also seen in the LRC cells of the primary root (Figure 3.3B). Notably, *PDLP4pro:erYFP* seedlings revealed that the sporadic expression patterns seen in the *PDLP4pro:GUS* seedlings were in fact endodermal lateral root primordia overlying cells that overlay early stage LRP (Figure 3.3C).

Early stage LRP can be defined as stages I-III. To determine whether the *PDLP4* expression pattern was also seen in lateral roots, I examined the lateral roots of 8-day-old seedlings. Figures 3.3E-E'' shows that endodermal and LRC expression was in fact present in mature lateral roots. But omitted from recently emerged lateral roots smaller than 1 mm in size (Figure 3.3D).

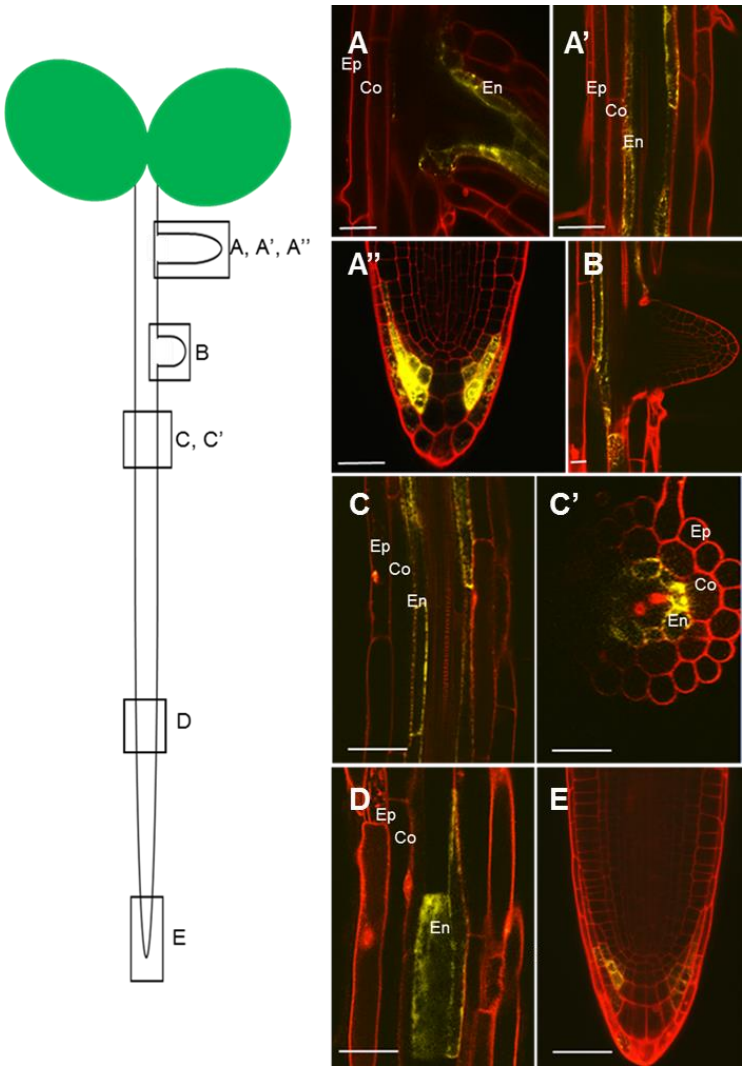

Figure 3.3 *PDLP4pro:erYFP* expression pattern of an 8-day-old seedling, shown via erYFP.. (A) Primary root, medial longitudinal section. (A') Primary root, transverse section. (B) Primary root tip. (C) Early stage lateral root primordia. (D) Recently emerged lateral root. (E, E', E'') Emerged and matured lateral root and lateral root tip. Ep; epidermis, Co; cortex, En; endodermis. White arrowheads; lateral root cap. Red; PI stain. Yellow; YFP expression. Size bars, 50µm

3.1.2 PDLP6 is Expressed in the Shoot Apical Meristem and Sporadically throughout the Root.

GUS staining assays preformed on seedlings expressing GUS under the *PDLP6* promoter revealed consistent expression in the SAM Figure 3.4B) and some inconsistent staining patterns seen in the CRC cells and the vasculature (Figure 3.4C, D). The

reporter line expressing erYFP under the *PDLP6* promoter, revealed transient expression in the roots that was rapidly disappearing. Without consistent images from *PDLP6pro:erYFP* lines, I was unable to determine the cellular expression of *PDLP6* in the roots. The SAM is hidden under several layers of cells and thus difficult to capture under a microscope.

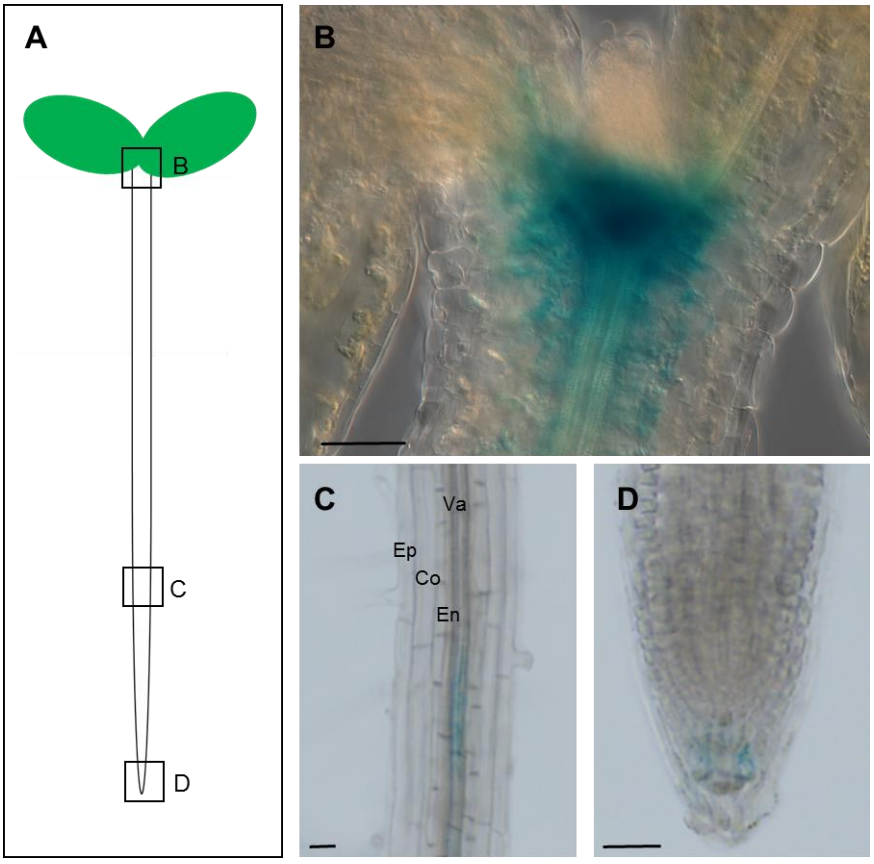

Figure 3.4 *PDLP6pro:GUS* expression pattern of a 5-day-old seedling, shown via GUS stain. (A) Schematic diagram of whole seedling. (B) Expression in the shoot apical meristem. (C) *PDLP6* expression pattern in the lower primary root. (D) Expression in the primary root tip. Ep; epidermis, Co; cortex, En; endodermis, Va; vasculature. Size bars, 50µm

3.1.3 PDLP7 is Expressed in the Vasculature of the Root, Cotyledon, Young True Leaves and the Columella Root Cap.

Staining of the *PDLP7pro:GUS* lines indicate expression in the vasculature of the root, cotyledons, young true leaves and the CRC, consistently (Figure 3.5). Figure 3.5B highlights vascular expression in the hypocotyl and newly forming true leaves. GUS assays conducted on older leaves (8 days and older) resulted in no staining, indicating the promoter is not active in mature leaves. Root images taken with a higher objective lens exposed vascular staining was excluded from the xylem and instead resembled phloem-specific staining (Figure 3.5C).

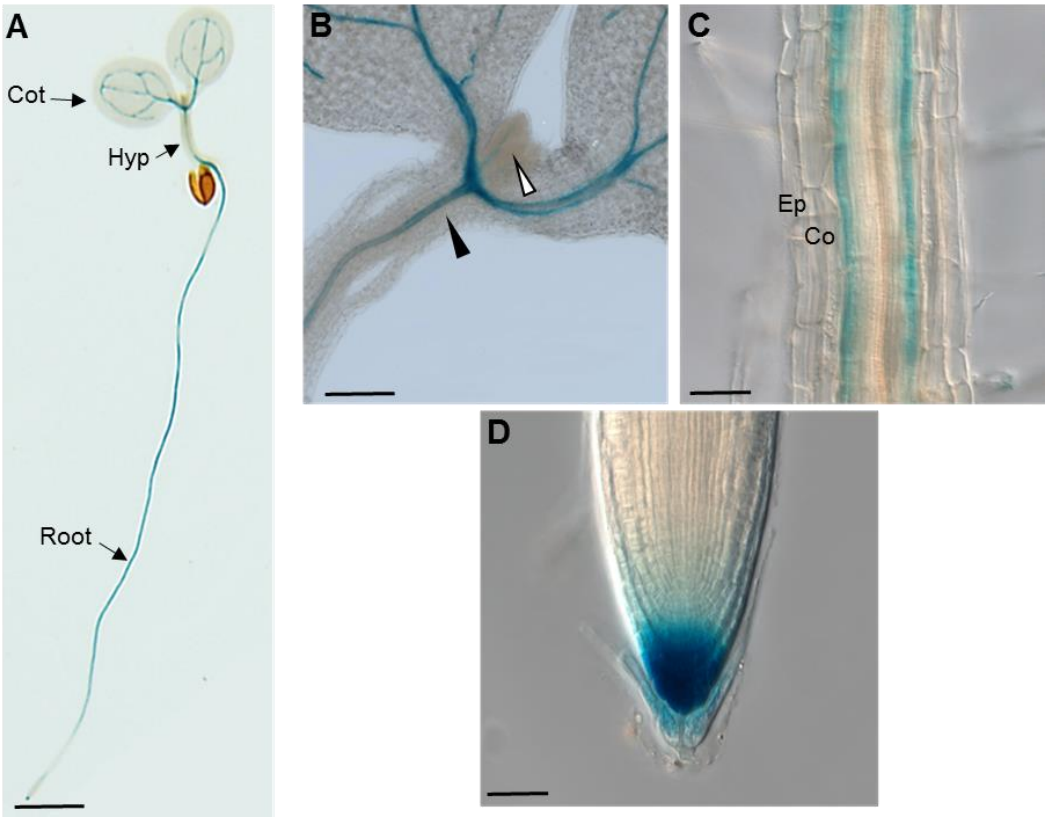

Figure 3.5 *PDLP7pro:GUS* expression pattern of a 5-day-old seedling, shown via GUS stain. (A) Lower magnification image showing whole seedling. Cot; cotyledon, Hyp; hypocotyl. Size bar, 3mm. (B) *PDLP7* expression pattern in the hypocotyl and newly developing true leaves. White arrowhead; true leaves. Black arrowhead; hypocotyl. Size bar, 200µm. (C) Expression in the primary root. (D) Expression in the primary root tip. Size bars, 50µm.

PDLP7pro:erYFP reporter lines confirmed that expression was in fact in the CRC and what seemed to be the phloem-pole pericycle (Figures 3.6B and 3.6D). A clearer image revealed YFP expression not only in the phloem-pole pericycle but the phloem companion cells as well (Figure 3.6C).

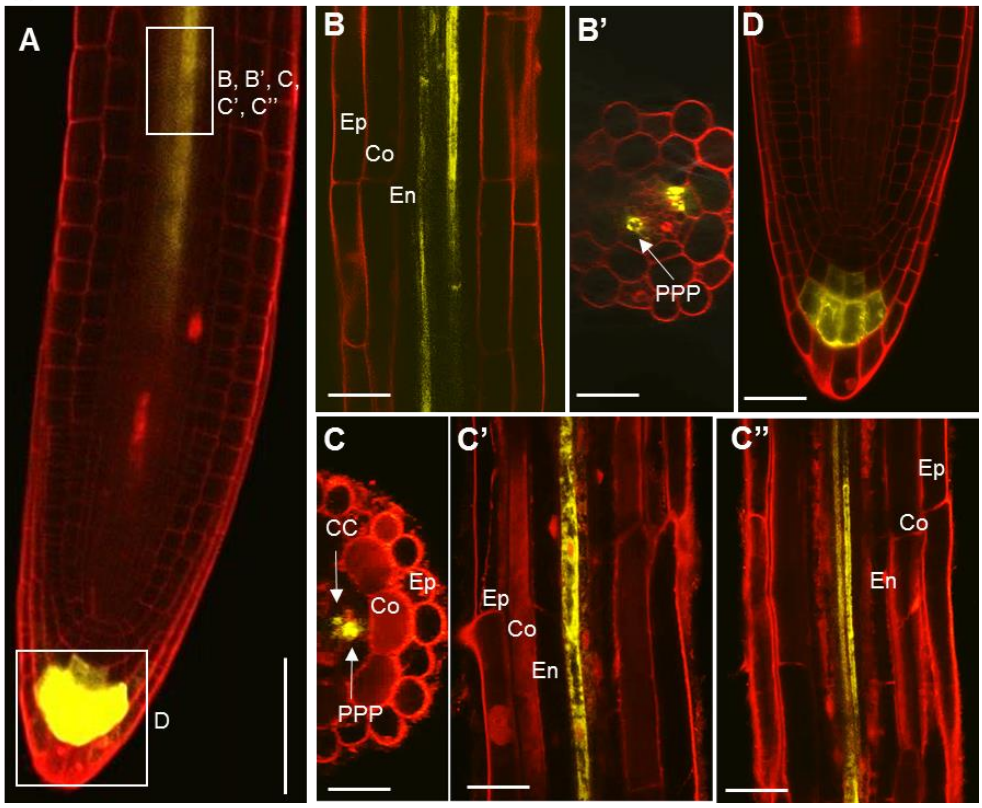

Figure 3.6 *PDLP7* expression pattern of a 5-old-seedling, shown via erYFP. (A) Primary root, medial longitudinal section. Size bar, 200 µm. (B) Primary root, medial longitudinal section. (B') Primary root. Transverse section. Medial longitudinal section. (C) Primary root. Transverse section. (C', C'') Primary root. Medial longitudinal section. Two different planes. (D) Primary root cap. Ep; epidermis, Co, cortex, En; endodermis. CC; companion cells. PPP; phloem pole pericycle. Red; PI stain. Yellow; YFP expression. Size bars, 50µm.

3.1.4 PDLP8's is Expressed in the Vasculature of the Cotyledons and the Root.

PDLP8pro:GUS lines resulted in no staining at all. However, the *PDLP8pro:erYFP* line resulted in YFP expression patterns similar to *PDLP7pro:erYFP*.

Figure 3.7A revealed vascular staining in the cotyledon, possibly companion cells, and Figure 3.7B very clearly shows YFP expression in the phloem-pole pericycle.

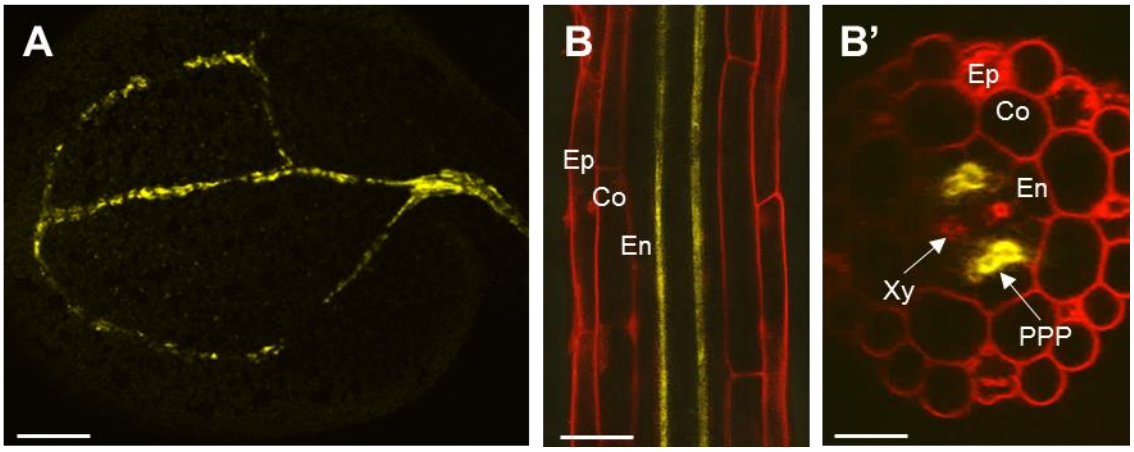

Figure 3.7 *PDLP8* expression in 5 -old-seedling, shown via erYFP. (A) Cotyledon. Size bar, 200µm. (B) Primary root. Medial longitudinal section. (B') Primary root. Transverse section. Ep; epidermis, Co; cortex, En; endodermis, Xy; xylem, PPP; phloem pole pericycle. Red; PI stain. Yellow; YFP expression. Size bars, 50µm.

3.2 PDLP4 and PDLP7 Promoter Activity is Enhanced by Exogenous Auxin Treatment.

To determine whether any of the promoters were responsive to auxin, I treated each working GUS lines with auxin (NAA) for 12 hours and then performed the GUS staining assay described in chapter 2.5. Immediately after vacuum infiltrating staining could be seen in the roots of the *PDLP4pro:GUS* and *PDLP7pro:GUS* lines. However, the same protocol for staining was to be used, so the seedlings were incubated at 37℃ for 1 hour. For *PDLP4pro:GUS* lines, staining was darkest in the endodermal LRP overlying cells that overlay early stage LRP. Staining in overlying cells of late stage LRP was not as dark, indicating a lower level of expression and *PDLP7pro:GUS* lines reacted in a similar way. The *PDLP7pro:GUS* lines stained very darkly and all throughout the root vasculature and increased staining was seen in the LRC along with the CRC (Figure

3.8).

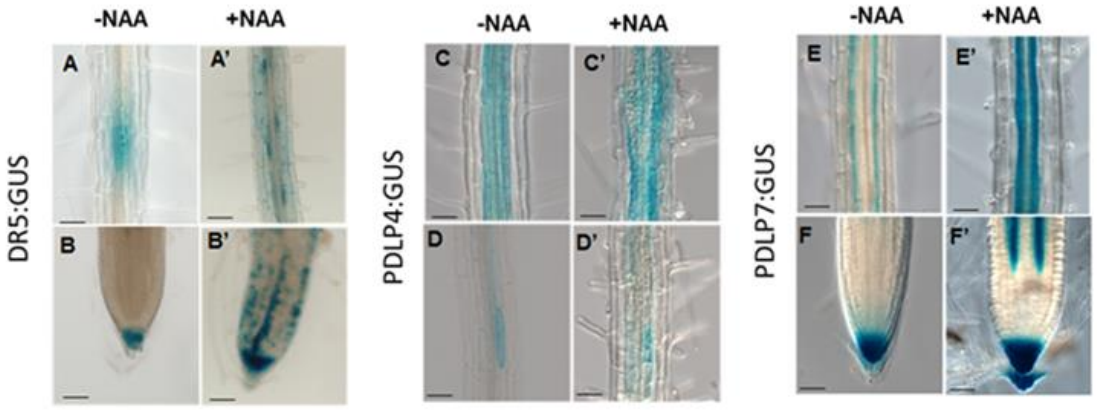

Figure 3.8 *PDLP4* and *PDLP7* propagation via auxin. (A) DR5 expression in primary root. (B) DR5 expression in primary root cap. (A') DR5 expression in primary root post auxin treatment. (B') DR5 expression in primary root tip post auxin. (C, D) *PDLP4* expression in primary root. (C', D') *PDLP4* expression in primary root post auxin treatment. (E) *PDLP7* expression in primary root. (F) *PDLP7* expression in primary root tip. (E') *PDLP7* expression in primary root post auxin treatment. (F') *PDLP7* expression in primary root tip post auxin treatment. Size bars, 50µm.

3.3 PDLP4 and PDLP7 Knock Out Phenotypes Show Altered Root Length, Lateral Root Emergence and Altered Root Tip Morphology

To study the possible functionality of the *PDLP4* and *PDLP7* proteins I employed different quantifiable measurements. A study of the primary root lengths at 5 days old, indicated that *pdlp7-1* lines were significantly longer on average (Figure 3.9A, B). Figure 3.9A', B' specified that the primary roots of 14-day-old *pdlp7-1* knockouts were significantly longer than that of wildtype plants. The primary roots of 14-day-old *pdlp4-1* knock outlines were also shown to be significantly longer in comparison to wildtype. No significant variance was found when comparing the two knockout lines to each other. While looking for primary root phenotypes, I also noticed that *pdlp7-1*knock outlines possessed an increased number of lateral roots in comparison to WT and *pdlp4-1*

knockout lines. Therefore, a gravitropic study (as described in Chapter 2.7) was conducted to give insight to the progression of lateral root emergence. Lateral root progression was quantified by analyzing the stage of the root bend LRP. I found that lateral roots emerged faster in *pdlp7-1* lines in comparison to WT seedlings. *pdlp4-1* lateral roots emerged significantly slower than WT (Figure 3.10).

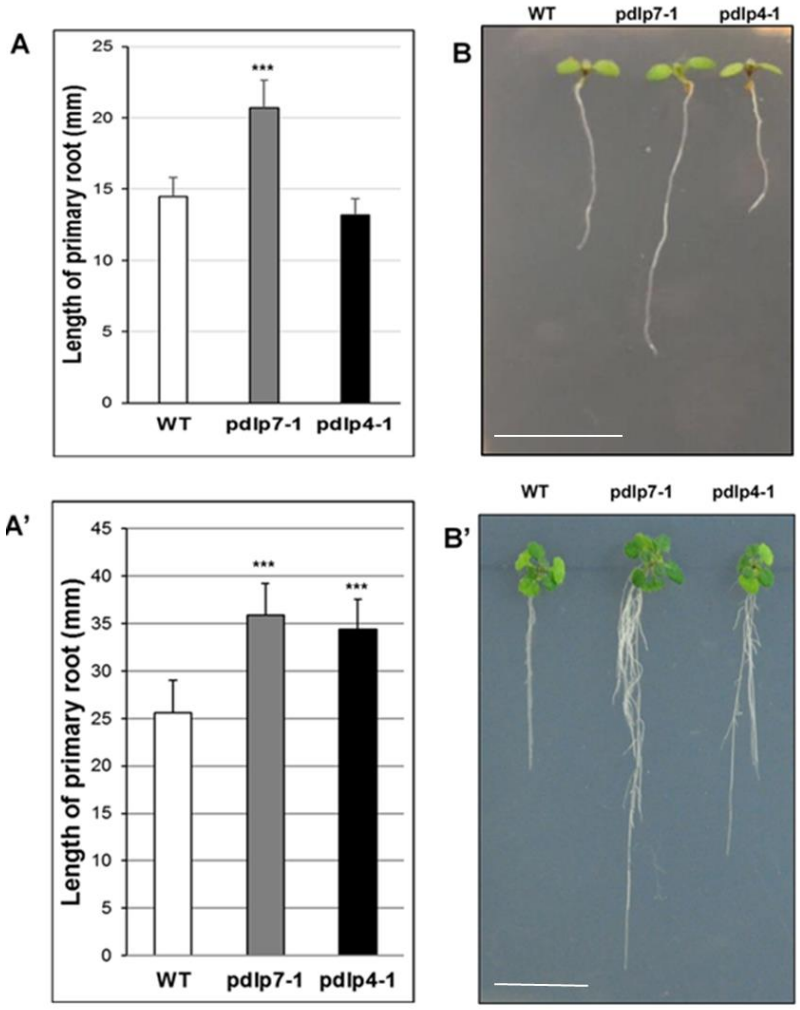

Figure 3.9 PDLP members regulate root elongation. (A) Mean length (±s.e.,; n=60) of 5-day-old seedling roots of *pdlp7-1*, *pdlp4-1* and wild-type (Col-0) plants. (B) Representative 5-day-old seedling roots of selected genotypes. Mean lengths were determined (±s.e.,; n=60): *pdlp7-1*, 20.72 ± 1.91 mm; *pdlp4-1*, 13.18 ± 1.123.141 mm; WT, 14.45 ± 1.37 mm. (A') Mean length (±s.e.,; n=60) of 14-day-old seedling roots of *pdlp7-1*, *pdlp4-1* and wild-type (Col-0) plants. (B') Representative 14-day-old seedling roots of selected genotypes. Mean lengths were determined (±s.e.,; n=60): *pdlp7-1*, 35.875 ± 3.324 mm; *pdlp4-1*, 34.438 ± 3.141 mm; WT, 25.625 ± 3.442 mm. Size bars, 10 mm.

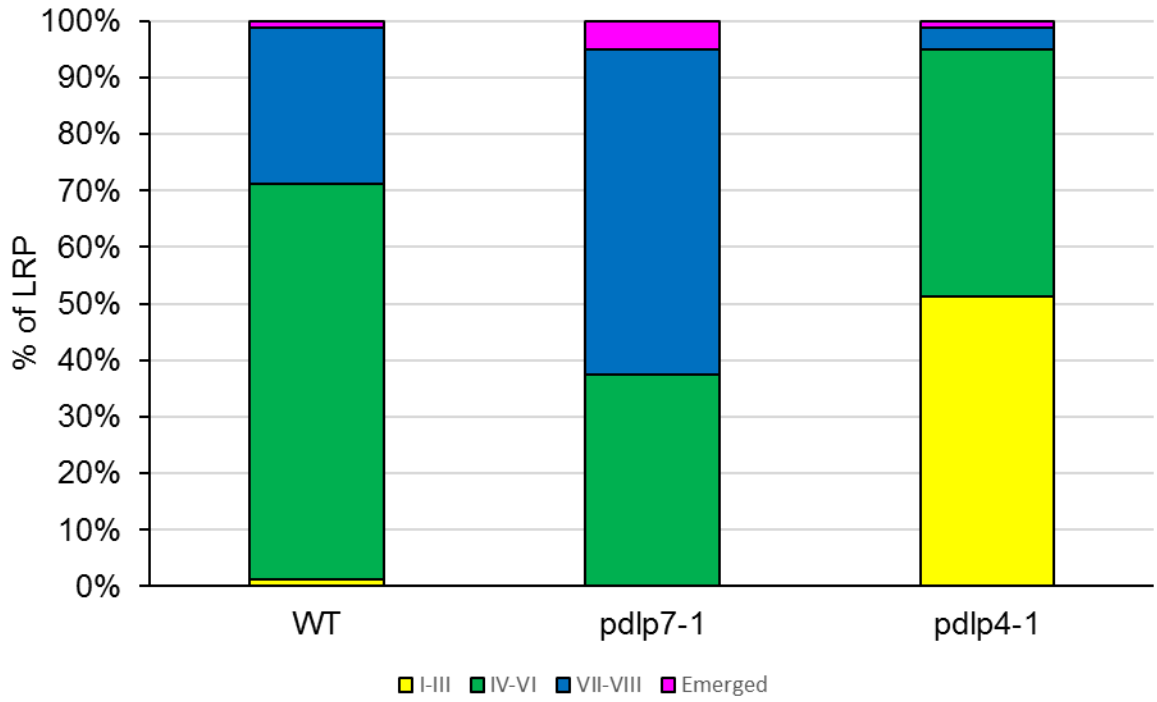

Figure 3.10 Graph represents the progression of lateral root primordia in the *pdlp7-1* and *pdlp4-1* knock out lines in comparison to wild-type plants. n=80 seedlings.

DIC images of ethanol cleared and confocal images of propidium iodide stained knockout lines revealed a morphological phenotype seen in the root tip of knockout lines (Figure 3.11). The *pdlp4-1* knockout line displayed thickened root tips, altered morphology root cap cells that were not sloughing off. The *pdlp7-1* knockout line presented with CRC cells that were not detaching from another. Both lines, presented with altered root tip cell morphology, as seen by the difference in sizes.

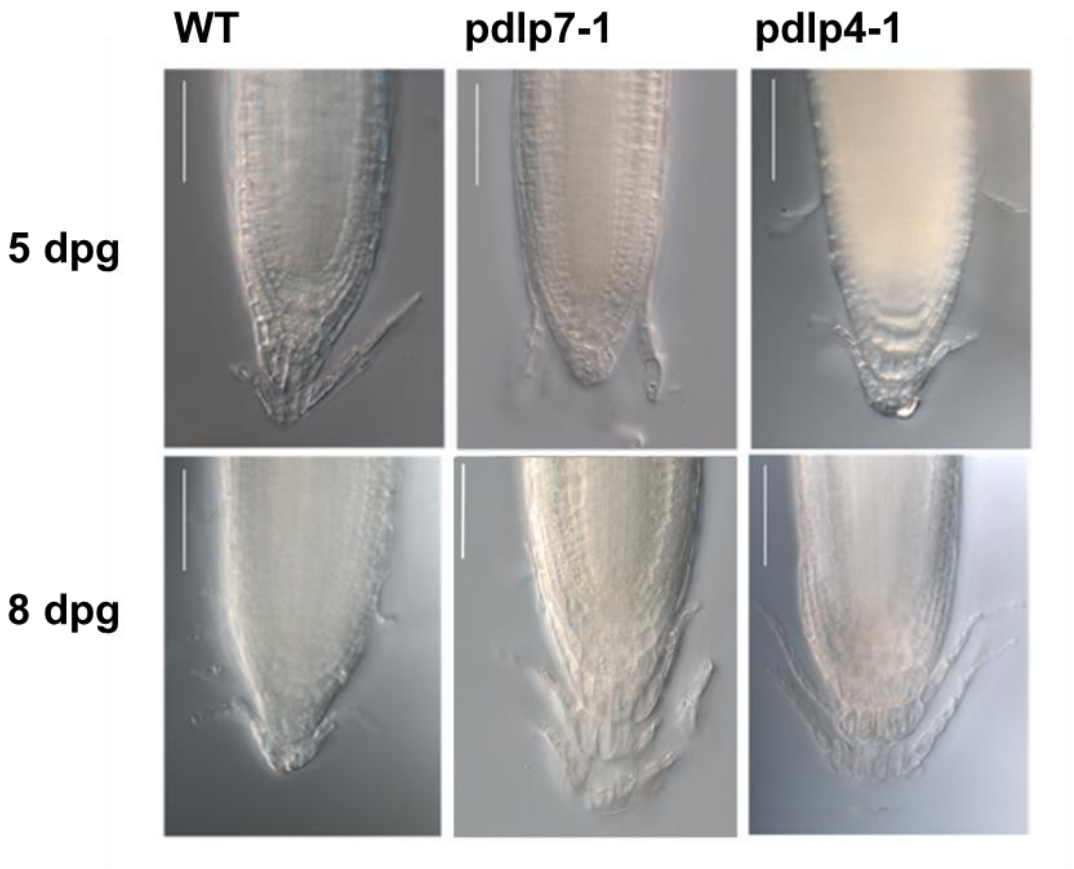

Figure 3.11 *pdlp7-1* and *pdlp4-1* knockout lines have defects in root cap maturation. WT, *pdlp7-1* and *pdlp4-1* were imaged at 5 and 8 dpg. Medial longitudinal sections. Size bars, 50 µm.

Chapter 4

DISCUSSION

4.1 Employing PDLP4 to Study Endodermal Function

PDLP4pro:GUS lines indicated that the promoter was active in the upper half of the root, excluding the hypocotyl, consistently and sporadically in the lower root, in 5-day-old seedlings (Figure 3.2). Examination of the *PDLP4pro:erYFP* lines revealed that the root expression was limited to the endodermal layer, which is specific to the root and omitted from the hypocotyl. Furthermore, promoter activity of *PDLP4* was also seen in the LRC cells via the YFP reporter. Confocal imaging of the lateral roots exhibited the same endodermal and LRC pattern, as observed in primary roots.

It is notable that expression is confined to the LRC and not the entire root cap. The LRC is made up of a group of cells with high turnover rate, as new cells are formed, old ones are sloughed off. LRC also has significant functionality in root gravitropism. Upon examining older seedlings, I determined that promoter activity was seen only in mature endodermal cells that were most likely surrounded by a suberin cell wall modification known as the casparian strip. The casparian strip formation occurs after the endodermal cells have fully differentiated. Thus, only the top half of the root is stained (Figure 3.2). The casparian strip modification makes the endodermal layer inaccessible apoplastically and is involved in water uptake, ion selectivity and plant homeostasis. Water moving through the xylem, which is actually part of the apoplastic pathway, can thereby be selectively regulated since it has no choice other than to enter the symplast in the endodermis via PD. It is possible that *PDLP4*, like its homolog: PDLP5, is regulating PD aperture in the mature endodermal layer and possibly implicated in water uptake and

retention. The sporadic staining pattern seen in lower roots in the GUS lines were also seen in the YFP lines. Confocal imaging along with histochemical staining confirmed that this sporadic expression was confined to the endodermal layer of early stage LRP overlying cells (Figures 3.8). Late stage (IV-IIIV) LRP did not present with endodermal overlying cell staining in the lower roots. In contrast, current research in our lab has shown that PDLP5 expression is seen in all layers of LRP overlying cells: endodermal, cortical and epidermal. LRP overlying cells are involved in the auxin transport/gradient that is necessary for lateral root formation and emergence. Collectively, these findings led us to question whether auxin had any effect on *PDLP4* expression and whether *PDLP4* is involved in lateral root emergence. This was addressed by treating the *PDLP4pro:GUS* and *PDLP7pro:GUS* lines with NAA. In response to NAA treatment, *PDLP4* and *PDLP7* promoter activity was enhanced.

4.2 PDLP6: A Model for Studying Symplastic Communication in the Shoot Apical Meristem

PDLP6 expression was always seen consistently in the SAM via the GUS staining assay. Staining of the root, however, resulted in inconsistent sporadic staining. Several seedlings of the same line gave different histochemical results. To image the SAM of *PDLP6pro:erYFP* plants, which is buried deep inside several layers of tissue, one would have to dissect apart the shoot tip. It was technically difficult to observe the *PDLP6pro:erYFP* lines because YFP signal in the root tip was rapidly appearing and disappearing or often too faint to image. This highly suggests that *PDLP6* promoter activity throughout the root is transiently controlled.

PDLP2 and PDLP3, also expressed in the SAM, indicate a role for defined symplastic domains in shoot development (Bayer et.al., 2008). Expression of *PDLP6* in the SAM then leads one to ask, is symplastic trafficking is necessary for the function of the SAM. The SAM stem cells give rise to all cell types in the shoot such as flowers, siliques and even leaves. Proper shoot development is necessary for reproduction and consequently species survival. One study shows that PD frequency in the SAM increases while the plant is flowering, indicating a presumable role of PD in SAM function (Ormanese et. al., 2000). A transcription factor known as KNOTTED1 (KN1) is known to regulate PD aperture in the SAM. mRNA of KN1 is synthesized in the L2 layer of the SAM, but the protein is found to function in the L1 layer. KN1 was found to change PD SEL to regulate its own movement through the cell layers (Lucas et. Al., 1995). Another example of PD involvement in stem cell niches is the WUS transcription factor. WUS is transcribed in the organizing center of the SAM and is transported, unknowingly, to the central zone cells where it activates CLV3 to maintain stem cell integrity and keep the cells from differentiating (Yadav et. Al. 2011). Interestingly, CLV3 a membrane bound receptor-like protein is found to sub-localize in the cell membrane at PD. This suggests that WUS may be trafficked symplastically via PD. What guides the WUS transcription factor through the layers of the SAM remains unknown. It is hypothesized that WUS movement, regulated by PD aperture and selectivity, can lead to the formation of symplastic subdomains within the SAM, which are most likely necessary to regulate stem cells. In the future expressing free GFP under the *PDLP6* promoter in WT and *PDLP6* KO backgrounds will give insight to *PDLP6*'s ability to alter PD aperture in the SAM. If *PDLP6* is in fact able to close PD in its expression domain, like its known counterpart

PDLP5, then the movement of WUS in the SAM through PD can be studied using a GFP tagged WUS fusion (as done in Yadaav et. Al 2011) expressed in WT and *pdlp6-1* KO backgrounds. Finally putting an end to the question of whether WUS is transported through PD or not.

4.3 PDLP7 and PDLP8 in Floral Transitioning

Our studies revealed that *PDLP7* and *PDLP8* promoter activity was seen in phloem pole pericycle and companion cell domains. The phloem is made up of companion cells and sieve elements, amongst other types of cells. The phloem is a vascular tissue that transports sugars, proteins and mRNA throughout the plant. The sieve element cells are responsible for transport of sugars, specifically. However, sieve element cells rely heavily on their companion cell counter parts because mature sieve element cells lack a nucleus and other organelles. Interestingly enough, sieve element cells are connected to their companion cell counterparts via PD. Sieve areas are large pores made up of modified and enlarged PD. Companion cells are often referred to as the "life support" for the sieve element, without which the phloem would not properly function. Companion cells provide ATP and nutrients and carry out all the cellular functions for the sieve element cells. Like *PDLP7pro:erYFP*, *PDLP8pro:erYFP* expression was observed in the phloem pole pericycle (Figure 3.7). However, it is probably in the companion cells as well, because the expression is continuous in the cotyledons, cotyledons do not have pericycle cells. The *PDLP8pro:GUS* reporter lines resulted in seedlings that produced no stain post incubation with X-Gluc. I was unable to determine in which tissues the promoter was active via GUS. This is probably due to the low level of *PDLP8* promoter activity.

If, PDLP7 functions similarly to the known PDLP members, then PDLP7 can be used as a tool to study symplastic communication between sieve elements and their

companion cells. One example of this communication is the FLOWERING LOCUS T (FT) protein which is produced in the companion cells of cotyledons and leaves and moves into the SAM where it initiates floral transition. FT is loaded into sieve element cells by FT INTERACTING PROTEIN 1 (FTIP1), an ER and PD localized protein. FT in the SAM, interacts with FLOWERING LOCUS D to upregulate LEAFY, which in turn leads to floral transitioning. Overexpression of FT leads to early flowering. I propose the hypothesis that *PDLP7* and *PDLP8* are redundantly regulating PD in companion cells to sequester FT in companion cells, which is why *PDLP7* and *PDLP8* expression is seen in cotyledons and young leaves but not in the leaves of older plants (Figure 4.2).

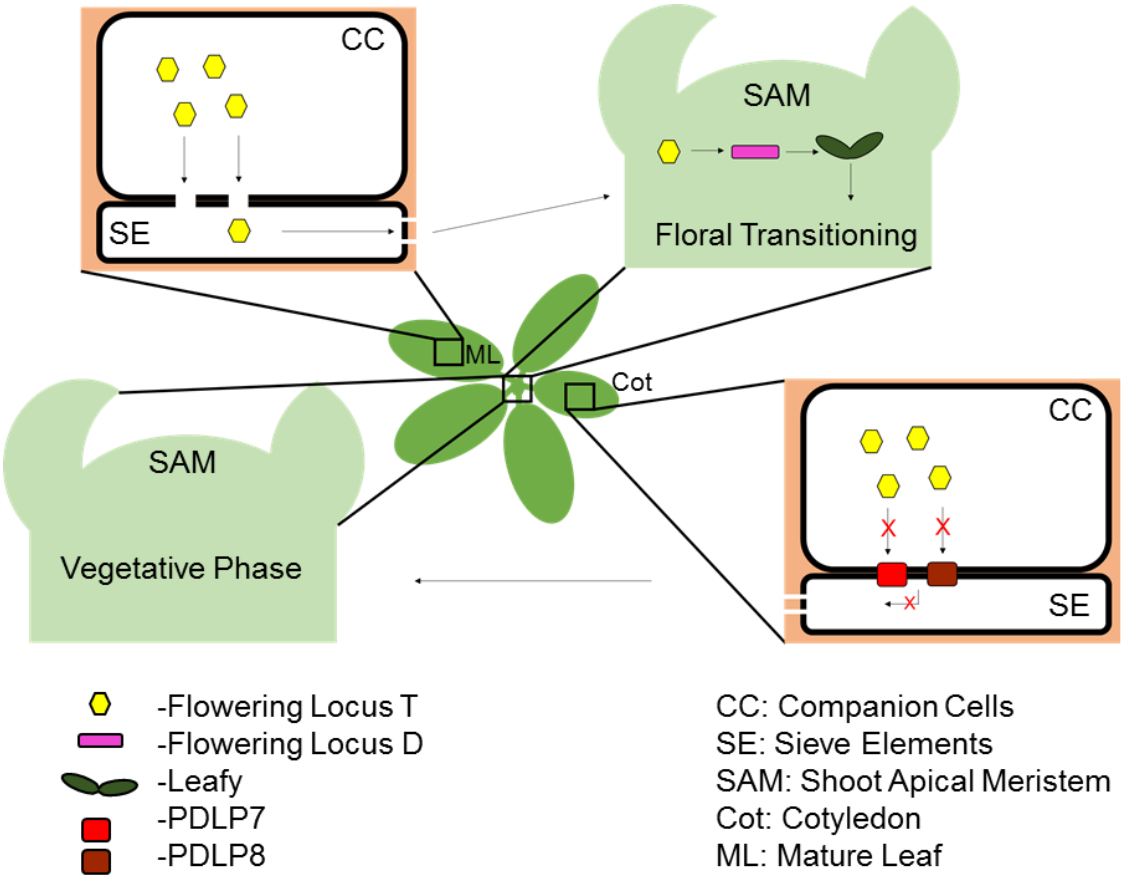

Figure 4.1 Schematic diagram of FLOWERING LOCUS T movement through PD. FLOWERING LOCUS T in cotyledons and young leaves is sequestered in the companion cells due to *PDLP7*/*PDLP8* mediated PD closure. In mature leaves, FLOWERING LOCUS T moves through open PD from the companion cells into the sieve elements and is transported into the shoot apical meristem where it activates FLOWERING LOCUS D, which then activates LEAFY, initiating floral transitioning.

4.4 Symplastic Isolation of Phloem Pole Pericycle Cells

What sets *PDLP7* activity apart from *PDLP8* is the expression observed in the CRC. The CRC stores auxin, which is then transported to the xylem pole pericycle to initiate anticlinical divisions to initiate lateral root primordia. The CRC cells are also rich in statoliths, which are involved in gravity sensing. Most importantly CRC cells produce

CLE40, a peptide involved in stem cell maintenance in a similar pathway as seen in the SAM. The QC expresses WOX5, a WUS homolog, that promotes CSC maintenance. CLE40 promotes CSC differentiation into CC, which are eventually sloughed off. It was found that secreted CLE40 interacts with the ACR4/CLV1 complex to limit WOX5 expression. WOX5 mutants result in extra CC layers and ACR4 mutants result in extra CSC layers. Both ACR4 and CLV1 localize to PD in the root apical meristem (Stahl et. al., 2013). Even though CLE40 moves apoplastically, it is hypothesized that an "unknown stemness" factor is trafficked through PD and interacting with ACR4/CLV1 complexes. If this is true, *PDLP7* is most likely the player involved in regulating PD aperture for the trafficking of this unknown stemness factor.

Analysis of *PDLP7* promoter expression pattern revealed that it is limited to the vasculature and CRC. The GUS stain was observed in the vasculature of cotyledons, and young true leaves. However, no histochemical stain was observed in mature leaves. This suggests that *PDLP7* is active only in the vasculature of young leaves and cotyledons. The staining is seen in the vasculature of the cotyledons, hypocotyl and root, as well as in the CRC. Comparing the histochemical staining patterns to known staining patterns suggested the expression was consistent with phloem expression. I then used the YFP lines to look at the cellular activity of *PDLP7*. Figure 3.6 suggests an expression pattern consistent with phloem pole pericycle cells. The pericycle is a non-vascular tissue that is divided into two cell types one being the phloem pole and the other being the xylem pole. Xylem and phloem pole pericycle cells are distinguished through their differing cell morphology and difference in gene expression. Lateral root initiation in *Arabidopsis* occurs in the xylem pole pericycle however it is still unclear how exactly interactions with the xylem and phloem poles governs the separate pericycle identities with differing abilities; one can give rise to lateral roots and the other cannot (Peret et. Al., 2009). Based on the expression patterns, I propose that regulation of PD aperture via *PDLP7* in the phloem pole pericycle symplastically isolates it from receiving auxin signals that

could cause the pericycle cells to give rise to lateral root progenitor cells (Figure 4.1). This idea is also consistent with the phenotype shown in the *PDLP7*-1 KO (Figure 3.9).

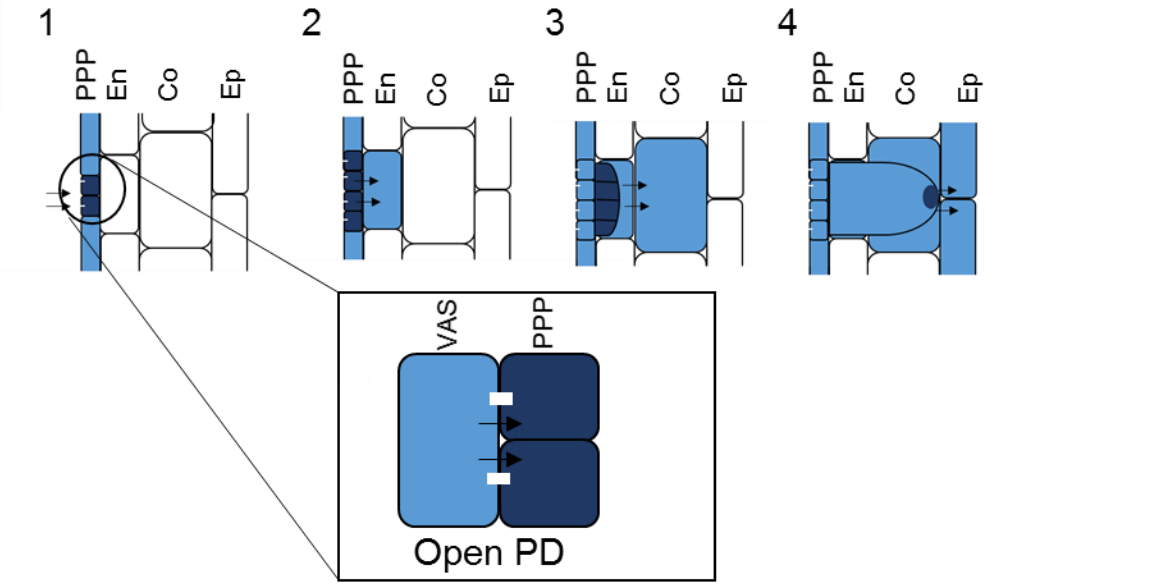

Figure 4.2 Proposed mechanism for lateral root propagation from phloem pole pericycle cells in *PDLP7* knockout lines. Auxin, once it leaks into the phloem pole pericycle (PPP) via open PD, in *PDLP7* knockout plants, allows for the first round of anticlinical divisions necessary for early LRP formation. This allows lateral roots to form from phloem pole pericycle progenitor cells. Cellular auxin responses are represented as a blue color gradient. Arrows depict auxin movement. En, endodermis. Co, cortex. Ep, epidermis. VAS, vasculature.

4.5 Increase in PDLP4 and PDLP7 Promoter Activity in the Presence of Auxin Point Towards Their Possible Role in Root Development

The auxin hormone is essential in lateral root development and emergence. Auxin is synthesized in the shoot and transported into the root via phloem to accumulate in the CRC. Auxin is then redistributed in pulses to the xylem pole pericycle where designated lateral root founder cells can initiate lateral root primordia. Although, influx and efflux

transporters such as AUX1 and PIN1 regulate the concentration of auxin in the primordia as well as the auxin gradient in the overlying tissue, an alternative hypothesis is that Auxin may be able to leak in and out of cells via PD. I observed, *PDLP4* promoter activity in the endodermal LRP overlying cells and *PDLP7* expression in the CRC, both of which are known cell domains of auxin accumulation. Based, on the overlapping cell domain localization patterns of the *PDLP4* and *PDLP7* family members and auxin, I tested whether promoter driven expression of GUS would be induced by exogenous auxin (NAA). NAA, the synthetic form of auxin is most commonly used in propagation experiments because Indole-3-acetic acid (IAA), the naturally occurring form, is rapidly degraded in vivo.

GUS staining conducted post 12 hours of NAA treatment revealed staining, in *PDLP4pro:GUS* lines, was darkest in the endodermal LRP overlying cells that overlay early stage LRP. Staining in overlying cells of late stage LRP was not as dark, indicating a lower level of *PDLP4* promoter expression at that time point in LRP development (figure 3.19). The *PDLP7pro:GUS* lines, however, stained very darkly and all throughout the root vasculature and increased staining was seen in the LRC along with the CRC. Upregulation of *PDLP4* and *PDLP7:GUS* activity in response to Auxin led me to question, whether there were any known Auxin binding motifs in the promoters. Promoter analysis tool, *Arabidopsis* cis-regulatory element database (http://arabidopsis.med.ohio-state.edu/AtcisDB/), confirmed no known auxin binding motifs in the promoter regions. With this information, for future studies the promoters can be analyzed for a yet unknown auxin binding motif or perhaps a secondary molecule,

such as a transcription factor, that is first activated by auxin and then acts on the promoters.

To further study the role of *PDLP4* and *PDLP7* in auxin mediated mechanisms, such as root growth and lateral root development I studied the *pdlp4-1* and *pdlp7-1* knockout lines. I studied primary root growth in the knockout lines by measuring root length at two time points, 5 days post germination (dpg) and 14 dpg. At 5 dpg, *pdlp7-1* knockout lines were significantly longer than WT or *pdlp4-1*. There was no significant difference between WT and *pdlp4-1* growth at 5dpg. However, *pdlp4-1* lines tended to display slightly shorter roots. At 14 dpg, both *pdlp7-1* and *pdlp4-1* lines had significantly longer roots than WT but no significant difference in length.

4.6 *pdlp4-1* and *pdlp7-1* Knockout Lines Resemble SMB, BRN1, BRN2 Mutant Phenotypes

The phenotypes of *PDLP4*-1 and *PDLP7*-1 knockout lines were similar to knockout lines of SOMBRERO, BEARSKIN1 and BEARSKIN2, (SMB, BRN1 BRN2, respectively) a group of closely related class IIB NAC domain transcription factors (Bennet et. al., 2010). NAC domain transcription factors activate genes involved in stem cell differentiation. The SMB knockout, *smb-3*, upregulates FEZ, another transcription factor that regulates root cap stem cells. *smb-3* presents with an extra layer of LRC cells that are unable to mature properly. Mature LRC and CRC cells are eventually sloughed off and replaced by newer cells. However, in the *smb-3* mutant the LRC do not properly mature and therefore do not slough off. Analysis of SMB-like genes identified two other genes later termed BRN1 and BRN2. BRN1 and BRN2 knockouts, *brn1-1* and brn2-2 double mutants present with deficiency in CRC cell sloughing. The triple mutant, *smb-3 brn1-1 brn2-1* displayed a strong phenotype displaying large masses of CRC cells still attached at the root tip. SMB, BRN1 and BRN2 are speculated to activate enzymes

involved in cell wall modifications that occur prior to the cells being shed (Bennet et. al., 2010). *pdlp4-1* exhibits a similar phenotype as *smb3* and *pdlp7-1* exhibits a similar phenotype to the *brn1-1 brn2-1* double mutant as seen in Figures 3.11. The *pdlp4-1* and *pdlp7-1* phenotypes, however, seem to be less severe, and the cells easily detach with mechanical manipulation. SMB, BRN1 and BRN2 transcription factors are most likely working upstream of *PDLP4* and *PDLP7*, with SMB. BRN1 and BRN2 acting in other pathways that lead to the same cell fate (Figure 4.4). Thus, the *pdlp4-1* and *pdlp7-1* phenotypes are not as severe as their upstream players. To explore the possible relationship between the NAC domain TF and the PDLP members, a chromatin immunoprecipation (ChIP) assay would reveal any direct interaction between the TFs and PDLP genes.

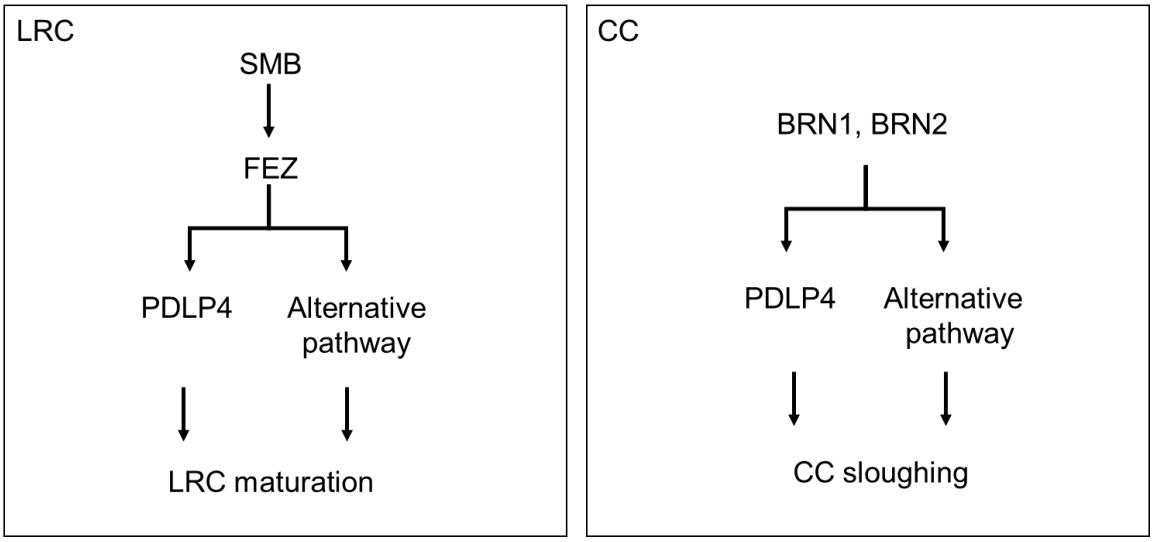

Figure 4.3 Proposed pathway to cell maturation and sloughing in root cap. In the lateral roto cap (LRC), SMB activates FEZ which can then upregulate *PDLP4* and also other unknown genes involved in lateral roto cap maturation. In the columella cells (CC) BRN1 and BRN2 act redundantly upstream of *PDLP4* and alternative pathways to target columella cells for sloughing.

4.7 Conclusion

The results acquired from this study have led to the following conclusions:

1. PDLP member promoter activity is tightly regulated spatiotemporally throughout the *Arabidopsis* life cycle.
2. *PDLP4* promoter expression is seen in the mature endodermis for primary and lateral roots, the endodermal overlying cells of early stage (I-III) LRP and the LRC of primary and lateral roots.
3. *PDLP6* promoter expression is seen in the SAM and transiently in the primary root and root tip.
4. *PDLP7* promoter expression is seen in the CRC, phloem pole pericycle and companion cells of primary and lateral roots and the companion cells of cotyledons and young leaves.
5. *PDLP8* promoter expression is seen in the phloem pole pericycle and of primary and lateral roots and most likely the companion cells of primary and lateral roots and cotyledons and young leaves.
6. *PDLP4* and *PDLP7* promoter activity is upregulated by exogenous NAA.
7. *pdlp4-1* knockout lines have a slower rate of lateral root emergence and initially slower rate of primary root growth. Eventually, *pdlp4-1* knockout primary root growth exceeds that of WT plants.
8. *pdlp7-1* knockout lines have an increased rate of lateral root emergence and an increased rate of primary root growth.
9. *pdlp4-1* knockouts present with defects in LRC maturation and subsequently thicker root tips, along with some difficulty of CRC cell detachment.
10. *pdlp7-1* knockouts present with defects in CRC cell detachment.

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