GENERATION AND SCREENING OF T-DNA INSERTION MUTANTS THAT ALTER LOCALIZATION OF PDLP5

by

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LIST OF ABBREVIATIONS

BASTA	Glufosinate Ammonium
DPG	Days Post Germination
KAN	Kanamycin
MP	Movement Protein
MS	Murashige and Skoog
NCAPs	Non-Cell Autonomous Proteins
PD	Plasmodesmata
PDLP5	Plasmodesmata-located Protein 5
PM	Plasma Membrane
SEL	Size Exclusion Limit
TMV	Tobacco Mosaic Virus

ABSTRACT

Plasmodesmata (PD) are structures unique to plants, which serve as a signaling pathway between distinctly separate neighboring cells and facilitate cell-to-cell communication. PD has been shown to play a role in plant growth, developmental processes, and plant defense. Recently a family of proteins was found to localize to the plasmodemata, indicating a potential role in these multiple functions. The fifth isoform of the family has been implicated in a plant defense role via the regulation of components associated with PD, facilitation of cellular signaling, and alteration of PD size exclusion. Else wise, little is known about PDLP5 including the molecular players that interact with the protein to aid in function or the specific localization to PD. In order to elucidate these players, a genetic screen was designed utilizing the molecular mechanisms of a certain bacteria, *Agrobacterium tumefaciens*.

Mutants were generated using *A. tumefaciens* to insert transfer DNA into transgenic *Arabidopsis thaliana* lines with fluorescent proteins fused to PDLP5. The resulting plants were screened for any alteration of normal fluorescent signal. Based on the probability of an insertion into the DNA sequence of molecular player that interacts with PDLP5 and subsequent disruption or altered expression of such a player, few mutants were found to alter the localization of our protein of interest. Albeit, the study provided proof of concept indicating the methodology is sound for future investigations of this nature, as continued study would result in the isolation of additional mutants.

Chapter 1

INTRODUCTION

1.1 Plant Structure

A distinct feature of plant cells is the rigid cell wall, which is a critical component in multiple facets of cellular life. The cell wall acts as a physical barrier that completely encompasses the cell membrane. To lend structural support to the cell, the cell wall acts as a firm backstop to the plasma membrane as it fills with water in a hypotonic solution. The enlargement of the plasma membrane to press against the cell wall is termed turgidity, which is essential for the plant to maintain rigidity, upright structure, and essential in photosynthesis via guard cells. The cell wall also acts as a semi-permeable blockade, which only allows the movement of small molecules and proteins. This physical barrier (between 200-1000 nm) presents a problem for the intercellular transport of larger molecules and signaling molecules that are essential for interaction with neighboring cells. In order to overcome this obstacle and facilitate communication between these distinctly separate plant cells, two signaling pathways were evolved. As the cell wall is semi-permeable, the first pathway relies on small signaling molecules diffusing through the structure and binding to receptors on the surface of the plasma membrane thereby activating a signaling cascade; it has been found that G Protein-Coupled Receptors interact with some phytohormones through this pathway (Lucas et al., 2009). The second signaling pathway involves a unique structure that acts as a cytoplasmic bridge between adjacent cells, the plasmodesmata (Lucas et al., 2009).

1.2 Plasmodemata

Biogenesis of plasmodesmata occurs in two distinct ways, dependent on the classification of PD being created. Primary PD is formed while a cell is undergoing division and this pathway is relatively well understood. In comparison, secondary PD are formed independently of cell division and they are inserted into cell walls which are already established (Lucas et al., 2009). During primary PD biogenesis, a portion of the smooth endoplasmic reticulum is trapped in the phragmoplast, a plant structure which acts as a guide and scaffold for the cell plate, while cytokinesis is occurring (Lee and Sieburth, 2010). The endoplasmic reticulum is appressed and forms a membranous structure as the mother cell almost completely divides. The appressed ER is encapsulated in plasma membrane that is contiguous with both daughter cells and crosses through the newly formed cell wall. Between the appressed ER and the plasma membrane, a multitude of proteins reside which are though to interact and inhibit or allow the passage of molecules (Alfonso et al., 2010). Of these proteins, actin is found to surround the appressed ER, which may impart constraints on the PD to limit flow of molecules (Alfonso et al., 2010). A study by Ding et al. 1996 carried out treatment with chemical disruptors of actin that led to an increase in the size exclusion limit of plasmodesmata (Alfonso et al., 2010). This passage allows for a symplasmic exchange of materials through the PD as opposed to the apoplastic exchange via the cell wall (Lucas et al., 2009). During the early stages of primary PD biogenesis and development, the PD is relatively simple and unbranched with a single sheath of cytoplasm. As the tissues mature the PD becomes more complex characterized by the addition of branches and cavities, as well as twinned structures (Lucas et al., 2009; Lee and Sieburth, 2010). Secondary PD is thought to be inserted into the cell wall after cell division in a process that requires the cell wall to thin in order to form the channel.

It is speculated that the secretion of cell wall material accompanies this process (Lee and Sieburth, 2010). While a certain phytohormone, cytokinin has been seen to play a role in the formation of secondary PD; the mechanism of biogenesis is unknown (Lee and Sieburth, 2010).

As the role of plasmodesmata is to facilitate the communication of adjacent cells, studies of symplasmic pathways have been extensively studied. It was originally believed that PD acted merely as 'holes' in the cell walls that facilitated signaling by passive diffusion of small molecules (Lee and Lu, 2011). From the structure of PD, regarding the inclusion of plasma membrane, endoplasmic reticulum membrane, and cell wall it is possible for soluble molecules as well as membrane-associated molecules to be transported (Alfonso et al., 2010). The size exclusion limit (SEL) of PD is the size of which a molecule can pass through the PD without any alterations. The basal resting size exclusion limit of PD is between 0.8-1.2 kDa, severely limiting the number of molecules that can transfer through the PD, but under certain conditions can dilate to accommodate proteins over 20 kDa in size (Lucas and Lee, 2004). It has been clearly shown in numerous studies that PD have the ability to mediate movement of protein and RNA molecules from cell to cell, both endogenous and exogenous (Thomas et al., 2008; Ueki et al., 2010). Of particular importance, PD have been shown to be an important pathway in the transport of non-cell-autonomous proteins (NCAPs) or proteins that function in another cell other than the one in which the mRNA encoding the protein was transcribed and the protein synthesized. Among other functions, NCAPs have been shown to be important in developmental processes (Lucas et al., 2009). While the importance of these signaling bridges is extensive in terms of developmental processes in plants, the exploitation of PD by microbial

pathogens is an exciting study that contributes to the understanding of plant communication (Lee and Lu, 2011).

Tobacco Mosaic Virus (TMV) is a single-stranded positive sense RNA virus, commonly used in studies of PD function. To facilitate the spread of the virus from cell-to-cell, the virus encodes a movement protein (MP). In order for the virus to spread though it must travel through the plasmodesmata (Ueki et al., 2010). The TMV MP functions by associating with the viral RNA to form a ribonucleocomplex that is targeted to the PD. This complex interacts with the PD in such a way that it increases the size exclusion limit of PD to allow the complex to move across adjacent cells (Ueki et al., 2010). Studies have shown that movement proteins associate with PD in one of two ways: direct interaction and by interactions with cytoskeletal components or secretory pathways (Lee and Lu, 2011). While studies have used TMVMPs to study the regulation of PD SEL, these studies also bring to light another interesting point. Plasmodesmata must be considered an important factor in plant defense, namely to prevent the systemic spread of viral particles from the point of infection to distal sites. An important player in the alteration of PD to allow for transport of molecules and viral particles is β-1,3-glucan, which is commonly studied using plant viruses including Tobacco Mosaic Virus.

Callose, or β-1,3-glucan, is a plant polysaccharide which plays a critical role in the function of PD (Maule, 2008). Regulation of symplasmic pathways falls partly to callose via callose synthases and glucanases (Lucas et al., 2009). As callose is not typically located in the cell walls at the neck of the PD, the formation or degradation of callose either pinches or relaxes the entry to the PD (Maule, 2008). These molecular sphincters at the neck region of PD change the SEL of PD and thereby adding an

additional level of trafficking regulation (Ueki et al., 2010). The deposition of callose can seal the PD completely and block trafficking through the plasmodesmata (Lee and Lu, 2011). Studies have shown the SEL can be increased by the presence of both endogenous NCAPs and viral movement proteins (Lucas et al., 2009).

Figure 1.2.1 The overall structure of plasmodesmata spanning two adjacent cells (Image from Lee and Lu, 2011).

Figure 1.1.1 shows the general structure of PD spanning the cell wall to connect adjacent plant cells. The appressed ER forms the central element of the plasmodesmata. The neck of the PD is the area in which callose accumulates.

1.3 Plasmodesmata-located Proteins

In 2008, a study by Thomas et al. facilitated further investigation in the field of plasmodesmata. A novel family of proteins was found to localize to the plasmodemata (Thomas et al., 2008). This family of eight proteins was characterized as type I membrane receptor-like proteins (Thomas et al., 2008; Lucas et al., 2009). The first isoform discovered was PDLP1 in 2008 by Thomas et al., by surveying cell wall proteomics of *Arabidopsis thaliana* suspension cultured cells (Lee et al., 2011). Analysis of sequence homology to PDLP1 was used to identify other members of the PDLP family (Lee et al., 2011). PDLPs are thought to range from 30 - 35 kDa in size (Lee et al., 2011). The family contains an N-terminal signal peptide, two domains of unknown function, a single transmembrane domain, and a C-terminal tail (Thomas et al., 2008; Lucas et al., 2009). The C-terminal tail resides in the cytoplasm while the DUF26 domains are extracellular, being in the apoplast (Thomas et al., 2008).

The transmembrane domain was found to be necessary for the localization of PDLP family members to the PD, while the C-terminal tail could be deleted and still localize to the PD (Thomas et al., 2008). The transmembrane domain is composed of only twenty-one amino acids (Thomas et al., 2008). The domains of unknown function (DUF26) are characterized by cysteine residues and are found in a protein superfamily exclusive to plants, which include Cysteine-rich Receptor-like Kinases (CRKs) and cysteine-rich secretory proteins (Lee et al., 2011). These DUF26 domains are part of a massive gene family in which approximately one hundred DUF26 genes are encoded

by *A. thaliana*, and are also prevalent in rice (Lee et al., 2011). Previous studies have indicated a possible link between CRKs containing the DUF26 domain, and a role in plant defense, namely the sialyic acid dependent pathways (Lee et al., 2011).

Recently DUF26 domain studies have implicated the Cysteine-rich region in multiple functions. A secreted protein called ginkbilobin-2 (Gnk2), isolated from Ginko seeds, has been shown to have some level of antifungal effect (Lee et al., 2011). Gnk2 has a 28-31% sequence homology to the extracellular domain of CRKs from *A. thaliana* (Miyakawa et al., 2009). The domain has also been shown to be part of the stress response/antifungal family and has been found to play a role in salt stress response in rice (Punta et al., 2012).

By proteomic analysis, Lee et al., (2011) showed 30% amino acid sequence identity of the isoform PDLP5 to the originally isolated PDLP1. This fifth member of the family is the basis for our studies.

Figure 1.2.1 The general domain structure of PDLP family members. A signal peptide is located at the N-terminus followed by two domains of unknown function (DUF26). A single pass transmembrane domain follows the region containing the domains of unknown functions and culminates in a short tail at the C-terminus (adapted from Thomas et al., 2008).

To understand the biological role that PDLP5 plays, morphology was examined for *Arabidopsis* for varied levels of PDLP5 expression (Lee et al., 2011). For an upper limit, substantial over-expression of the protein, PDLP5 was expressed under the constitutive cauliflower mosaic virus 35S promoter. In the 2011 study by

Lee et al., 50 transgenic lines were produced using the 35S promoter and about 40% of the first generation progeny (transformants) demonstrated growth as well as morphological phenotypes. These plants over-expressing PDLP5 exhibited growth inhibition as well as chlorosis, a condition in which plants do not produce sufficient chlorophyll (Lee et al., 2011). A severe knock down mutant, pdlp5-1, was found from a T-DNA insertion in the first intron of PDLP5 and was studied in a similar fashion to determine changes in morphological phenotype but it was found that these plants did not show obvious signs of growth inhibition when compared to the wild type (Lee et al., 2011). Concerns about using a strong, constitutive promoter during the early stages of plant life led Lee and co-workers to generate inducible PDLP5 over-expression plants, under the control of estradiol, a non-plant steroid (Lee et al., 2011). The studies resulting from this construct gave credence to the idea that PDLP5 over-expression negatively impacted the growth and health of plants, regardless of the life stage in which the protein is upregulated.

In a 2008 study by Thomas et al., it was shown that the eight members of the PDLP family, when fused with fluorescent tags, localize to the cell periphery as punctate structures, giving the impression of specific localization to the plasmodemata. To confirm the localization of PDLP5 to the PD, Lee et al., (2011) examined transgenic 35S:PDLP5-GFP seedlings that had a lower expression level of PDLP5, due to concerns of PDLP5 causing a distinct growth phenotype. In both the constitutive 35S:PDLP5-GFP and the estradiol inducible PDLP5-GFP lines, fluorescent punctate signals were found at cellular boundaries of leaf epidermal cell, only at cross-wall boundaries, indicating the signal was likely associated with the plasmodesmata (Lee et al., 2011). Upon determination that PDLP5 localizes to the PD,

Lee et al., (2011) attempted to determine the subdomain of PD to which PDLP5 reports by an immunogold labeling method. Using the immunogold labeling method with 35S:PDLP5-GFP plants and *Tobacco mosaic virus* movement protein (TMVMP-GFP) tagged GFP plants, Lee et al., (2011) showed that both TMVMP-GFP and PDLP5-GFP constructs localized to the central region of PD and raised the possibility that TMVMP and PDLP5 might localize to the same subdomain of PD.

Based on studies by Thomas et al., (2008) in which GFP was used as a reporter for cell-to-cell diffusion to show that PDLP1 altered PD permeability, Lee et al., (2011) believed that based on homology, PDLP5 might also modulate PD permeability. The over-expression of PDLP5 was shown to restrict the movement of GFP while the reduction of PDLP5 showed the inverse, which coincides with the outcome of PDLP1 over-expression and reduction reported by Thomas et al., 2008 (Lee et al., 2011).

As mentioned earlier, part of the regulation of symplastic pathway falls party to the action of callose, either the deposition of callose via callose synthases or the degradation of callose via glucanases (Lucas et al., 2009). Based on studies of other PD associated proteins (although not members of the PDLP family), Lee et al., studied how 35S:PDLP5 and pdllp5-1 mutants were able to affect PD permeability via interaction with callose (2011). By callose staining, Lee et al., determined samples of 35S:PDLP5 plants demonstrated a four-fold increase in PD callose over wild type while pdlp5-1 showed a two-fold decrease in the accumulation of callose at the PD (2011). This study demonstrated the ability of PDLP5 to alter PD callose accumulation, which would be necessary for the function of PDLP5 in controlling the permeability of PD (Lee et al., 2011).

As it has been shown that PD can be utilized by microbial pathogens, it would be thought that plants should have a counter active strategy to combat these pathogens (Lee et al., 2011). Lee et al., found that overexpression of PDLP5 correlated to necrotic cell death via high levels of salicyclic acid and the associated pathway, which is known to mediate defense against pathogens (2011). This role for PDLP5 in the SA pathway indicated that PDLP5 was likely involved in defense response (Lee et al., 2011). To test this role of PDLP5, 35S:PDLP5 and pdlp5-1 plants were infected with *Pseudomonas syringae* while bacterial growth was monitored in conjunction with a callose staining assay, showing a susceptibility in pdlp5-1 plants to bacterial infection but a reduction in bacterial growth in the 35S:PDLP5 plants (Lee et al., 2011). The outcome lent credence to the notion that innate defense involves PD permeability which functions via some interaction of PDLP5, indicating the importance of PDLP5 as a defense protein (Lee et al., 2011).

1.4 *Agrobacterium tumefaciens* and Transfer DNA

A. tumefaciens is a Gram-negative soil bacterium, which in nature produces crown gall, a disease of dicotyledonous plants causing large tumors (Zupan and Zambryski, 1995). Tumors from *Agrobacterium* infection were found to result from the expression of genes from a DNA segment of bacterial origin that was transferred into the plant and managed to be a stable mutation in the plant genome (Zupan and Zambryski, 1995). *A. tumefaciens* is the only naturally occurring organism that is capable of interkingdom gene transfer, more specifically transforming a plant cell, and as such has been adapted for use in experimental gene transfer (Pitzschke and Hirt. 2010; Zupan and Zambryski, 1995). When a plant is infected by *A. tumefaciens*, a piece of DNA is transferred (hence transfer DNA or T-DNA) from a specific plasmid

known as the tumor-inducing plasmid (Ti-plasmid), which is found in a low number of natural populations of *Agrobacterium* (Zupan and Zambryski, 1995). The T-DNA is 'capped' on the ends by short 25-bp direct repeats which direct T-DNA processing; any sequence between these borders are transferred into the target plant cell (Zupan and Zambryski, 1995). As the sequence between the borders is transferred, irrelevant to what the sequence is, the replacement of the native T-DNA sequence with DNA of interest allows for experimental studies (Tzfira et al., 2004). In nature the T-DNA sequence encodes for enzymes necessary for the synthesis of plant growth regulators including auxin and ctyokinin, which cause the tumorous growths, associated with Crown Gall disease, as well as amino acid derivatives known as opines (Zupan and Zambryski, 1995). The Ti-plasmid carries sequences for expression of enzymes of catabolism in one region, while also on the Ti-plasmid is the virulence region, which encodes the components necessary for the transfer of T-DNA (Zupan and Zambryski, 1995).

The virulence region of the Ti-plasmid encodes two virulence genes that are only expressed in the presence of wounded plant cells, and after induction of these genes a T-strand (single stranded copy of T-DNA) is generated (Pitzschke and Hirt, 2010; Zupan and Zambryski, 1995). Two viral proteins, VirD1 and VirD2, interact with the 25-bp border of T-DNA to create a single stranded endonucleolytic cleavage in the bottom strand of the borders, which are used as the start and stop sites for T-strand production (Zupan and Zambryski, 1995). VirD2 nicks the T-DNA at the 25 bp borders and is covalently bound to the 5'-end of the T-DNA (Pitzschke and Hirt, 2010). It is thought the T-strand forms an ssDNA-protein complex (T-complex) with a protein VirE2 (single-stranded DNA-binding protein), coating the T-strand and

protecting it from nucleolytic degradation, to allow for travel through membranes (Pitzschke and Hirt, 2010; Zupan and Zambryski, 1995). VirE2 is also thought to function in nuclear import and integration of T-DNA (Pitzschke and Hirt, 2010). Other virulence factors, VirB and VirD4, form a channel from the *Agrobacterium* cell to the target plant cell, which allows the T-strand to enter the cytoplasm (Tzfira et al., 2004).

The VirB complex is composed of a minimum of 12 proteins including VirB1-11 and VirD4, all of which are essential for bacterial virulence (Pitzschke and Hirt, 2010). These proteins interact with the cell envelope to form a multi-subunit envelop-spanning structure, which allows Vir proteins and T-DNA to pass through the bacterial membrane components, inner membrane then peptidoglycan layer then out membrane, and also pass the plant cell wall and plasma membrane (Pitzschke and Hirt, 2010). The VirD2-T-DNA conglomerate is transported through this complex.

After the T-strand enters the host cell, integration into the host chromosome is necessary for expression of transferred DNA as part of the plant genome. In order for the integration event, the T-strand must enter through a nuclear pore, but proteins greater than 40kDA require a nuclear localization signal (NLS) (Zupan and Zambryski, 1995). VirD2 and VirE2 both contain NLS sequences, which function to direct chimeric proteins to the nucleus, while the deletion of these NLS regions result in a near complete loss of transformation (Pitzschke and Hirt, 2010). Studies done on the VirD2 NLS sequences indicate that deletions of the region still allow for a function protein as far as creating a nick in the T-DNA and binding, but an inability to pass through the nuclear pore (Pitzschke and Hirt, 2010). These studies indicate that VirE2 is the key player in the direction of DNA to the nucleus, with a study in 2008 by Grange et al., showing that VirE2 actively pulled ssDNA into the host cell (Pitzschke

and Hirt, 2010). Another virulence protein, VirF, is thought to play a role (in conjunction with host proteases) in the degradation of the T-complex and help to release the T-DNA for integration into the host chromosome (Pitzschke and Hirt, 2010).

The mechanism for integration of T-DNA into the host genome is relatively unknown although some predictions can be made. T-DNA integrates into plant chromosomes in a manner similar to non-homologous end-joining (Pitzschke and Hirt, 2010). This process is fairly precise at the 5' end resulting in the loss of only a few 5' nucleotides, and it is speculated that is in part the action of VirD2 capping the T-DNA and protecting it from exonucleases (Pitzschke and Hirt, 2010). As T-DNA enters the cell in a single-stranded state, the synthesis of the complementary strand is necessary at some point, although it is not known if the integration occurs as ssDNA or dsDNA (Pitzschke and Hirt, 2010). VirD2 likely plays a role in T-DNA integration as it covalently bonds to the strand (Pitzschke and Hirt, 2010). On the converse, the ligation process of T-DNA is known to be mediated by host proteins (Pitzschke and Hirt, 2010).

1.5 *Agrobacterium*-mediated *Arabidopsis* Transformation

The ability of *A. tumefaciens* to transfer DNA into a plant genome via the mechanism above is termed insertional mutagenesis, a means of disrupting gene function by insertion of foreign DNA into the gene of interest or another gene which functions in relation to the gene of interest (Krysan, 1999). The inserted foreign DNA serves a dual role: first it disrupts the expression of the gene into which it is inserted, and it also serves as a marker for identification of the insertion (Krysan, 1999).

T-DNA insertional mutagenesis is advantageous, as the insertions will not transpose after the insertion, creating a stable mutant through multiple generations (Krysan, 1999). Subsequent transformed generations via *A. tumefaciens* mediated T-DNA insertional mutagenesis are typically hemizygous for the transgene at a particular locus (Clough and Bent, 1998). The T-DNA insertion on one chromosome indicates that the transformation occurs in the later stages of floral development, after the divergence of pollen and ovules (Bent, 2000). Studies by three different groups, Ye et al., 1999, Bechtold et al., 2000, and Desfeux et al., 2000, were able to determine the primary target for transformation was in fact the female germ-line (Bent, 2000). Two groups (Ye et al., 1999 and Desfeux et al., 2000) indicated the transformation in ovules using the GUS reporter gene to show the site of T-DNA delivery (Bent, 2000). The insertion of T-DNA into the plant genome is a random event and transformants from the same plant or silique are independent (Bent, 2000). As the *Arabidopsis* genome is relatively small, well defined, introns are small, and very little non-coding material mutagenesis by a T-DNA insert ranging from 5 - 25 kb is enough to have a significant impact on the expression of the gene of interest (Krysan, 1999). Use of this method has yielded favorable responses and transformed plants are obtained at high enough frequencies for use of the technique in random mutagenesis screening and gene-tagging (Clough and Bent, 1998). These factors have led to the use of a so-called floral dipping method of *A. tumefaciens* mediated T-DNA mutagenesis as the *de facto* protocol for large scale plant transformation over the previously used vacuum infiltration method. In this method, plants are grown to a specific life cycle point, dipped into an inoculation medium containing *A. tumefaciens*, and allowed to grow to maturity (Clough and Bent, 1998).

Studies, specifically Clough and Bent, 1998, have shown that the stage of plant growth is a critical factor in the transformation process (floral dipping) and that carrying out the procedure during a specific point in the life cycle results in a higher rate of transformation. After the primary central stem (colloquially bolts) emerge and are clipped, secondary bolts will grow. When the secondary bolts are between two and ten cm in length with only a few open flowers that is the ideal time perform the floral dip transformation (Clough and Bent, 1998). Clough and Bent found this to be the optimal life cycle for transformation in six of seven replications (1998). Another factor, which contributes to the success of the floral dip, is the inclusion of sucrose in the inoculation medium, in which the *A. tumefaciens* are resuspended and the plants are dipped. Clough and Bent found that between 0.5% and 5% sucrose concentrations of the inoculation medium results in no significant change but that the inclusion of sucrose does increase the transformation rate (1998). The final contributing factor outlined by Clough and Bent was the inclusion of surfactant, specifically Silwet L-77, in the floral dip protocol. Silwet L-77 aids in the entry of *A. tumefaciens* into plant cells at a relatively low toxicity to the plant (Clough and Bent, 1998). Their study showed the use of Silwet L-77 at low concentrations resulted in transformation rates similar to the previously used vacuum infiltration method of T-DNA insertional mutagenesis. Levels of Silwet L-77 between 0.02% and 0.1% yielded transformation results higher than the vacuum infiltration method but the use of higher concentration surfactant can cause necrosis (Clough and Bent, 1998). After transformation, the plants were covered with plastic domes to maintain high humidity for 24 hours, which proved to be advantageous and resulted in twice the number of transformants (Clough and Bent, 1998). Clough and Bent found that following the floral dipping technique

with mind to the factors mentioned could result in high levels of transform ranging from 0.5% to 3% (1998).

Chapter 2

MATERIALS AND METHODS

2.1 Transgenic Lines and Vectors

The J-Y Lee laboratory supplied parental plant lines used in this project and they have been previously constructed from a maintained seed stock. The first transgenic line used was PDLP5-GFP, which served as a control for visualization. This plant line is a construct in which enhanced green fluorescent protein (EGFP) was fused to the C-terminal end of PDLP5 (Figure 2.1.1). The plasmid vector pdGC was used for this project (Lee et al., 2005).

Figure 2.1.1 The domain structure of PDLP5-GFP.

The second transgenic plant line used was YFP-PDLP5, a construct in which yellow fluorescent protein (YFP) was fused to the N-Terminal end of PDLP5 (Figure 2.1.2). YFP-PDLP5 also served as a control for visualization.

Figure 2.1.2 The domain structure of YFP-PDLP5.

A. tumefaciens strain GV3101 was used in conjunction with a binary vector system to simplify the process to engineer the strain and carry out the transformation. GV3101 is resistant to the antibiotics gentamycin and rifampicin but is sensitive to kanamycin, which allows the use of GV3101 with binary vectors that confer kanamycin resistance (Indiana, 2012). GV3101 has an inactive Ti-plasmid with virulence genes necessary for transfer of T-DNA but contains no T-DNA region (Indiana, 2012). The binary vector system used in this project is the pBIN-pROK2 system with the GV3101 helper strain (Lee and Gelvin, 2008). The pROK2, which contains the T-DNA region including the left and right borders, is spliced into the pBIN vector (Salk Institute, 2012). The pROK2 also contains a multiple cloning site, the 35S promoter, and of particular importance the NPTII gene (Salk Institute, 2012). The NPTII gene confers kanamycin resistance when the T-DNA is integrated into the host plant cell genome (Salk Institute, 2012).

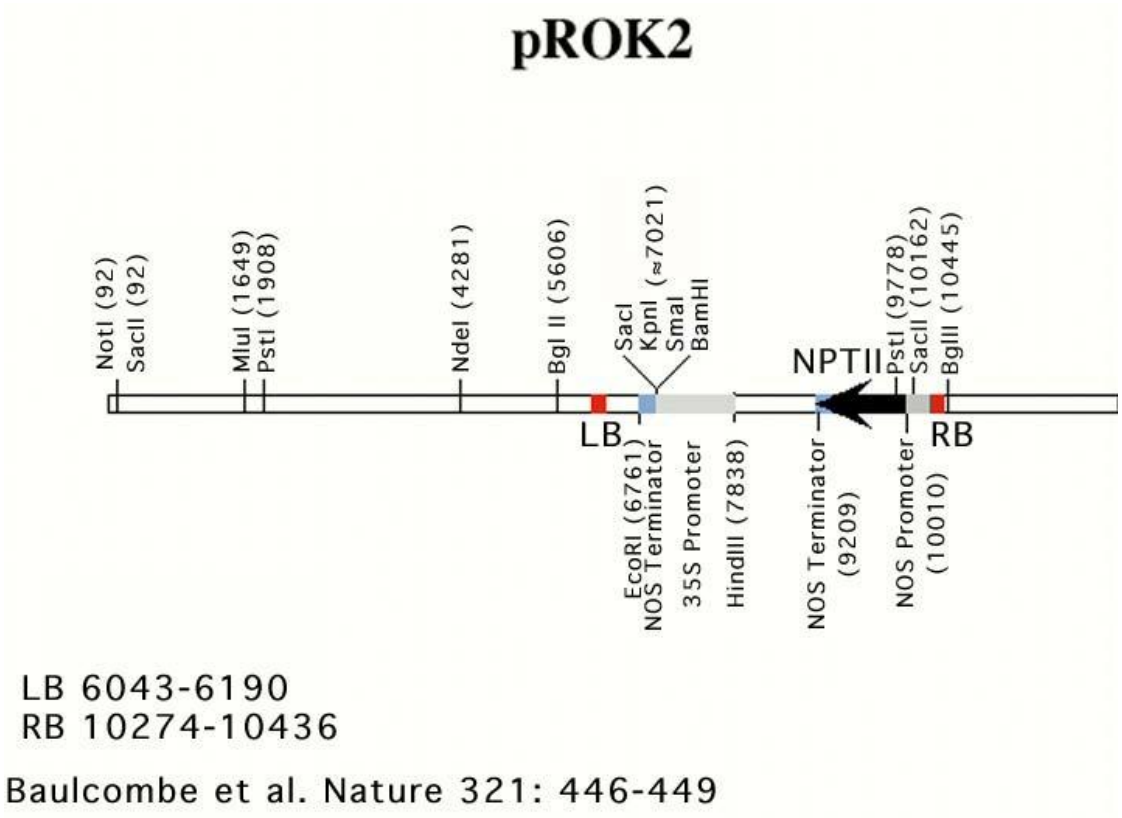

Figure 2.1.3 pROK2 vector map indicating restriction enzyme cut sites, both inside and outside of the T-DNA region. Everything between the red regions labeled LB and RB is part of the T-DNA and will be transferred into the plant cell. (Image from Salk Institute Genomic Analysis Laboratory website.)

2.2 Plant Growth and Conditions

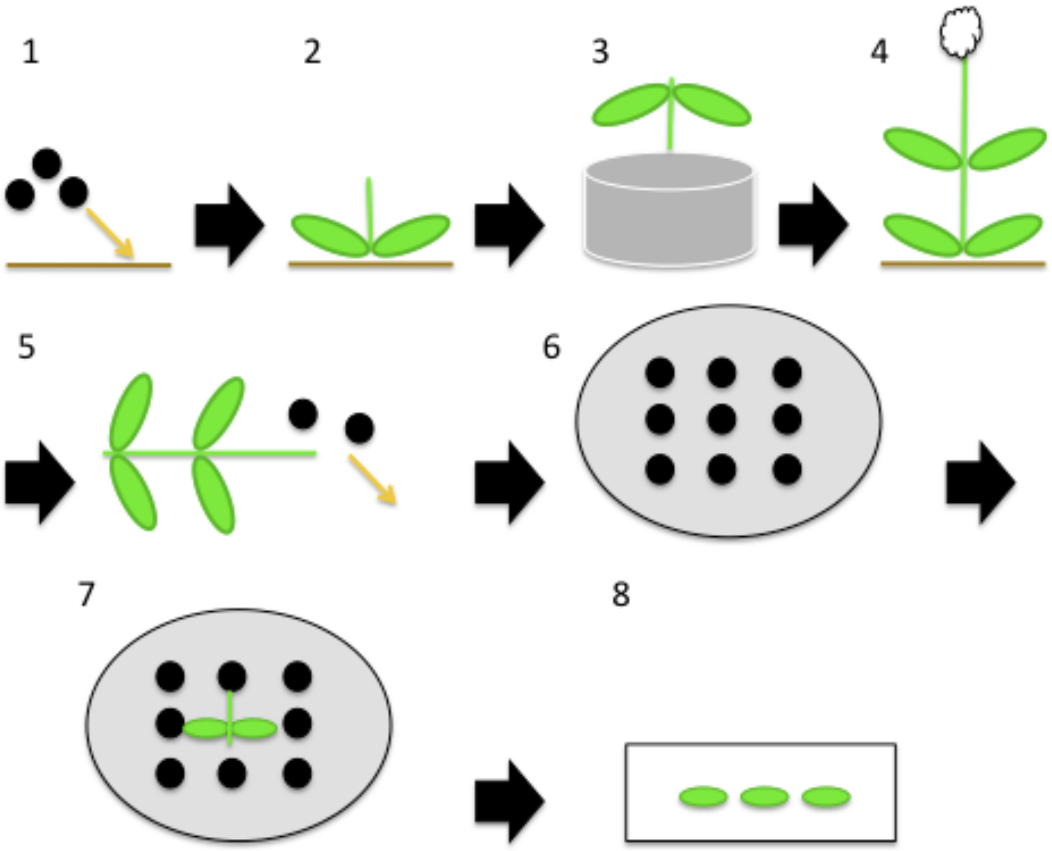

Figure 2.2.1 General flowchart of experimental procedures. Seeds are sown (1) and grown until the first inflorescences emerge (2) at which point the plants undergo the floral dipping procedure (3), a method of *Agrobacterium*-mediated transformation. Transformed plants are grown to maturity (4) and the seeds are harvested (5). These seeds are plated for selection (6) and only transformants survive (7). The cotyledons from the transformants are removed and prepared on slides (8) for viewing under the microscope.

Flats were prepared with 6 X 3 pot inserts. The pots were filled with Sunshine Mix soil (Sun Gro Horticulture) and soaked with water containing Gnatrol (larvicide).

After the soil was sufficiently damped, twenty flats of PDLP5-GFP and twenty flats of YFP-PDLP5 were sown. The target number was three plants per pot to yield over two thousand plants in total. After sowing seeds, flats were covered with translucent lids and stored in a 4°C room for 48 hours. The flats were then transported into a Conviron walk in growth chamber. The conditions were 16 hours of light and 8 hours of darkness at 22°C with 50% humidity. After approximately 2 and one half weeks of growth, the emerging central stem was clipped. When secondary shoots emerged, plants were tied to wooden stakes.

2.3 Transformation via Floral Dip

The protocol for floral dipping was adapted from Clough and Bent (1998). Plants were grown at the above conditions until inflorescences emerged. Three days prior to the transformation, 5 ml of liquid LB medium (tryptone, yeast extract, and NaCl) was inoculated with *A. tumefaciens* carrying the pROK2 vector. This liquid culture was incubated at 28°C with vigorous agitation (200 RPM) overnight. Two days after the initial inoculation, 3 ml of the preculture was used to inoculate 1 L of liquid LB media and grown for another day until the optical density was between 0.6-1.0. The culture was pelleted at 4000 rpm for 20 minutes and the cell pellet was re-suspended in a solution containing 5% sucrose and 0.2% silwet-77, a surfactant. The *A. thaliana* flowers were dipped into the solution and soaked with gentle agitation for about 60 seconds. Flats (for holding pots of plants) were lined with moist paper towels. The pots containing the plants were laid on their side and covered with a translucent lid for 24 hours. After the 24-hour period, the plants were returned to the normal growing conditions.

2.4 Seed Harvest

After the floral dip transformation, the plants continued to grow to maturity. Seeds were harvested before the dry matured siliques opened. Each pot was tipped on the side and the stake was removed. The plant was gently massaged to allow the siliques to break open and the seeds to fall onto paper below. A sieve was used to filter the seeds from the plant debris. The seeds were then air-dried in small envelopes, labeled with the date and transgenic line, and stored at room temperature to await selection and screening.

2.5 Transformant Selection and Screening

Selection of transformants was carried out on plates of ½ MS medium with 0.8% agar. The ½ MS medium was prepared with 2.17 grams of MS per liter of water and the pH was adjusted to 5.7. One half liter of ½ MS medium was transferred into a 1 L flask and 4 grams of agar was added. After autoclaving, the media was cooled before adding kanamycin and glufosinate ammonium to a final concentration of 50 μg/ml and 10 μg/ml respectively.

Seeds were sterilized in a biosafety cabinet. Approximately 300 μl (7500 seeds) of harvested seeds were aliquoted into 2 ml microfuge tubes. One ml of a 3% bleach solution was added to the microfuge tube, vortexed at high speed for 3 minutes, and spun down in a centrifuge. The bleach solution was decanted off. One ml of 70% ethanol was added, vortexed at high speed for 30 seconds, and spun down. The ethanol was decanted. The sample was washed 5 times with one ml of sterile water, while vortexing during each wash, ending with a resuspension in one ml of sterile water.

After sterilization, the microfuge tubes containing seeds were inverted multiple times to evenly resuspend the seeds. Using sterile one ml pipette tips, seeds were

deposited onto the ½ MS plates with 0.8% agar infused with Kan and Basta (described above). The seed density remained consistent throughout the plate, with approximately 100 ul of seeds (2500 seeds) per spot, and seeds were deposited in an orderly column fashion. After plating the seeds the Petri dishes were sealed with surgical micropore tape, labeled with pertinent information, and transferred to a 4°C cold room. After 48 hours the plates were moved to a 22°C incubator with 24-hour light conditions.

After 7 days in the incubator, the seedlings were ready for screening. Upon inspection the transformants were distinguishable from non-transformants based on pheontype, which prevents development of the seedling (Figure 2.6.1). The transformed seedlings were unaffected by the selection factors (Kan and Basta). This resulted in seedlings with a healthy green color and a cotyledon size larger than non-transformants, which was typical for seedlings 7 dpg. Additionally, emerging true leaves could be distinguished as seen below.

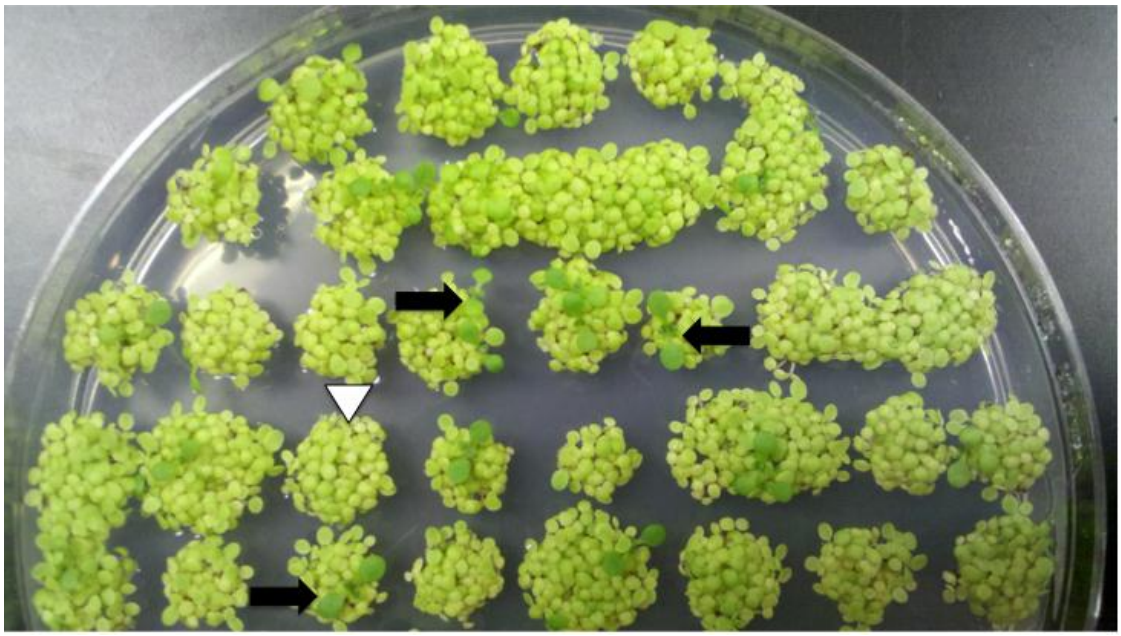

Figure 2.6.1 Photograph of transformants on a plate grown on ½ MS Media with 0.8% Agar infused with Kanamycin and Glufosinate Ammonium. At 7 days post germination, the transformed seedlings (black arrows) are clearly distinguishable from the non-transformed seedlings (white dart indicates a cluster of non-transformed seedlings).

The cotyledons from transformants were clipped without damaging the rest of the seedling; in particular the emerging true leaves. The cotyledons were placed on a microscope slide with the abaxial side facing towards the objective. The cotyledons were overlaid with water and a coverslip, and then slight pressure was applied. On a Zeiss Axioskop 2 in the Delaware Biotechnology Institute Bioimaging Suite, the cotyledons were observed with both 20X and 40X objectives under fluorescent conditions. Samples that showed an aberration of normal punctate structure distribution were transferred to soil and at 3 weeks of age, these samples were checked again with a Zeiss 5 LIVE DUO high-speed confocal microscope.

Figure 2.6.2 Photograph of cotyledons after removal from transformed seedlings and placed on slide with coverslip.

Chapter 3

Results

3.1 Controls and Images

To serve as a basis for comparison, the parental transgenic lines were grown on selection plates and images were taken. Images of PDLP5-GFP and YFP-PDLP5 were points of reference for the normal distribution of punctate signal, as well as intensity. In order to visualize the fluorescently-tagged protein, images were taken with the abaxial side facing towards the objective. This allowed for the best visualization of signal on the cell wall between adjacent leaf epidermal cells.

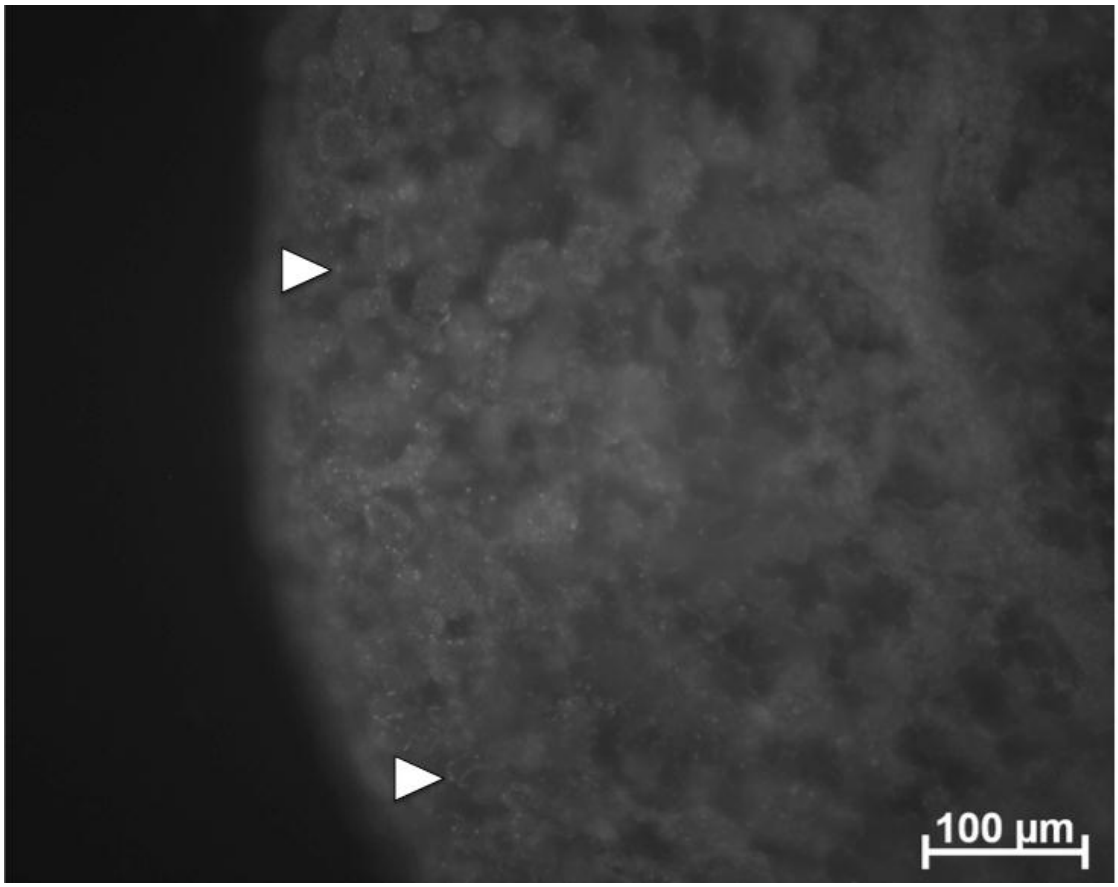

Figure 3.1.1 Fluorescent image of PDLP5-GFP taken on a Zeiss Axioskop 2 at 20X objective, viewing the cotyledon 7 dpg. Punctate signals (white dart) represent the fluorescently -agged protein embedded in the cell wall. The boundary of the cell can be seen around the lower white dart.

While difficult to see from captured images, the signal intensity of PDLP5-GFP plants was much higher than the YFP-PDLP5. The PDLP5-GFP seed stock was older than the YFP-PDLP5 and as such suffered some seed health-related consequences, such as a lower germination rate. For this reason, both lines were used in the project.

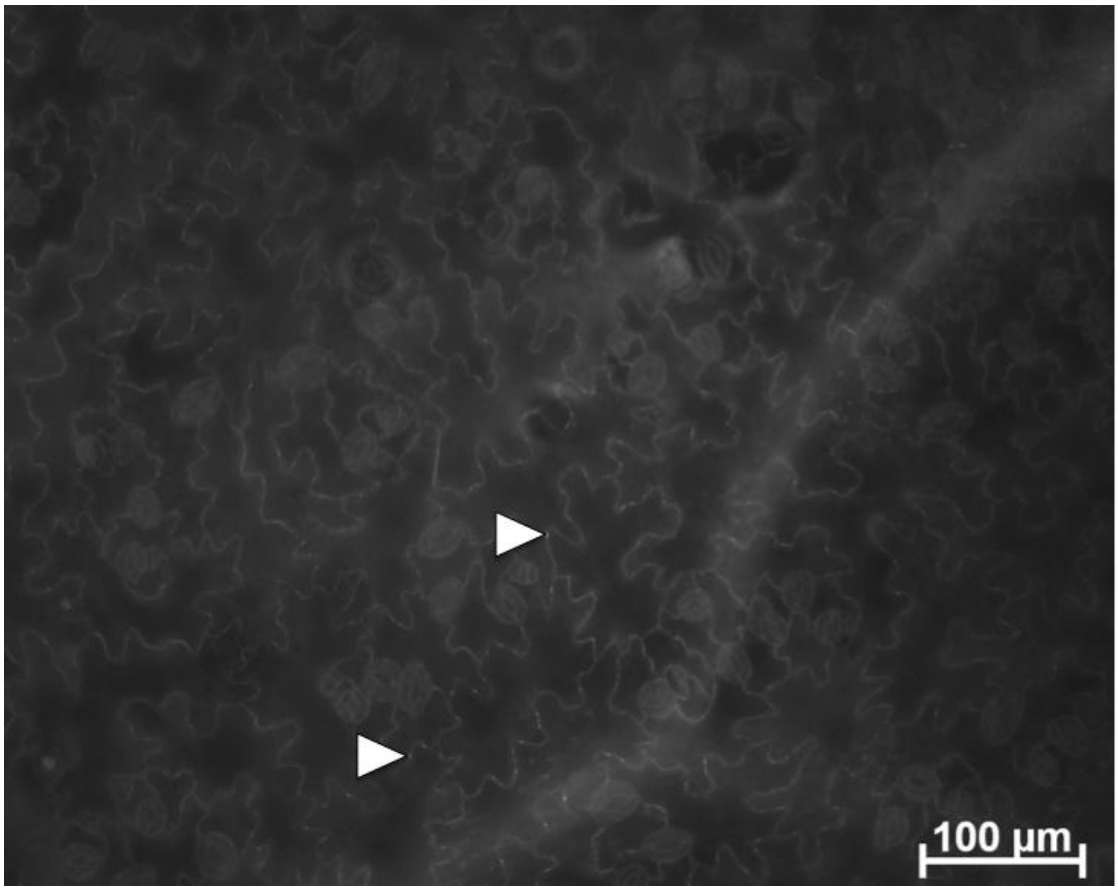

Figure 3.1.2 Fluorescent image of YFP-PDLP5 taken on a Zeiss Axioskop 2 at 20X objective, viewing the cotyledon 7 dpg. Punctate signals (white darts) represent the fluorescently-tagged protein embedded in the cell wall.

As mentioned above, the signal intensity is not as strong in the YFP-PDLP5 plants but the cell boundaries are easier to see, from the lower amount of fluorescence. Discrete puncta are easily distinguishable, which allows for a rapid screening process.

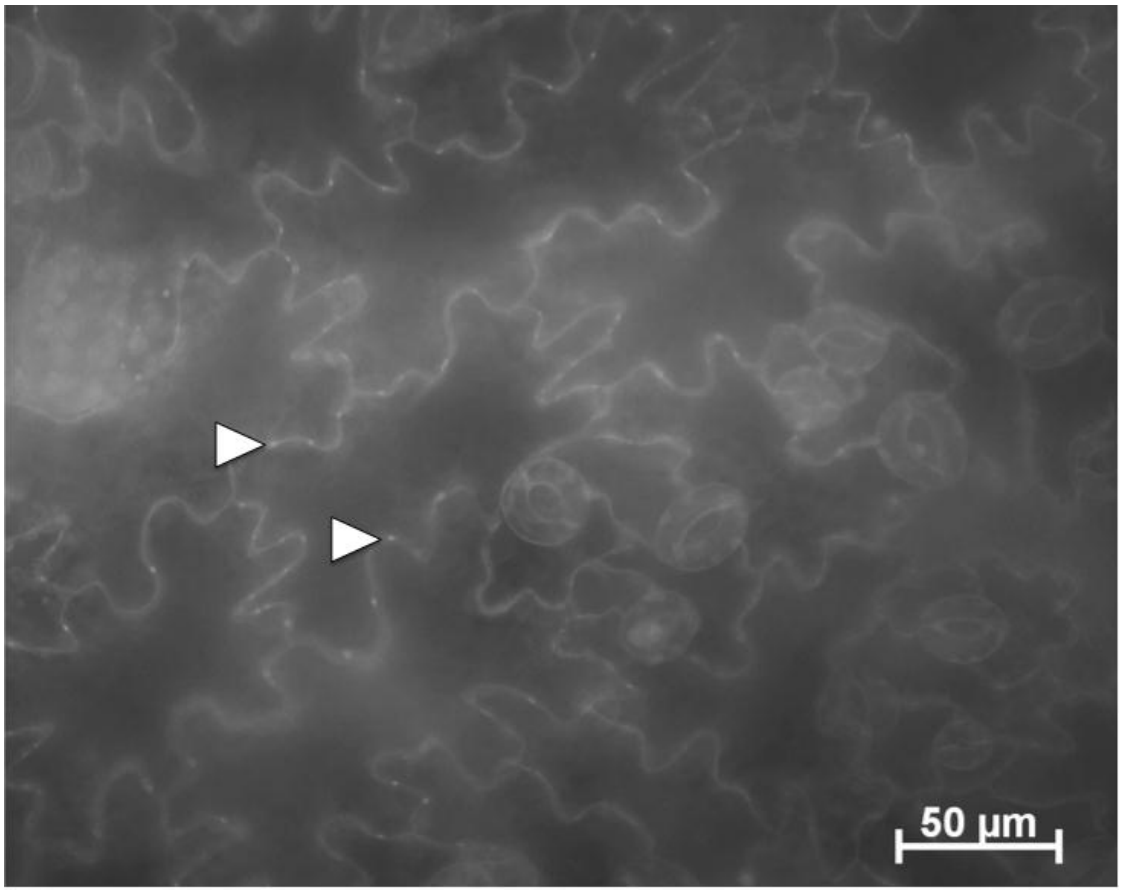

Figure 3.1.3 Fluorescent image of YFP-PDLP5 taken on a Zeiss Axioskop 2 at 40X objective, viewing the cotyledon 7 dpg. Punctate signals (white darts) represent the fluorescently-tagged protein embedded in the cell wall.

The increased magnification and resolution with the higher objective lens accentuates the individual punctate structure of the YFP-PDLP5 signal. The higher magnification is useful in identifying a signal that is tightly grouped, helping to differentiate large aberrant signals that may be the result of aggregates.

3.2 Other Visualizations

In order to appreciate alternate protein localizations, numerous other transgenic *Arabidopsis* lines were grown on selection plates and imaged under fluorescence. These other lines showed fluorescence when the signal was contained in the Golgi apparatus and the plasma membrane, or if the signal interacted with cytoskeletal elements. These marker lines were imaged so that any aberrations in the PDLP5 signal could be identified.

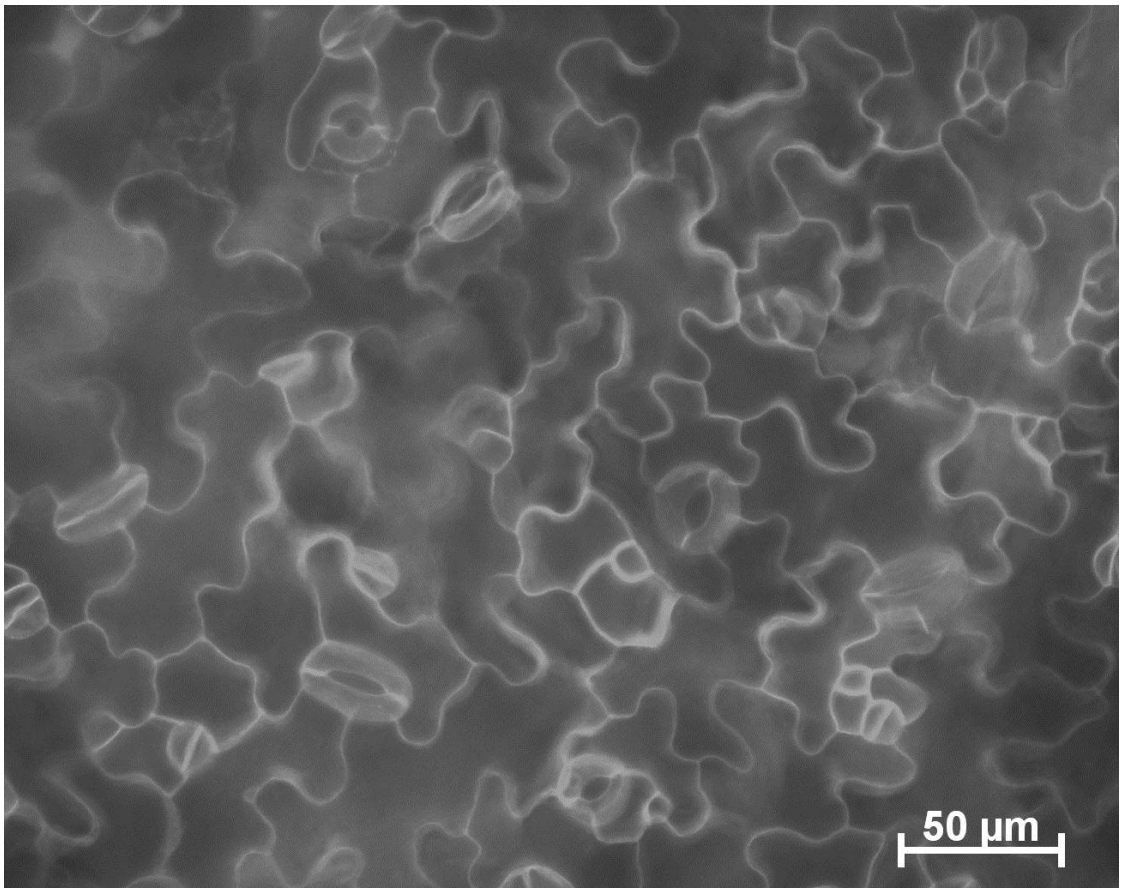

Figure 3.2.1 Fluorescent image of PM-GFP, a fusion construct in which the green fluorescent protein localizes to the plasma membrane.

The image was taken on a Zeiss Axioskop 2 at 40X objective, viewing the cotyledon 7 dpg. In this image, the fluorescence is not restricted to small, discrete areas (such as PD) and as such lack the punctate signal seen in PDLP5-GFP and YFP-PDLP5. Instead the fluorescence is seen around the boundary of epidermal cells.

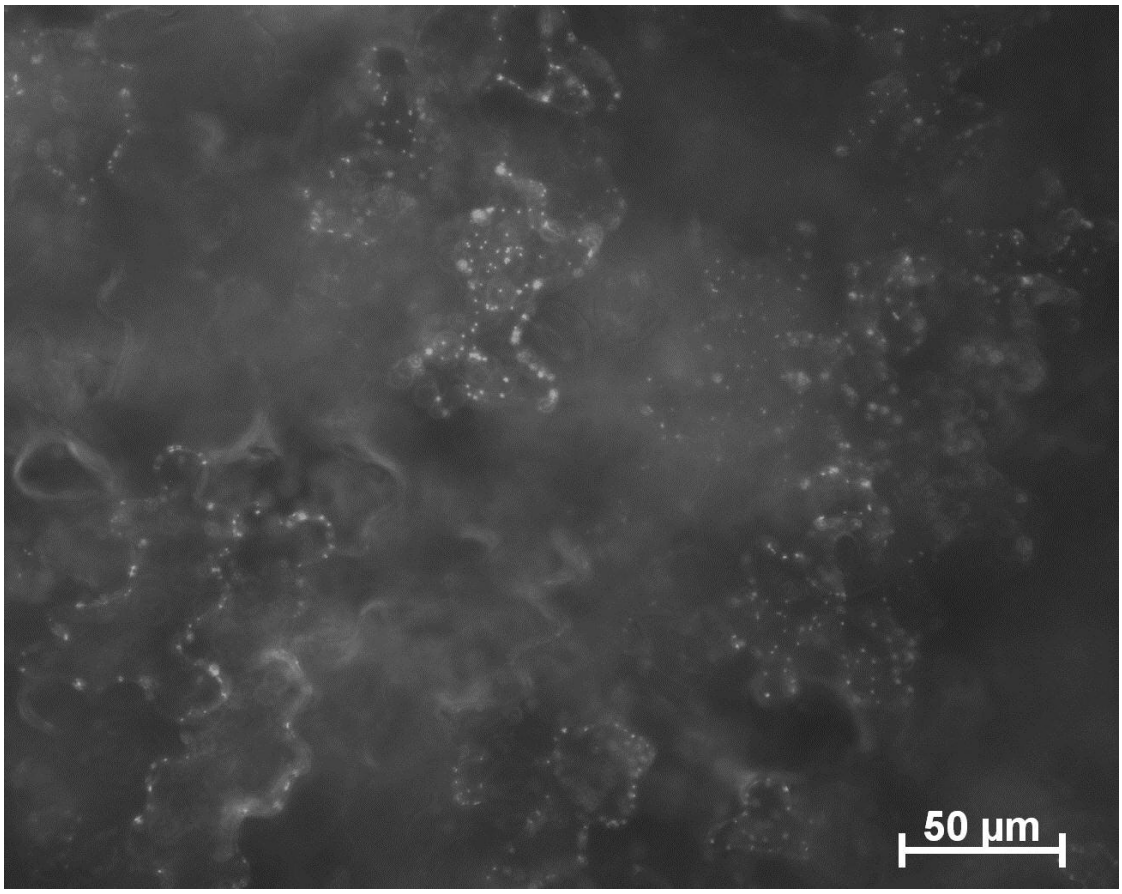

Figure 3.2.2 Fluorescent image of Golgi-GFP, a fusion construct in which the green fluorescent protein resides in the Golgi apparatus.

The image was taken on a Zeiss Axioskop 2 at 40X objective, viewing the cotyledon 7 dpg. At the magnification and resolution of this image, the cisternae

appear as numerous discrete punctate signals. When compared to the PDLP5 lines, the punctate structure is similar but the striking difference is the distribution. The PDLP5 signal was restricted to the cell periphery but the Golgi-GFP signal can be seen intracellularly. This distribution would be of particular interest if it were observed in a transformant.

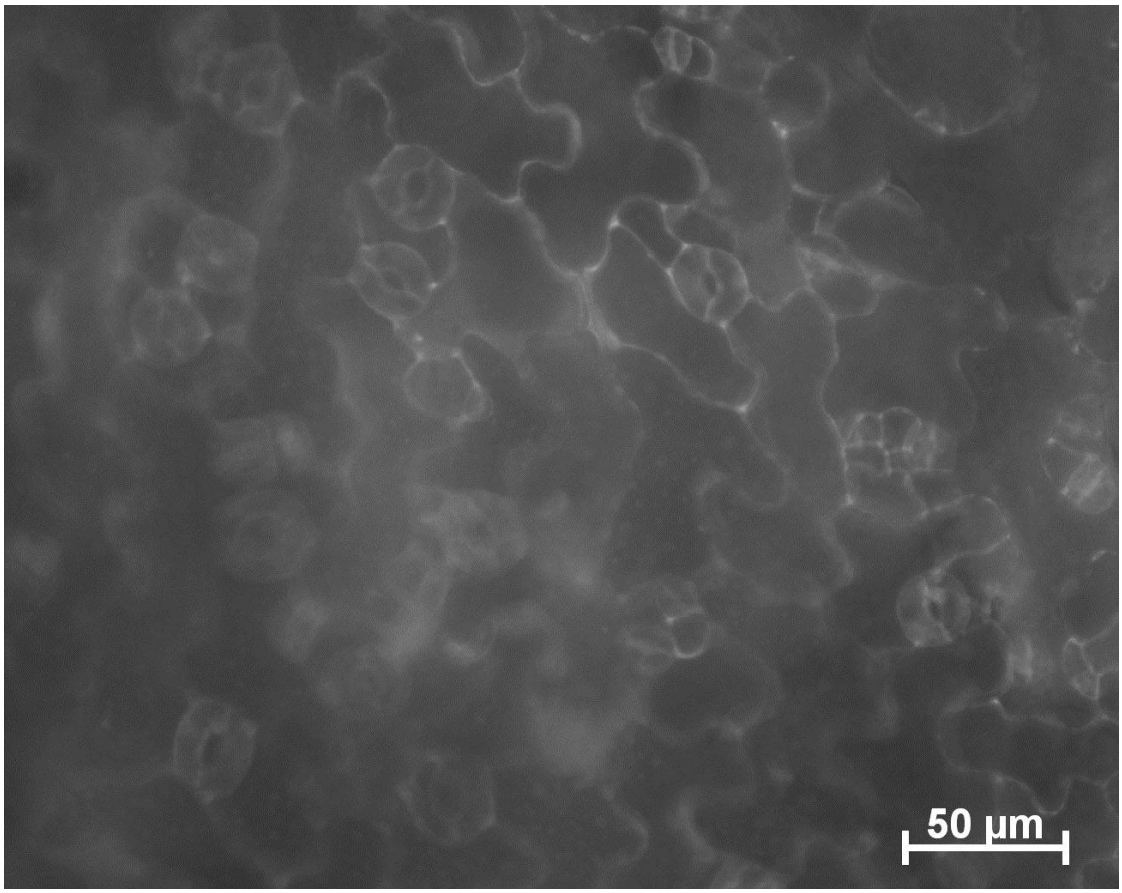

Figure 3.2.3 Fluorescent image of Tubulin-GFP, a fusion construct in which the tubulin subunits, which make up microtubules, are tagged with green fluorescent protein.

The image was taken on a Zeiss Axioskop 2 at 40X objective, viewing the cotyledon 7 dpg. Small filaments are visible within the cell but unlike animal counterparts, microtubules are rather dispersed without a distinct organizing center (Wasteneys, 2002).

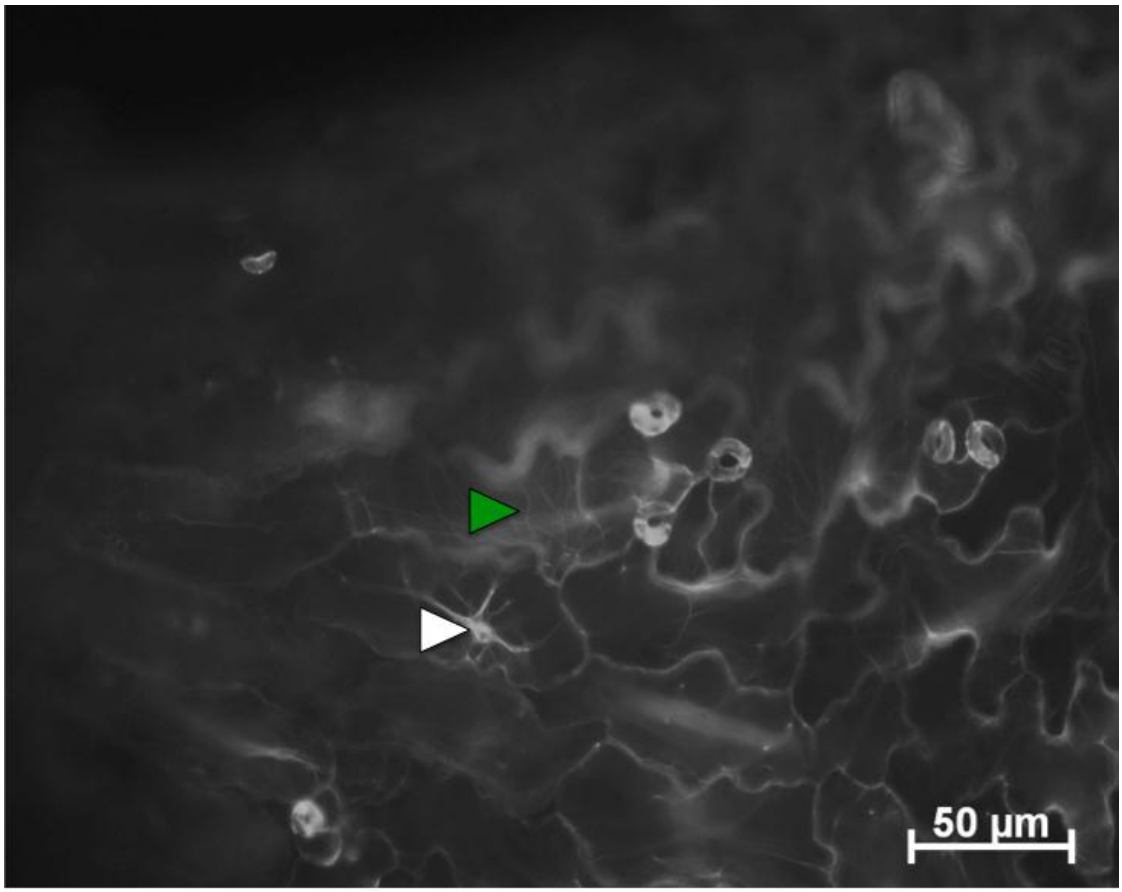

Figure 3.2.4 Fluorescent image of Actin-GFP, a fusion construct in which microfilaments are tagged with green fluorescent protein. Individual strands indicated by a green dart, while the condensed actin filaments are indicated by a white dart.

The image was taken on a Zeiss Axioskop 2 at 40X objective, viewing the cotyledon 7 dpg. In this image, the fibrillar structure can be seen, as individual strands and also in a highly condensed fashion.

Based on the information known about faithful localization of PDLP family members and PDLP5 in particular to the PD in studies by Lee et al. (2011), and Thomas et al. (2008), the control images above (Figure 3.1.1, 3.1.2, and 3.1.3) represent non-transformants. Almost all seedlings that receive a T-DNA insertion, which are able to grow on selection media, will exhibit the control phenotype shown above (Figure 3.1.1, 3.1.2, and 3.1.3). The rare transformant will exhibit some other phenotype. This may be similar to another of the images shown above, such as something similar to Figure 3.2.2, in which the signal remains inside the cell, instead of being exported to the cell periphery, or perhaps the signal is retained in an organelle. The phenotype might also appear as an aggregation of signal in an intracellular fashion as if tightly packed inside of vesicles. The lack of a signal at all or an extreme contrast in intensity of signal would be of interest. From experience, a small fault of the camera system used with the Zeiss Axioskop 2 is that the camera does not represent exactly what is seen through the oculars, especially in the sense of signal intensity.

3.3 Current Findings

Currently over 30,000 transformed seedlings have been observed on a Zeiss Axioskop 2 from selection plates. While the vast majority of these seedlings have been in line with the PDLP5 control lines, some interesting samples have arisen. Recently a small number of samples were found (5 individual plants) with a similar signal pattern, which was quite different from the YFP-PDLP5 control. Rather than the

punctate signal localizing to the cell walls between leaf epidermal cells, the signal appeared to be extracellular. Images of three samples were taken although the nature of the signal made it difficult to obtain good images. Upon transferring the mutants to soil, four of the five mutants died within three days. Only one seedling survived for longer than 3 days after transferring to soil, although during that time it was in very poor health (Sample 3). Other seedlings from the same selection plate were also transferred to soil. A marked difference in size could be observed between Sample 3 and the other seedlings (3 days after transfer), on the order of Sample 3 being approximately one-eighth the size of the other seedlings, which sprouted the first inflorescence.

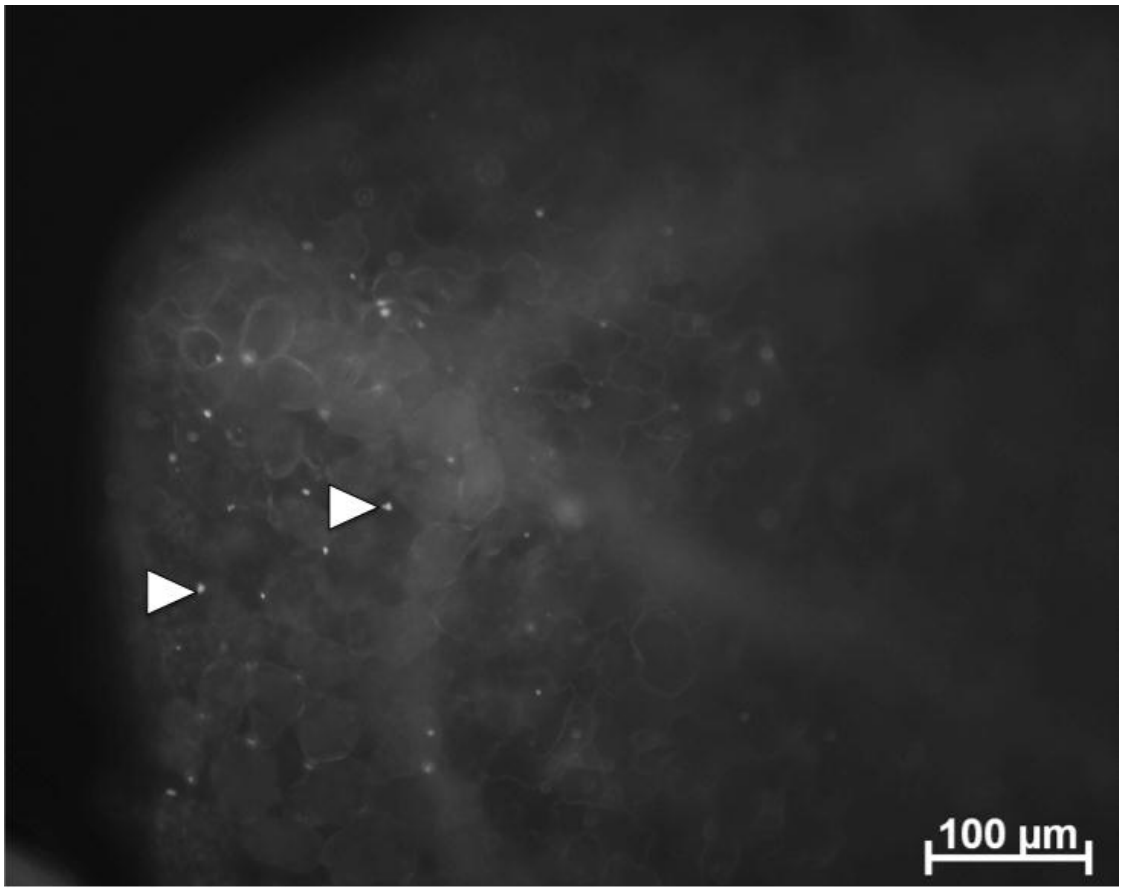

Figure 3.3.1 Fluorescent image of Sample 1, the first mutant of five found with similar signal localization. Punctate signal highlighted with a white dart.

The image was taken on a Zeiss Axioskop 2 at 20X objective, viewing the cotyledon 7 dpg of a YFP-PDLP5 transformant. The signal is visible as larger than normal structures, not the discrete punctate signal normally associated with YFP-PDLP5. The lack of resolution from the objective and camera make it difficult to see the distinct puncta forming the aggregates. Other images were obtained with a higher objective (see Appendix, Figure A.1.1). In an associated time-lapse video, the aggregates can be seen to undergo random motion.

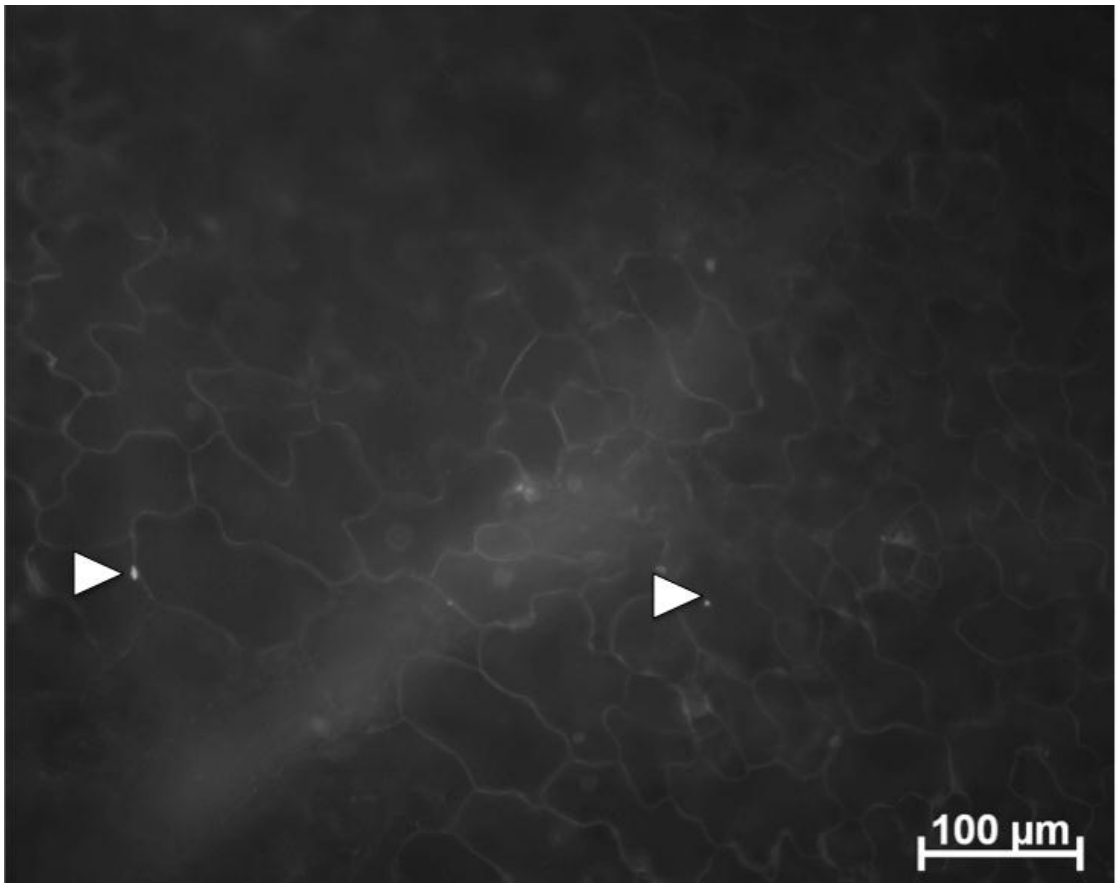

Figure 3.3.2 Fluorescent image of Sample 2, the second mutant of five found with similar signal localization. Punctate signal highlighted with a white dart.

The image was taken on a Zeiss Axioskop 2 at 20X objective, viewing the cotyledon 7 dpg of a YFP-PDLP5 transformant. A series of images was taken in the same X and Y plane but with varying Z points in an attempt to show where the signal appeared in comparison to common structure. Upon changing the Z plane, more signal becomes visible.

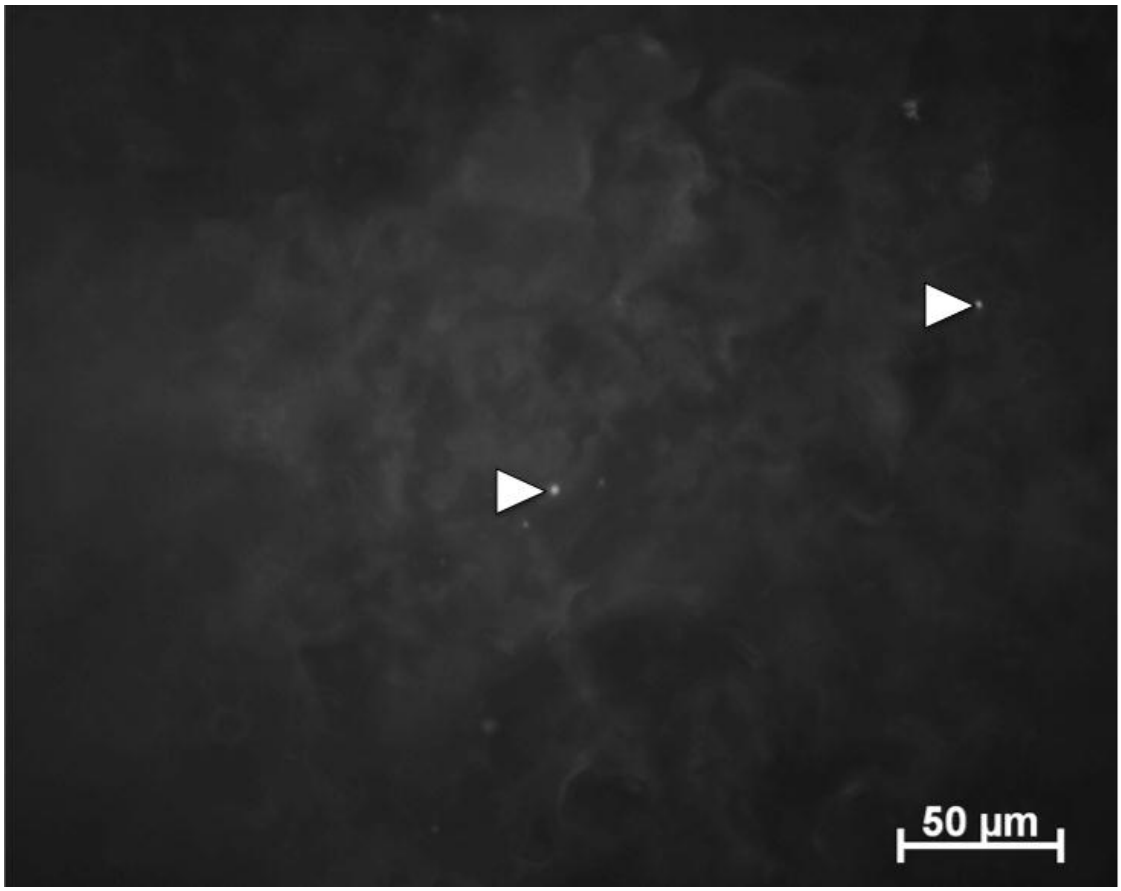

Figure 3.3.2 Fluorescent image of Sample 3, the third mutant of five found with similar signal localization. Punctate signal highlighted with a white dart.

The image was taken on a Zeiss Axioskop 2 at 40X objective, viewing the cotyledon 7 dpg of a YFP-PDLP5 transformant. This sample was the most difficult to capture quality images, but a series of images taken in rapid succession show the bright distinct signal actually to be multiple signals in close proximity which then move away from other puncta. Upon transferring to soil, these samples exhibited a significant phenotypic difference from other transformants of the same plate also transferred to soil. While other samples with some minor aberrations appeared healthy and vital, Samples 1-3 (plus 4 and 5, not imaged) appeared slightly chlorotic and

substantially reduced in size. While only Sample 3 lived past 3 days post transfer to soil, it is the best comparison for size to Sample 1 - 5. Sample 3 showed a significant reduction in size, compared to other seedlings transferred from the same plate on the same day, and they were roughly 8 times the size of Sample 3 after 9 days. Unfortunately, Sample 3 did not survive to maturity. Without the mature plant, future studies of the aberration cannot be continued. Ideally, progeny would have been procured and a T2 seed stock could have been established. At this point, the mature T1 could have been used for genomic DNA extraction and subjected to a procedure called Thermal asymmetric interlaced polymerase chain reaction or TAIL-PCR. This procedure utilizes the known 25 bp repeats that flanks the T-DNA insertion as primers for the PCR. In this way it is possible to determine where in the genome the T-DNA was inserted and as such the gene disrupted, likely causing the phenotypic change in PDLP5 localization.

Chapter 4

Discussion

4.1 Current Findings

Little is known about the localization of the PDLP family members including other molecules, which may aid in a particular step of the process. A study conducted by Thomas et al., 2008, showed that the isoform PDLP1 is transported to the plasmodesmata by utilizing the secretory pathway. From the endoplasmic reticulum, PDLP family members are transported to the Golgi apparatus, where the proteins are sorted and packaged for delivery to the target destination, the plasmodemsata.

A possibility of mislocalization may lie in the folding of the protein. A mutation in a chaperone protein would result in a misfolded protein. Chaperones are proteins that function in the folding/unfolding of other proteins (Chen and Shimamoto, 2011). There are multiple classes of chaperones that function in different situations, as well as co-chaperones, which usually assist chaperone proteins (Chen and Shimamoto, 2011). It is possible that PDLP5 is assisted in the folding process by these chaperone proteins. A misfolded protein may in turn lead to the altered localization of a protein, such as export to the cytosol for degradation.

A number of possibilities exist for the mislocalization of a protein. The protein may have a mutant that causes retention in the endoplasmic reticulum, and the protein would accumulate and not be transported out of the ER. The protein may successfully be transported to the Golgi but may remain in the organelle because of processing

issues. The protein might also be cleared from the Golgi but be exported into the cytosol and likely targeted for degradation.

Approximately 35,000 seedlings have been screened thus far. As would be expected, only a small number of seedlings showed an aberration from the control signal seen above (see Figure 3.1.1, 3.1.2, and 3.1.3). While the transformation efficiency is at a respectable 1.2%, the probability of the T-DNA insertion interacting with a gene that functions in the localization of PDLP5 is incredibly low. The five samples mentioned above all had a similar signal distribution that appeared extracellular. The images taken, in conjunction with time-lapse images, show the signal aggregates, rather than forms discrete punctate, and the, it undergoes movement.

While it is clear that the *Agrobacterium*-mediated transformation was a success, there is a caveat to the process. *A. tumefaciens* insert the segment of transfer-DNA randomly into the host plant genome. This is both the strength and downfall of the transformation event. As the other molecules involved with PDLP5 are unknown, the random insertion of the transfer-DNA is a way to discover the gene products that interact with PDLP5. This means the insertion may be in a region of DNA that encodes for a gene product which is responsible for transporting PDLP5 to the PD, or that the T-DNA is inserted in a region that plays no role in PDLP5 localization or function. The vast majority of T-DNA integration events will fall into the second category, strictly in adherence with probability. This explains why the majority of screened seedlings (of the greater than 30,000) have exhibited normal localization patterns similar to the parental control lines. It is also possible, although unlikely again according to probability, that the T-DNA could be inserted into the region of DNA

encoding PDLP5 itself. This integration event could have a range of possibilities including phenotypic changes at the morphological level as well as in the localization of the protein itself. It is important to note that the above-mentioned mutants (Sample 1 - 5) may have received the T-DNA insertion in this region.

PDLP5 over-expression plants have a distinct phenotype from both wild-type and the severe knock-down pdlp5-1. The over-expression plants display chlorosis, while the knock-down appears as wild-type, healthy and vital (Page 6; Lee et al., 2011). These over-expression plants also exhibit a stunted growth phenotype, in which the seedling is much smaller in size compared to wild-type, but is able to develop and reach maturity (Lee et al., 2011). The size reduction, along with fluorescent images of mislocalization, seen in Sample 3 could indicate that the T-DNA insertion did indeed have an effect on the PDLP5 pathway in some manner. It is tempting to believe that the developmental issues associated with Sample 3 are a direct result from impediment of the PDLP5 pathway, causing a more extreme phenotype as compared to the PDLP5 over-expression line. Alas, without the mature plant or progeny, it cannot be determined.

4.2 Further Work

Although this study did not result in the determination of molecular players involved in the function of PDLP5, from a probability standpoint, continued screening may yet result in isolation of additional mutants. The rapidity of the screening process is the limiting factor. The studies already conducted and described in this document report proof of concept although it requires a large investment of time to screen a large pool of transformants. The transformation of the parent lines (PDLP5-GFP and YFP-PDLP5) was successful when considering the efficiency, which fell between the

percentages of 1% to 3% as listed in the literature (Clough and Bent, 1998). The methodology of transformant selection also proved to be an effective method. The T-DNA insertion was shown to alter the localization of fluorescent signal in a small number of mutants dictating that the project was successful on all fronts except the genomic analysis of a mutant. Generation of additional T1 plants and continued screening would eventually result in additional mutants for genomic analysis.

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Appendix A

Supplementary Images and Protocol

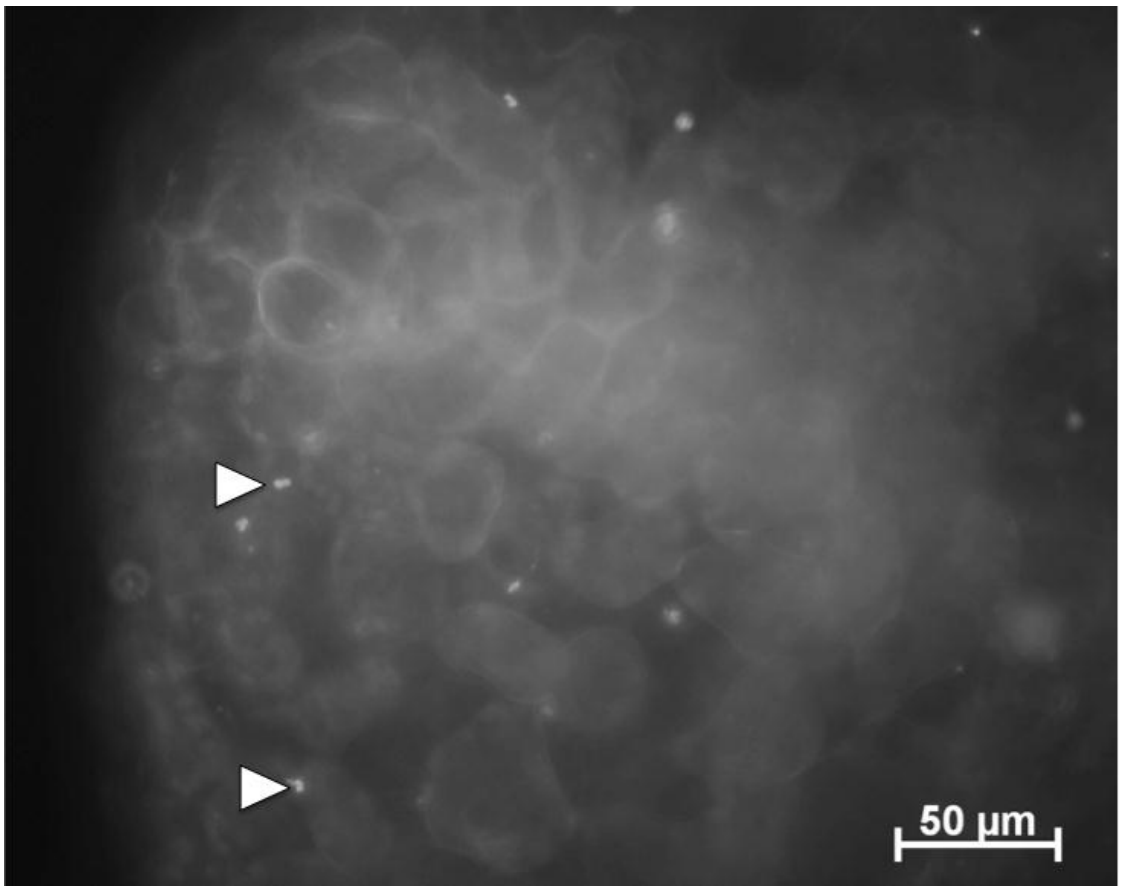

Figure A.1.1 Fluorescent image of Sample 1, with punctate signal highlighted with a white dart.

The image was taken on a Zeiss Axioskop 2 at 40X objective, viewing the cotyledon 7 dpg of a YFP-PDLP5 transformant. This image is an increased

magnification of Figure 3.3.1. The increased magnification and resolution shows the signal to be not only larger than normal structures of YFP-PDLP5, but also what appear to be multiple signals aggregating together.

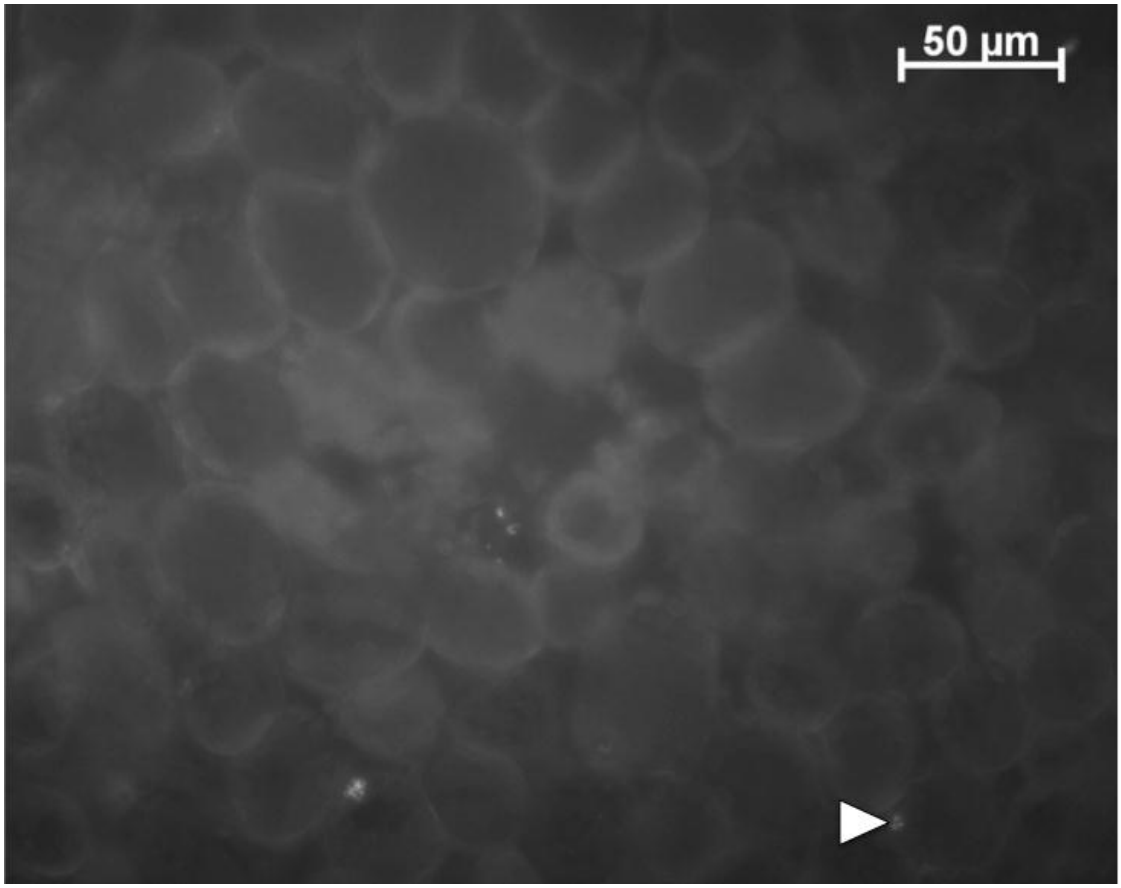

Figure A.1.2 Fluorescent image of Sample 2, with punctate signal highlighted with a white dart.

The image was taken on a Zeiss Axioskop 2 at 40X objective, viewing the cotyledon 7 dpg of a YFP-PDLP5 transformant. The dart in the figure points to a cluster of 3 distinct puncta.

Phase 1 of Protocol

Day 1: Prepare the appropriate amount of flats by filling the plastic inserts with soil of choice. Pour water with Gnatrol into the flat and allow the soil to soak for 15 minutes. If the top of the soil is no longer level with the top of the insert, add more soil and water if needed to saturate the soil. Fold a piece of white printer paper in half and pour a small quantity of seeds into the middle. Gently tap the paper until the seeds are at the edge of the fold and continue to tap the paper to deposit the seeds onto the saturated soil. After sowing the seeds, place translucent lids on the flats and transport to 4°C conditions for 48 hours.

Day 3: After 48 hours, transport to growth chambers with conditions of 16 hours of light and 8 hours of darkness at 22°C with 50% humidity.

Day 17: Allow the plants to grow about 2 weeks until the central stem emerges. When the stem is about two cm, clip the stem at the base. Allow plants to continue growing until inflorescences are between two and ten cms.

Day 28: Three days prior to transformation, innoculate 5 ml of liquid LB medium with *A. tumefaciens* carrying the desired vector (from frozen cell stock) and incubate 28°C with vigorous agitation (200 RPM) overnight.

Day 30: Two days after the initial inoculation, inoculate 1 L of liquid LB media with 3 ml of the preculture grown for another day until the optical density is between 0.6-1.0. Pellet the culture at 4000 rpm for 20 minutes and re-suspend in a solution containing 5% sucrose and 0.2% silwet-77, a surfactant. Dip the flowers into the solution and soak with gentle agitation for about 60 seconds. Line the flats with moist paper towels and lay the pots their side and cover with a translucent lid for 24 hours.

Day 31: After the 24-hour period, return the plants to upright, normal growing conditions.

Day 50: Continue to grow until plants are mature and harvest the seeds by gently massaging the siliques. Remove plant debris by using a sieve to separate out the seeds from debris and place seeds in packets for drying and storage.

Phase 2 of Protocol

Day 1: Prepare ½ MS medium with 2.17 grams of MS per liter of water and adjust the pH to 5.7. Transfer one-half liter of ½ MS medium into a 1 L flask and add 4 grams of agar, then autoclave. Add kanamycin and glufosinate ammonium to a final concentration of 50 μg/ml and 10 μg/ml respectively.

Day 2: Sterilize seeds in a biosafety cabinet. Aliquot approximately 300 μl of seeds into 2 ml microfuge tubes then wash with one ml of a 3% bleach solution, vortex at high speed for 3 minutes, and spin down in a centrifuge. Decant the bleach solution. Add one ml of 70% ethanol, vortex at high speed for 30 seconds, and spin down. Decant the ethanol. Wash the seeds five times with one ml of sterile water, while vortexing during each wash, ending with a resuspension in one ml of sterile water. Using sterile one ml pipette tips, deposit seeds onto the ½ MS plates in an orderly column fashion. Seal the plates with surgical micropore tape and move to a 4°C cold room.

Day 4: After 48 hours move the plates to a 22°C incubator with 24-hour light conditions.

Day 11: Remove the plates from the incubator and check for transformants. Carefully remove one cotyledon from the transformant with a sharp pair of forceps and place upside down on a microscope slide. Add some water and place a glass

coverslip over the cotyledons. Gently apply pressure to the coverslip. View the cotyledons under fluorescence. If the seedling exhibits an aberrant signal, transfer to a flat of prepared soil and grown to maturity.