PATELLIN1/2 interact with CVL1 and CVP2 to regulate vascular development in Arabidopsis thaliana

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PATELLIN1/2 interact with CVL1 and CVP2 to regulate vascular development in Arabidopsis thaliana

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Submitted in Partial Fulfillment of the Prerequisite for Honors in Biological Sciences

Wellesley College
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To Kaye Peterman, thank you so much for all of your patience and for pushing me to always keep improving. Working with you has shaped who I am as a scientist and I am so grateful for everything you have taught me.

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# TABLE OF CONTENTS

Abstract.................................................................4

List of Figures and Tables.........................................5

List of Abbreviations...............................................6

Introduction...........................................................8

Methods...............................................................25

Results.................................................................33

Discussion............................................................66

References............................................................79
ABSTRACT

Unidirectional flow of the phytohormone auxin through files of cells occurs due to polar localization of PIN1 and is responsible for vascular development in *Arabidopsis thaliana*. The endosomal recycling pathway that establishes and maintains proper PIN1 localization is coordinated by phosphoinositide species within the cell’s membranes. In order to determine if the Sec14-like proteins PATELLIN1/2 and the related PATELLIN6 are involved in regulating these phosphoinositide pools during vascular development, *PATELLIN1/2* and other known vascular genes in this pathway or *PATELLIN6* were knocked out and analyzed for evidence of genetic interactions. Analysis of the cotyledon venation of these higher order mutants revealed *PATELLIN1/2* interact with *CVL1* and *CVP2*, but only weakly with *VAB*. Additionally, evidence showed *PATELLIN6* plays a role in vascular development, but is not redundant with *PATELLIN1/2*. Therefore, this study provides evidence that *PATELLIN1/2* play a role in regulating the PtdIns(4,5)P2 pools that are necessary for PIN1 endocytosis, while *PATELLIN6* plays a unique role during vascular development.
LIST OF FIGURES AND TABLES

Figure 1: The cotyledon vascular pattern

Figure 2: Genotypic identification of T-DNA insertion mutants

Table 1: Primer Sequences for identification of vascular mutations

Figure 3: Scoring categories for cotyledon analysis

Table 2: Average number of vascular defects, aeroles, free ends, and branch points in the \( p1p2vab \) triple mutant

Figure 4: \( p1p2vab \) mutants show subtle differences from \( p1p2 \) and \( vab \) mutants

Figure 5: \( p1p2vab \) cotyledon venation forms slightly fewer areoles than \( vab \)

Figure 6: \( p1p2vab \) cotyledons have subtly reduced vascular complexity compared to \( vab \)

Table 3: Initial analysis of vascular defects in the \( p1p2cvl1 \) triple mutant

Figure 7: \( p1p2cvl1 \) triple mutants show a slight decrease in vascular complexity compared to \( cvl1 \) mutants

Figure 8: \( p1p2cvl1 \) triple mutants have a slightly less complex vascular pattern

Figure 9: Characterization of \( p1p2cvp2 \) and \( p1p2cvl1cvp2 \) mutants

Figure 10: \( p1p2cvp2 \) triple mutants have an increase in vascular defects and a decrease in vascular complexity compared to \( cvp2 \)

Table 4: Average number of vascular defects, aeroles, free ends, branch points and secondary veins in \( p1p2cvp2 \) and \( p1p2cvl1cvp2 \) mutants

Figure 11: The \( p1p2cvp2 \) cotyledon vascular pattern is less complex than \( cvp2 \)

Figure 12: \( p1p2cvl1cvp2 \) quadruple mutants do not have more severe phenotypes than \( cvl1cvp2 \) mutants

Figure 13: \( p1p2cvl1cvp2 \) and \( cvl1cvp2 \) show similar decreases in areoles

Figure 14: Characterization of \( patl-6 \) and \( p1p2p6 \) mutants

Table 5: Average number of vascular defects, aeroles, free ends, branch points and secondary veins in \( p6 \) and \( p1p2p6 \) mutants

Figure 15: \( p1p2p6 \) triple mutants do not show enhanced vascular defects or delays compared to \( p6 \)
Figure 16: *p6* causes a decrease in the complexity of the cotyledon vascular pattern compared to wild type.

**LIST OF ABBREVIATIONS**

5PTase: Phosphatidylinositol-5-phosphatase  
Col: Columbia  
ARF: ADP-ribosylation factor  
AGD3: ARF-GAP domain3  
CVL1: CVP2-like 1  
CVP2: Cotyledon vascular pattern 2  
EtOH: Ethanol  
FKD: Forked  
GAP: GTPase activating protein  
GEF: GTPase exchange factor  
GOLD: Golgi dynamic  
GSP: Gene specific primer  
GUS: β-glucuronidase  
PATL: Patellin  
*p1p2*: *patellin1 patellin2*  
PCR: Polymerase chain reaction  
PH: Pleckstrin homology  
PI: Phosphoinositide  
PIP: Phosphorylated phosphoinositide
<table>
<thead>
<tr>
<th>Acronym</th>
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<tr>
<td>PtdIns(3)P</td>
<td>Phosphatidylinositol-3-phosphate</td>
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<tr>
<td>PtdIns(4)P</td>
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<td>PtdIns(5)P</td>
<td>Phosphatidylinositol-5-phosphate</td>
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<tr>
<td>PtdIns(4,5)P$_2$</td>
<td>Phosphatidylinositol-4,5-biphosphate</td>
</tr>
<tr>
<td>PIN1</td>
<td>PINFORMED1</td>
</tr>
<tr>
<td>PtdIns</td>
<td>Phosphatidylinositol</td>
</tr>
<tr>
<td>SFC</td>
<td>SCARFACE</td>
</tr>
<tr>
<td>T-DNA</td>
<td>Transfer-DNA</td>
</tr>
<tr>
<td>TGN</td>
<td>trans-Golgi network</td>
</tr>
<tr>
<td>VAB</td>
<td>VAN3 binding protein</td>
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<td>VAN3</td>
<td>Vascular network defective 3</td>
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<td>WT</td>
<td>Wild type</td>
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INTRODUCTION

The structure and function of vascular tissue

In order for terrestrial plants to grow more than a few centimeters from the ground, evolution of specialized vascular tissue was required (Raven, 1993). The veins formed by the vascular tissue provide mechanical support and carry water from the parts of the plant in direct contact with the soil upward, allowing plants to grow vertically (Raven, 1993). In addition to water, veins also transport nutrients and signaling molecules throughout the plant, which allowed for the development of specialized organs in vascular plants (Cook and Graham, 1999; Graham, 1993; Niklas, 1997; Schulz, 1998; Taylor, 1990; van Bel et al., 2002). These molecules are transported by two types of specialized vascular tissue, the xylem and the phloem, which make up the vascular bundles. Phloem transports photoassimilates, the products of photosynthesis, and xylem transports water and mineral nutrients. Continuous files of these specialized cell types transport the water, sugars, nutrients and signaling molecules required for proper growth, development and response to the environment.

For effective transport, dicots have developed highly branched vascular systems that form predictable patterns in members of the same species. In the leaves of Arabidopsis thaliana, a primary midvein grows first, followed by a network of connected secondary veins (Bowman, 2012). Tertiary veins then extend from the secondary veins, either forming connections between secondary veins or ending freely, followed by quaternary veins that extend from the tertiary veins and almost always end freely (Bowman, 2012). However, the cotyledons (embryonic leaves) have a simpler vein pattern composed of only primary and secondary veins (Bowman, 2012). The primary vein grows
down the center of the cotyledon from the base to the apical end (Fig. 1). Once this midvein is formed, one set of secondary veins begins to grow out and down in the opposite direction and each connect with the midvein approximately halfway down the cotyledon to form two closed loops (areoles) (Fig. 1). A second set of secondary veins then begin to grow near this connection point in the same direction as the first set, connecting with the midvein just above the petiole to form two more closed loops (Fig. 1) (Bowman, 2012).

![Figure 1. The cotyledon vascular pattern.](image)

Figure 1. The cotyledon vascular pattern. Cotyledons were cleared in 9:3:1 (w/v/v) chloral hydrate: water: glycerol and imaged using darkfield microscopy. In a wild type cotyledon, the midvein (1) grows first, forming from the base to the distal end. The first set of secondary veins begin at the tip of this midvein and grow downward and connect with the midvein about halfway down the cotyledon (2). A second set of secondary veins form proximal to the first set and grow downward to connect with the midvein at the base of the cotyledon (3) forming the complete cotyledon vascular pattern.
The stages of vascular development

Vascular tissue undergoes several developmental stages before becoming mature vascular tissue in these predictable patterns. From a field of initially identical ground meristem cells, all cells develop into either premesophyll or preprocambial tissues (Nelson and Dengler, 1997). Although these cell populations still appear identical, they are determined for mesophyll or vascular cell fate (Nelson and Dengler, 1997). From these preprocambial cells, the structurally distinct, elongated procambial cells, which are the stem cell precursors to vascular tissue, are recruited (Nelson and Dengler, 1997). This procambial development begins in the embryo and is halted until germination. Once the procambium is fully established, which occurs in the cotyledon by one day after germination (Steynen and Schultz, 2003), the pattern of the procambial cells predicts the final vein pattern (Jurgens et al., 1994; Sieburth, 1999). The final differentiation of procambial cells into mature vascular cells is believed to release factors, such as xylogen, that induce the differentiation of neighboring cells, causing formation of the final vascular pattern (Motose et al., 2004).

Unidirectional auxin flow triggers vascular differentiation

The auxin canalization hypothesis attributes this recruitment of procambial cells and vascular differentiation to the unidirectional flow of the phytohormone auxin through files of cells that are destined to become the vascular tissue (Sachs, 1981). Auxin expression, which has been visualized through GUS expression under a D5 synthetic auxin responsive promoter, is initially broad (Scarpella et al., 2006) and high levels of auxin are found in the leaf margin, which serves as a significant auxin source during early embryonic development (Mattsson et al., 2003; Sieburth, 1999; Steynen and Schultz,
2003). As embryonic development continues, auxin expression narrow to files of cells that will become the procambium (Scarpella et al., 2006).

Once auxin expression has narrowed, positive feedback mechanisms cause auxin to promote its own transport, creating unidirectional auxin flow specifically through these files of cells (Sachs, 1981, 1991). By investigating auxin response genes and the effect of exogenously applied auxin, researchers have identified auxin flow as a trigger for the gene expression patterns necessary for vascular differentiation (Mattsson et al., 2003; Mattsson et al., 1999; Sieburth, 1999). Specifically, auxin flow helps regulate the activity of MONOPTEROS, a transcription factor that binds to the promoters of auxin-responsive genes necessary for vascular differentiation (Deyholos et al., 2000; Hardtke and Berleth, 1998; Przemeck et al., 1996). If auxin efflux is inhibited with naphthylphthalamic acid, preventing proper auxin flow, discontinuous vein patterns form (Scarpella et al., 2006; Wenzel et al., 2007). Therefore, the unidirectional flow of auxin is necessary for activating proper development and patterning of vascular tissue.

PIN1 polarity establishes unidirectional auxin flow

Proper unidirectional auxin flow is established through control over its influx into and efflux from the cell (Sachs, 1991). The PINFORMED (PIN) proteins are a family of auxin efflux carriers that influence auxin distribution to regulate cell division and growth (Blilou et al., 2005). These PIN proteins become polarly localized to one side of the plasma membrane, limiting auxin efflux to one direction and consequently creating a polar flow of auxin (Galweiler et al., 1998). One member of this family, PIN1, has been connected to vascular patterning in Arabidopsis. PIN1 is initially expressed diffusely then narrows to single files of preprocambial cells that predict the final vein pattern.
Within these cells, PIN1 is initially localized apically to create a distal maximum of auxin (Benkova et al., 2003; Reinhardt et al., 2003) before switching to basal localization to initiate midvein (Bayer et al., 2009; Hou et al., 2010; Scarpella et al., 2006; Wenzel et al., 2007) and secondary vein (Bilsborough et al., 2011; Scarpella et al., 2006; Wabnik et al., 2010; Wenzel et al., 2007) formation.

Genetic removal or disruption of PIN1 causes defects in polar auxin flow and disorganization of the vascular pattern, demonstrating the importance of proper PIN1 function (Galweiler et al., 1998; Mattsson et al., 1999; Steinmann et al., 1999). As a result, pin1 knockout mutants have increased vascularization in the leaf margins with no clear vein pattern, as well as other developmental defects such as smaller cotyledons and fused leaves (Mattsson et al., 2003; Steinmann et al., 1999). Furthermore, auxin flow is necessary for maintenance of PIN1 polarity, causing a positive feedback loop in which auxin promotes its own polar transport (Geldner et al., 2001). Mechanistically, auxin controls the localization of PIN1 in preprocambial cells by regulating its internal trafficking (Geldner et al., 2001; Paciorek et al., 2005; Pan et al., 2009).

**PIN1 polarity is established and maintained by an endosomal recycling pathway**

PIN1 polarity is not established or maintained without continual trafficking through an endosomal recycling pathway (Geldner et al., 2003; Jaillais and Gaude, 2007). Therefore, proper PIN1 localization relies on a dynamic process in which PIN1 is endocytosed and transported to the trans-Golgi network (TGN) and the recycling endosome before returning to the plasma membrane. In addition to this recycling, vesicles containing PIN1 can also be targeted to the lysosome via the late endosome/pre-vacuolar...
complex (Kleine-Vehn et al., 2008; Laxmi et al., 2008; Oliviusson et al., 2006; Spitzer et al., 2009). This degradation pathway is important for regulating several developmental processes, including vascular development and patterning (Pahari et al., 2014; Spitzer et al., 2009). The targeting of PIN1 for degradation is regulated by UNHINGED-1, which encodes a component of the Golgi-associated retrograde complex that returns important targeting molecules to the Golgi following vesicle fusion with the lysosome (Pahari et al., 2014). unhinged-1 knockout mutants have discontinuity in their vascular patterns and decreased distal connections between secondary veins, indicating that PIN1 degradation is important for proper auxin flow and vascular patterning (Pahari et al., 2014). Therefore, the regulation of PIN1 levels and localization can both be regulated by internal PIN1 trafficking.

One factor that is known to influence the trafficking pathways responsible for both the initial establishment and the maintenance of PIN1 polarity is GNOM (Geldner et al., 2003; Steinmann et al., 1999). GNOM is an ARF GEF that has been shown to regulate the endocytosis of PIN1 and the transport of PIN1 from the recycling endosome to the plasma membrane (Geldner et al., 2003; Koizumi et al., 2005). gnom mutants have increased vascular differentiation in the leaf margin and disorganization of the vein pattern, indicating that auxin produced in the leaf margins cannot be properly transported toward the center of the leaves (Galweiler et al., 1998; Mattsson et al., 2003; Steinmann et al., 1999). This aberrant venation pattern resembles that seen in pin1 mutants, which suggests that GNOM is necessary to establish PIN1 polarity early in vascular development (Mattsson et al., 2003; Steinmann et al., 1999).
Vesicle trafficking at the trans-Golgi network is necessary for continuous vein formation

In contrast to gnom mutants, other vascular mutants show discontinuity in the venation without changes to the overall vascular pattern, suggesting that some vascular genes are only required for maintenance of PIN1 polarity. Several of these vascular gene products have been localized to the trans-Golgi network (TGN) (Carland and Nelson, 2009; Deyholos et al., 2000; Hou et al., 2010; Koizumi et al., 2005; Steynen and Schultz, 2003). Proper vesicle formation and targeting from the TGN is important for the PIN1 recycling and degradation pathways (Pahari et al., 2014; Spitzer et al., 2009). Therefore, the TGN localization of these gene products and the disruption of vascular continuity observed when they are mutated suggest that these vascular genes function in vesicle formation and targeting pathways at the TGN (Carland and Nelson, 2009; Deyholos et al., 2000; Hou et al., 2010; Koizumi et al., 2005; Steynen and Schultz, 2003).

**Phosphoinositides**

One class of molecules that plays an important role in the membrane trafficking pathways that are responsible for vascular patterning is phosphoinositides (PIs) (Heilmann, 2016a; Ischebeck et al., 2013). PIs are phosphorylated derivatives of the membrane lipid phosphatidylinositol (PtdIns) that can be reversibly phosphorylated and dephosphorylated by specific PI kinases and phosphatases respectively (Munnik and Nielsen, 2011; Strahl and Thorner, 2007). PIs are composed of fatty acid tails that are embedded in membranes and polar inositol head groups that extend into the cytosol (Di Paolo and De Camilli, 2006). There are several locations on the inositol head that can be phosphorylated, and so the extent and localization of phosphorylation creates different PI molecules that serve specific signaling and regulatory functions (Munnik and Nielsen, 2011; Strahl and Thorner, 2007). The phosphorylation pattern of PIs allows them to bind
with specificity to lipid binding domains of proteins, such as the family of Pleckstrin homology (PH) domains (Lemmon, 2008). It has also been proposed that PIs are further distinguished into microdomains due to the levels of unsaturation in their fatty acid tails, adding an extra layer of specificity in PI-target binding (Heilmann, 2016a).

These binding events can impact the localization and catalytic activity of proteins (Cullen et al., 2001; Itoh and Takenawa, 2002) and since PI phosphorylation is reversible, PIs can help regulate pathways responsible for dynamic and complex events such as growth and development (Gujas and Rodriguez-Villalon, 2016; Heilmann, 2016a). In the context of vascular development, phosphoinositides have been show to act with other phospholipids (Markham et al., 2011; Men et al., 2008) to regulate PIN1 trafficking. While several phosphoinositides have been implicated in this PIN1 trafficking pathway, they have distinct and limited patterns of subcellular localization (Heilmann and Heilmann, 2015). This spatial separation suggests that different phosphoinositides play particular, non-redundant roles in PIN1 trafficking by recruiting specific effector proteins or initiating distinct signaling pathways (Heilmann and Heilmann, 2015).

One specific PI that has been linked to PIN1 trafficking is phosphatidylinositol-4-phosphate (PtdIns(4)P). Using the PH domains of FAPP1 and OSBP, which are known PtdIns(4)P-binding proteins, PtdIns(4)P was localized to the plasma membrane and to a lesser extent to the TGN/early endosome (Simon et al., 2014; Simon et al., 2016; Vermeer et al., 2009). The pool of PtdIns(4)P at the plasma membrane creates a highly negative charge, which electrostatically interacts with positively charged proteins to localize them to the plasma membrane (Simon et al., 2016). Specifically, PINOID, a polar auxin transport regulator that phosphorylates PINs and other auxin efflux carriers, localizes to
the plasma membrane due to interactions with this electrostatic field, which demonstrates the importance of this PtdIns(4)P pool in PIN trafficking (Simon et al., 2016). In contrast, the pool of PtdIns(4)P at the TGN is much smaller (Simon et al., 2014). Therefore, effector proteins need to bind to additional factors in the TGN, such as Arf1, to increase their avidity for the TGN in order to localize to this membrane over the plasma membrane (Simon et al., 2016). Therefore, PtdIns(4)P may play an important role in regulating membrane trafficking of PIN1 at the plasma membrane and at the TGN through recruitment of specific effector proteins.

In addition to recruiting important effector proteins, PtdIns(4)P helps regulate PIN1 trafficking by serving as a substrate for production of phosphatidylinositol-4,5-biphosphate (PtdIns(4,5)P$_2$) (Heilmann and Heilmann, 2015). PtdIns(4,5)P$_2$ is localized to the apical and basal ends of the plasma membrane (Simon et al., 2014) and the PIP kinases that produce it colocalize with PIN1 in the embryo, in the lateral root primordium, and in the root apical meristem (Ischebeck et al., 2013; Tejos et al., 2014). When these PtdIns(4,5)P$_2$ levels are depleted, fewer clathrin coated vesicles are formed off of the plasma membrane (Ischebeck et al., 2013) and PIN1 polarity is lost in the vascular tissue (Ischebeck et al., 2013; Tejos et al., 2014). This subcellular defect corresponds to reduced auxin transport, abnormal cell division, vascular discontinuities, growth defects, and an increase in embryonic lethality (Ischebeck et al., 2013; Tejos et al., 2014). Therefore, both PtdIns(4,5)P$_2$ (Ischebeck et al., 2013; Tejos et al., 2014) and PtdIns(4)P (Simon et al., 2014; Simon et al., 2016) appear to influence auxin transport by regulating PIN1 trafficking to and from the plasma membrane.
CVL1/CVP2

In Arabidopsis thaliana, PtdIns(4,5)P_2 is dephosphorylated by the 5TPases COTYLEDON VASCULAR PATTERN 2 (CVP2) and CVP2 LIKE 1 (CVL1) (Carland and Nelson, 2009; Carland and Nelson, 2004). These 5PTases can therefore help regulate the relative levels of PtdIns(4,5)P_2 and to a lesser extent PtdIns(4)P necessary to activate or localize a protein target (Stevenson et al., 2000). Other 5PTases play a role in terminating inositol(1,4,5)P_3 signaling, but CVP2 and CVL1 have no activity against IP_3s, suggesting their role is specific to regulation of PtdIns(4,5)P_2 levels (Carland and Nelson, 2009; Kaye et al., 2011). While the cvp2 and cvll single mutants have weak or no aberrant phenotypes, the cvllcvp2 double mutant has open vein patterns due to discontinuity in the vascular tissue, suggesting that these two 5PTases have functionally redundant roles in vascular patterning (Carland and Nelson, 2009; Naramoto et al., 2009). However, CVP2 has higher catalytic activity than CVL1 in vitro and the cvp2 mutant shows early termination of vascular elongation, while the cvll mutant resembles wild type, suggesting an unequal redundancy in which CVP2 plays a more significant role in PtdIns(4,5)P_2 degradation and PtdIns(4)P production (Carland and Nelson, 2009; Carland et al., 1999; Carland and Nelson, 2004). These defects in vascular patterning in the cvp2 and cvllcvp2 mutants are seen in the preprocambium and procambium, indicating that CVP2 and CVL1 play a role in proper preprocambium formation (Carland et al., 1999; Carland and Nelson, 2004). Similar discontinuities are observed in the leaves and roots of overexpression mutants, suggesting that tight regulation of PtdIns(4,5)P_2 and PtdIns(4)P levels are necessary for proper preprocambial formation (Rodriguez-Villalon et al., 2015).

Consistent with a role in preprocambial formation, CVP2 and CVL1 follow the expression patterns of auxin and PIN1, narrowing from broad expression to specific files
of cells that will develop into the procambium (Carland and Nelson, 2004). Therefore, the regulation of PtdIns(4,5)P_2 levels by CVP2 and CVL1 likely establishes proper auxin flow by regulating endocytosis of PIN1 (Naramoto et al., 2009). This proposed role of CVP2 and CVL1 is supported by the inability of cvl1cvp2 double mutants to maintain PIN1 polarity after it is initially established (Naramoto et al., 2009), suggesting CVP2 and CVL1 are necessary for the endosomal recycling of PIN1. Through their regulation of PtdIns(4,5)P_2 levels, CVP2 and CVL1 can maintain PIN1 polarity by controlling the recruitment and activity of downstream effectors responsible for vesicle trafficking of PIN1 (Naramoto et al., 2009).

**VAN3/SFC/FKD2/AGD3**

A discontinuous vein pattern is also observed when VASCULAR NETWORK DEFECTIVE 3 (VAN3) is mutated (Deyholos et al., 2000; Koizumi et al., 2005; Koizumi et al., 2000). VAN3 is also known as SFC (Deyholos et al., 2000), FKD2 (Steynen and Schultz, 2003), and AGD3 (Vernoud et al., 2003), but we will refer to this gene as VAN3. VAN3 is expressed in the vascular tissue of the leaves and roots and encodes an ADP-ribosylation factor GTPase activating protein (ARF GAP) (Carland and Nelson, 2009; Koizumi et al., 2005; Sieburth et al., 2006). The family of ARF proteins plays important roles in regulating the recruitment of coat proteins that are necessary for proper vesicle formation (Donaldson and Klausner, 1994; Moss and Vaughan, 1995, 1998) and ARF GAPs help regulate this activity. VAN3 binds to PtdIns(4)P through its PH domain, which increases its ARF GAP activity and causes localization to the TGN (Koizumi et al., 2005; Naramoto et al., 2009; Sieburth et al., 2006). Therefore, CVP2/CVL1 likely help regulate the role of VAN3 in vesicle formation at the TGN through production of appropriate PtdIns(4)P pools (Naramoto et al., 2009).
This ARF GAP activity of VAN3 has been connected to auxin transport and PIN1 recycling (Carland and Nelson, 2009; Deyholos et al., 2000; Koizumi et al., 2005; Naramoto et al., 2010). Similar to auxin and PIN1, VAN3 expression is initially broad and then narrows to the procambial cells (Carland and Nelson, 2009). Since van3 mutants have discontinuous procambial patterns (Deyholos et al., 2000), VAN3 appears to play a role in procambial development. Combined, these findings suggest that VAN3 helps regulate polar auxin flow, which is supported by the altered auxin transport and response in van3 mutants (Deyholos et al., 2000; Koizumi et al., 2005; Sieburth et al., 2006). Given that VAN3 interacts with DRP1A, a dynamin related protein, this role in auxin transport appears to be connected to proper vesicle formation (Fujimoto et al., 2010; Sawa et al., 2005).

Studies of genetic interactions involving VAN3 suggest that VAN3 specifically plays a role in PIN1 vesicle trafficking (Deyholos et al., 2000; Koizumi et al., 2005; Naramoto et al., 2010; Sieburth et al., 2006). Genetic interactions can be identified by comparing the phenotypes of single mutants to those of double or higher order mutants in which those mutations are combined. If the double mutant phenotype is an additive combination of the two single mutant phenotypes, it suggests that the two mutations are acting independently and that they have no functional relationship (Martienssen and Irish, 1999). However, if the double mutant displays a phenotype that is more severe than the additive effects of the single mutations, it indicates a genetic interaction (Perez-Perez et al., 2009). Given the high level of genetic redundancy found in plants, single knockouts are unlikely to completely disrupt a biological pathway, but rather simply reduce the level of certain products or complexes (Jaillon et al., 2007). Therefore, a synergetic effect in
double mutants can occur when two mutations cause reductions in the same products or complexes, causing a more severe impairment than the additive effect of each mutation (Perez-Perez et al., 2009). For that reason, synergistic genetic interactions observed in double mutants suggest that two genes interact directly, act in the same pathway or complex, and/or act on the same substrate (Perez-Perez et al., 2009).

Through these methods, genetic interactions were identified between VAN3 and GNOM (Deyholos et al., 2000; Koizumi et al., 2005; Naramoto et al., 2010; Sieburth et al., 2006), MONTOPEROS (Deyholos et al., 2000), and CVL1/CVP2 (Naramoto et al., 2009). Given these interactions and the colocalization of VAN3 and CVL1/CVP2 at the TGN, VAN3 appears to regulate the formation of vesicles that transport PIN1 at the TGN (Naramoto et al., 2009). Without this regulation of vesicle formation, PIN1 polarity would not be properly maintained, leading to discontinuities in the venation like those seen in van3 mutants (Deyholos et al., 2000; Koizumi et al., 2005; Sieburth et al., 2006).

**VAB/FORKED1**

First identified as a binding partner of VAN3, VAN3-binding protein (VAB), also known as FORKED1 (Steynen and Schultz, 2003), is required for distal vein connections that complete the proper closed vein pattern in cotyledons, leaves, sepals and petals (Hou et al., 2010; Naramoto et al., 2009; Steynen and Schultz, 2003). VAB is expressed at high levels in preprocambial cells, predicting the vascular pattern, and its expression narrows and decreases upon vascular differentiation (Hou et al., 2010). Furthermore, vab mutants have decreased distal connections and lowered auxin response in the provascular and mature vascular tissue (Hou et al., 2010; Steynen and Schultz, 2003). Therefore, VAB appears to play a role in auxin transport and response necessary for a closed vascular pattern during early development (Hou et al., 2010; Steynen and Schultz, 2003). vab
mutants have initial PIN1 polarity in the vascular tissue, but this polarity is lost as vascular differentiation progresses (Hou et al., 2010; Naramoto et al., 2009). Similarly, the pin1vab double mutant has increased vascularization in the leaf margin when compared to the pin1 single mutant, including at the distal end, indicating that loss of PIN1 prevents the distal defects associated with vab mutation (Steynen and Schultz, 2003). These results suggest that VAB acts in a PIN1 localization pathway, causing vab mutations to be negated when there is no PIN1 to act on (Steynen and Schultz, 2003). Therefore, VAB appears to regulate auxin transport through PIN1 trafficking (Hou et al., 2010; Naramoto et al., 2009; Steynen and Schultz, 2003).

Specifically, VAB was shown to interact with VAN3 in order to regulate the vesicle trafficking necessary for maintaining PIN1 polarity (Hou et al., 2010; Naramoto et al., 2009; Steynen and Schultz, 2003). van3vab-1 double mutants have increased vascular discontinuity in the cotyledons and leaves when compared to either single mutant, suggesting the two proteins work in the same pathway (Naramoto et al., 2009). Similar to VAN3, VAB localizes to the TGN and contains a PH domain that binds to PtdIns(4)P (Hou et al., 2010; Naramoto et al., 2009). Yeast two hybrid systems have shown direct binding between VAN3 and VAB, and proper VAN3 localization to the TGN is dependent on VAB expression (Naramoto et al., 2009). Given that proteins with PH domains often need to form complexes before they have sufficient affinity to their target membranes (Lemmon and Ferguson, 2000), this evidence suggests that VAB functions to guide VAN3 to its proper subcellular location through PtdIns(4)P binding, allowing for vesicle budding off the TGN (Naramoto et al., 2009).
**PATL1/2**

Another set of proteins that may be involved in this PIN1 trafficking pathway at the TGN is the pair of redundant proteins Patellin 1 and Patellin 2 (PATL1/2). A total of six patellin proteins have been identified in *Arabidopsis* and phylogenetic analysis separates them into four distinct clades, PATL1/2, PATL4, PATL6, and PATL3/5 (Peterman et al., 2006). Despite these distinctions, each member of the patellin family contains a Sec14-like domain, a Golgi dynamics (GOLD) domain, and a variable N terminus domain (Peterman et al., 2004). The presence of the GOLD domain suggests a role in vesicle trafficking and Golgi function (Anantharaman and Aravind, 2002).

Similarly, the Sec14-like domain, whose amino acid sequence shares 26% similarity and 48% identity with that of Sec14p from *Saccharomyces cerevisiae*, suggests a role in membrane trafficking (Bankaitis et al., 1990; Bankaitis et al., 1989; Peterman et al., 2004). Sec14p is involved in vesicle formation and exit from the TGN, and its homologs in plants, mammals and fungi have been shown to play a role in phosphoinositide signaling and transfer, and in membrane trafficking (Bankaitis et al., 1990; Bankaitis et al., 1989; Bankaitis et al., 2010).

Evidence suggests that Sec14-like proteins can help regulate PI metabolism by helping to “present” PtdIns to PI-4 kinases during abortive PtdCho/PtdIns exchange events (Bankaitis et al., 2010; Mousley et al., 2012; Schaaf et al., 2008). Since PtdIns can be difficult to access while embedded in the membrane, the temporary exposure of PtdIns that is not yet incorporated in a membrane during abortive PtdCho/PtdIns exchange events would allow PI4-kinases to efficiently phosphorylate its substrate to produce PtdIns(4)P (Bankaitis et al., 2010; Mousley et al., 2012; Schaaf et al., 2008). The areas of Sec14p that were shown by mutational analysis to be important for this PI binding and transfer are
fully conserved in all the plant patellins, suggesting that this role in PI signaling is likely conserved in PATL1/2 (Peterman et al., 2006).

In addition to these Sec14-like and GOLD domains, PATL1 has several PXXP sequences at its N terminal that are believed to be involved in SH3 protein interactions that regulate membrane trafficking. Additionally, PATL1 has a YGEFQ motif that is believed to recognize clathrin adaptor proteins. Therefore, the sequence analyses of PATL1/2 suggest that these proteins play a role in vesicle trafficking between the plasma membrane and the TGN/endosome through their regulation of PI signaling (Peterman et al., 2004).

PATL1 has been shown to play a role in the membrane trafficking necessary for cytokinesis (Peterman et al., 2004), but more recent evidence strongly suggests a role for PAT1/2 in vascular development (Rackaityte, 2013). Phylogenetic analysis suggests that the patellins evolved in the first vascular plants and PATL1::GUS lines revealed that PATL1/2 are expressed in vascular tissue, both in developing procambial cells and mature vascular tissue (Rackaityte, 2013). Additionally, *patl1patl2* (*p1p2*) double mutants were initially reported to have more free ends, fewer areoles and a higher frequency of gaps in the vascular pattern of their leaves and cotyledons than the wild type (Rackaityte, 2013). Collectively, these results suggest that PATL1/2 play a role in procambial development by regulating proper auxin flow (Rackaityte, 2013).

Given this potential role of PATL1/2 in procambial development and the subcellular actions suggested by the domains and motifs in PATL1/2, we predict that PATL1/2 play a role in PIN1 trafficking by regulating PI levels at the TGN. PATL1 localizes to endosomal compartments and *in vitro* studies show that it binds preferentially
to PtdIns(5)P, PtdIns(4,5)P$_2$ and PtdIns(3)P (Peterman et al., 2004). The affinity of PATL2 for these PIs was demonstrated to be dependent on phosphorylation by a mitogen-activated kinase pathway, which suggests that PATL1/2 may help link extracellular signals to the effector responses at subcellular membranes during development (Suzuki et al., 2016). Based on this evidence, we hypothesize that PATL1/2 play a role in regulating PtdIns(4)P production, which could generate the PtdIns(4)P pool that recruits VAN3 and VAB, or create the substrate for PtdIns(4,5)P$_2$ production, thereby influencing the balance between CVL1/CVP2 and PIP-kinases that regulates PIN1 endocytosis (Ischebeck et al., 2013).

In order to test this hypothesis, we have used triple and quadruple mutants to evaluate potential genetic interactions between $PATL1/2$ and $VAN3$, $VAB$, and $CVP2/CVL1$. Synergistic interactions between $PATL1/2$ and these known vascular genes would support that PATL1/2 play a role in PIN1 trafficking at the TGN (Martienssen and Irish, 1999; Perez-Perez et al., 2009). Similarly, genetic interactions between $PATL1/2$ and $PATL6$, another patellin gene that is expressed during vascular development (Kondo et al., 2015) have been evaluated to determine if there is further redundancy in the patellin family during vascular development. If this is the case, analysis of the defects in $patl1/2/6$ mutants would allow us to better characterize the roles of these patellin genes in vascular development. We have found that $PATL1/2$ interact weakly with $VAB$ and $CVL1$, and more strongly with $CVP2$, suggesting that they play a role in regulating PtdIns(4,5)P$_2$ levels during PIN1 trafficking. In contrast, no genetic interactions between $PATL1/2$ and $PATL6$ were found, suggesting that $PATL6$ plays a unique role in vascular development and that there is more redundancy in the patellin family that has yet to be identified.
METHODS

Plant Material and Growth Conditions

The Columbia (Col) ecotype of *Arabidopsis thaliana* was the wild type strain used in this study and all mutations were made in this background. All seed was obtained from the Arabidopsis Biological Research Center unless otherwise noted. The T-DNA insertion mutants *pat1pat2-1* (SALK_080204 (*patl1*), SALK_086866 (*patl2-1*)), *patl4* (SALK_139423), *patl6* (SALK_099090), *vab1* (SALK_142575), and *cvl1-1* (SALK_029945) were used to generate higher order mutants (Alonso et al., 2003). Additionally, *cvp2* single mutants contained a point mutation in the *cvp2* gene that results in expression of a nonfunctional protein (Carland and Nelson, 2004), and *cvl1-1cvp2* double mutants with the above mutations were obtained. These single and double mutants were crossed to produce triple and quadruple mutants with *patl1patl2-1*.

These crosses generated an F1 generation that was heterozygous at all loci of interest. The F1 generation was bagged upon sexual maturation to ensure self-fertilization. The resulting F2 generation segregated out the desired higher order mutants, which were identified using genotyping techniques described below. If the appropriate mutant could not be identified, an F2 individual that was homozygous recessive for 2 or 3 of the desired mutations and heterozygous for the remaining mutations were allowed to self-fertilize and the resulting F3 generation was genotyped in order to identify the mutant of interest. Once the desired triple or quadruple mutants were identified, they were bagged and allowed to self-fertilize to produce progeny with the same mutant genotype.

All the seeds that were grown for analysis and use in subsequent crosses were sterilized in a laminar flow hood. Sterilization involved two washes with 1% (v/v) Tween 20 to separate the seeds, one wash with 70% (v/v) EtOH, a wash with sterile water, 10
minute incubation in 30% (v/v) bleach, and 4-6 sterile water washes. All sterilized seeds were air dried and stored in sealed Petri dishes on sterile filter paper. Once sterilized, seeds were vernalized on ½x Murashige and Skoog medium with vitamins, pH 5.5-5.7 (PlantMedia™ #30630067-3), 1% (w/v) sucrose at 4°C in the dark for 2 to 3 days.

Following vernalization, seeds were transferred to a climate controlled growth chamber (22°C, 16-h-light/8-h-dark cycle, ~100 µE s⁻¹ m⁻² light intensity) for germination and growth. All plants intended for cotyledon analysis or seed collection were grown horizontally. When plants were to be grown for seed collection or use in crosses, the plants were transferred to pots and grown in 75% ProMix PGX, 25% Pro-Field Conditioner that had been soaked in a fertilizer solution (1g/L Peter’s 15-5-15 Excel Cal-Mg and 0.08ml/L 93% sulfuric acid).

**Genotyping Higher Order Mutants**

The genotypes of all mutants used in our analysis were confirmed by PCR amplification of the gene of interest followed by analysis of fragment size for genes with T-DNA insertions, or by sequence analysis for the point mutation in the CVP2 gene.

Crude DNA extracts were taken from leaf tissue using the Extract-N-Amp™ Extraction Solution (Sigma Aldrich, Cat No. XNAPS-1KT) according to manufacturer’s instructions. In order to genotype each plant for CVP2, this extracted DNA was amplified using a forward and a reverse primer (Table 1) to create the template for subsequent sequencing. Sequence analysis was performed using the Geneious software in order to identify the presence of the relevant point mutation.

To identify T-DNA insertion mutations, extracted DNA was used to amplify each gene via PCR using a T-DNA specific forward primer (LBa1) and two gene specific primers (Table 1). The gene specific primers bind to either end of the target gene so that
approximately 1000 bp of the wild type allele can be amplified (Fig. 2). The PCR cycles were designed so that if the T-DNA insertion was present, there was not enough extension time for the fragment between the two gene specific primers to be amplified successfully. Therefore, the presence of an approximately 1000 bp PCR product suggests the presence of a wild type allele. The LBa1 primer was complementary to the left border sequence of the T-DNA insertion. Therefore, if the T-DNA was present, the LBa1 and the reverse gene specific primer would anneal, resulting in the amplification of the sequence between the two primers (Fig. 2). The fragment resulting from this amplification is noticeably shorter than the gene-specific sequence so it can be easily identified by gel electrophoresis. If the T-DNA insertion were absent, the LBa1 primer would not bind to the template and so no smaller T-DNA fragment would be produced (Fig. 2). In a heterozygous individual, fragments of both sizes are seen. Using the LBa1 and appropriate gene specific primers (Table 1), the genes of interest were amplified using the REDExtract N-Amp Plant PCR kit according to manufacturer’s instructions. Once amplified, the resulting fragments were run on a 1% agarose, 1x TAE (40mM Tris, 20mM acetic acid, and 1mM EDTA (pH8)) gel and visualized using SYBR™ Safe DNA gel stain (Invitrogen™, Cat No. S33102). Gels were imaged using a ChemiDoc™ MP imaging system with Image Lab™ software.
Figure 2. Genotypic identification of T-DNA insertion mutants. (A) T-DNA insertion mutants were identified through PCR amplification. Two gene specific (GS) primers (black arrows) and one T-DNA primer that bound to the Left Border (LB) sequence that is specific to the T-DNA insertion (gray arrow) were used. If the T-DNA insertion was present, the LB primer and the GS primers could anneal. This would cause the fragment between this LB primer and the complementary GS primer to be amplified. However, the PCR conditions were designed so that the entire gene plus the T-DNA insertion was too long to be successfully amplified using the GS primers. Therefore, only the smaller T-DNA fragment will be amplified from the DNA of insertion mutants. If the T-DNA insertion is not present, the LB primer will not anneal, and the fragment between the GS primers will be amplified. (B) DNA was crudely extracted from a cvl1 mutant and a wild type control (WT) using Extract-N-Amp™ Plant Tissue PCR Kit. 20% extract, 50% REDExtract-N-Amp™ PCR Ready Mix and 0.4 µM each of two gene specific primers and a TDNA specific primer was used to amplify CVL1. Equal volumes of each reaction and of a 100 bp ladder were run on a 1% agarose gel.
Table 1: Primer sequences for identification of vascular mutations

<table>
<thead>
<tr>
<th>Gene</th>
<th>Left Primer</th>
<th>Right Primer</th>
<th>LBa1</th>
</tr>
</thead>
<tbody>
<tr>
<td>PATL1</td>
<td>5’-TCCTGAATTTGCCTGCTAAAGA-3’</td>
<td>5’-TCCTGAATTTGCCTGAAGA-3’</td>
<td>5’-TGGTTCACGTAAGAAGAAGA-3’</td>
</tr>
<tr>
<td>PATL2</td>
<td>5’-CTCTGACCAAATCTCGAGCTAGAGAGAGGGA-3’</td>
<td>5’-CGTCGAGGAGAAGACGAGCAG-3’</td>
<td></td>
</tr>
<tr>
<td>PATL4</td>
<td>5’-CGAAGAGACAGGACG-3’</td>
<td>5’-TGGGAGCTCATCAGCTGGAAT-3’</td>
<td></td>
</tr>
<tr>
<td>PATL6</td>
<td>5’-TGCGGGAAGACGAGGAGAGAGGGA-3’</td>
<td>5’-CCGACAGTGTAACGGGTAAGC-3’</td>
<td></td>
</tr>
<tr>
<td>VAB</td>
<td>5’-CGTTCGAGGAGAAGACGAGGGA-3’</td>
<td>5’-TAAGCTCGAGGTACG-3’</td>
<td></td>
</tr>
<tr>
<td>CVL1</td>
<td>5’-TGATCAGGAAACCCTGAGCTCC-3’</td>
<td>5’-AGCACATTTTGTAACG-3’</td>
<td></td>
</tr>
<tr>
<td>CVP2</td>
<td>5’-CCTCGTCTGCTTGGTGAGATAG-3’</td>
<td>5’-ATCCACGGAGTAAGGTAAGA-3’</td>
<td></td>
</tr>
</tbody>
</table>
Cotyledon Venation Analysis

Cotyledon venation patterns were analyzed in seven-day-old mutant and wild type *Arabidopsis* plants. Either dissected cotyledons or whole seedlings were fixed in 3:1 (v/v) ethanol: acetic acid for two hours, followed by incubation in 70% (v/v) ethanol for at least 30 minutes to remove chlorophyll. In some cases, the seedlings were incubated in 70% ethanol overnight. The seedlings were then rehydrated through incubation in 50% (v/v) ethanol and 25% (v/v) ethanol for 30 minutes each. Seedlings were stored in 25% (v/v) ethanol prior to analysis.

Once rehydrated, the cotyledons were dissected from the rest of the plant under a dissecting microscope and mounted in a chloral hydrate clearing solution (9:3:1 (w/v/v) chloral hydrate: water: glycerol). Slides were sealed using clear nail polish. The mounted cotyledons were analyzed and imaged using a ©Leica M65 FC dissecting microscope and the ©Leica Application Suite v4.4 software. Cotyledons were scored for developmental and complexity characteristics such as the number of fully closed areoles (Fig. 3A), the number of free ends in the vascular pattern (Fig. 3B), the number of secondary veins (Fig. 3A), and the number of connections formed between veins (branch points) (Fig. 3B).

Cotyledons also were scored for the frequency vascular defects such as discontinuities in the vascular tissue (gaps) (Fig. 3C), secondary veins growing from the proximal to the distal ends (reversals) (Fig. 3D), and vascular elements that were not connected to the continuous vascular network on either end, creating two free ends (vascular island) (Fig. 3E).

In order for a vascular defect to be scored as a gap or a vascular island, a clear absence of vascular tissue had to be identified along the length of a secondary vein (Fig. 3C, 3E). Any small gaps in the vasculature were assumed to be breaks that occurred
during the mounting procedure rather than true gaps. The veins with these breaks were scored as if they were continuous, making our frequencies of gaps and vascular islands conservative. If there was uncertainty about the validity of a gap or vascular island, it was confirmed using a Nikon 80i DIC microscope. If a true gap disrupted a secondary vein that was connected to the midvein at both ends, as in Figure 3C, any free ends created were scored and the vein was not considered a fully closed areole, but it was still scored as one secondary vein. Reversals were defined as secondary veins that connected to the midvein at the proximal and not the distal end. These reversals could only be identified if the areole was not yet closed and if the secondary vein was properly connected to the midvein, making our reversal frequencies conservative as well. Images were assembled and labeled using Adobe Photoshop CS Pro.

The frequencies of gaps, reversals, vascular islands, branch points, secondary veins, closed loops, and free ends were compared between genotypes by one-way ANOVA analysis and Tukey-Kramer HSD statistical analysis. In addition to these frequencies, a “complexity score”, defined as number of closed areoles: number of free ending secondary veins was determined for each cotyledon in order to evaluate cotyledon vascular complexity. The frequencies of all possible areoles: free ending secondary veins values were evaluated and compared between genotypes in order to assess vascular complexity.
Figure 3. Scoring categories for cotyledon analysis. One-week-old cotyledons were cleared in chloral hydrate and scored to assess the presence of vascular defects and complexity changes. All scale bars represent 1mm. (A) The fully developed wild type cotyledon pattern was defined as the presence of four secondary veins (arrowhead) that connect to the primary midvein (arrow) to form four closed areoles. (B) Free ends (arrowhead) and branch points, where connections formed between two veins (arrow). (C) Discontinuities (gaps). (D) Reversals in the direction of vein growth. (E) Sections of vein that are not connected to the vascular pattern (Vascular islands).
RESULTS

Analysis of genetic interaction between *PATL1/2* and *VAB*

In order to identify any potential interactions between the *PATELLIN1/2* and *VAB* genes, *p1p2vab* triple mutants were obtained through a series of crosses. Initially, a *p1p2* and a *vab* mutant were crossed to generate a triple heterozygous F1 generation. Individuals from this generation were self-fertilized to produce an F2 generation that could be screened for *p1p2vab* triple mutants. DNA extracts were obtained from leaf tissue and used for PCR genotyping reactions. Using two gene-specific and one T-DNA specific primer for each amplification reaction, T-DNA insertions in the *PATL1/2* and *VAB* genes were determined by the presence of a T-DNA band and the absence of a gene specific band (data not shown).

Once *p1p2vab* triple mutants were identified, they were re-potted and self-fertilized to produce *p1p2vab* progeny. To determine if combinations of *p1p2* and *vab* mutations resulted in a more severe phenotype, seedlings of *p1p2vab* (n=184), *p1p2* (n=98), *vab* (n=177), and wild type (n=190) were grown. Cotyledons from each genotype were fixed and cleared after 7 days of growth for analysis. The seven-day-old cotyledon vascular pattern was chosen for analysis because its simplicity makes it easy to quantify and after seven days of growth, the vascular pattern should be near completion in wild type cotyledons, allowing us to identify any developmental delays caused by our mutations. Defects and developmental characteristics were scored as previously described (Fig. 3) (Rackaityte, 2013; Roschzttardtz et al., 2014; Steynen and Schultz, 2003).

Throughout all of our experiments, consistent values were obtained for the wild type and *p1p2* controls in each of our scoring categories (Tables 2-4). The frequencies of gaps and reversals found within our wild type sample, however, were noticeably higher
than the 0.043 gaps and 0.023 reversals reported by Rackaityte (2013). In the p1p2 mutant, our frequency of reversals, about 0.05 to 0.06 (Tables 2-4), agrees well with the 0.059 frequency of reversals reported by Rackaityte (2013). However, our proportion of gaps, 0.05 to 0.08 (Tables 2-4), was much lower than the frequency of 0.185 reported by Rackaityte (2013). These differences in the frequency of gaps is likely due to differences in our fixation and clearing techniques as well as our assumption that small gaps were breaks rather than true gaps. Because of these differences, any increase in fragility of the vascular tissue in response to the fixation and clearing techniques could have skewed the number of gaps reported. However, the average number of gaps should be comparable as long as the fixation protocol is kept consistent, suggesting that gap values within our experiments can be compared. Differences in plant growth conditions may also have contributed to differences in observed defects due to the potential effect of stress responses.

On the other hand, no vascular islands were found in p1p2 or wild type (Tables 2, 4), which is consistent with the absence of vascular islands in wild type cotyledons previously reported (Carland and Nelson, 2009). Similarly, the average number of areoles, free ends and branch points were about 3.0, 1.0 and 5.5 respectively across this study with overlapping means ± one standard deviation (Tables 2-4). These values are also consistent with published values of 2.8 to 3.2 areoles (Carland and Nelson, 2009; Garrett et al., 2012; Rackaityte, 2013) and 0.7 to 1.1 free ends (Garrett et al., 2012; Rackaityte, 2013) previously reported in the literature. Our branch point values of about 5.5 were approximately 1 lower than those reported by Carland and Nelson (2009) and Garrett et al (2012), but agreed with the average value of approximately 5 reported by Rackaityte.
(2013). Since the number of areoles and free ends were consistent between all of these studies, this may be due to a difference in the characterization of branch points rather than a developmental difference. When two secondary veins met the midvein at the same point, it was characterized as one branch point in our study. If previous studies had classified this junction as two separate branch points, that would account for the observed differences. This consistency suggests that our scoring methods are reliable and can reasonably be used for comparisons between genotypes as long as the same clearing techniques and scoring methods are used.

Once the validity of the scoring techniques had been determined, the cotyledon vascular pattern was compared between \textit{p1p2vab}, \textit{p1p2}, \textit{vab}, and wild type. The number of gaps and vascular islands in all of the genotypes were not significantly different (Table 2) (Gaps: one-way ANOVA, DF=648, F=0.6185, p=0.6032; Vascular islands: one-way ANOVA, DF=648, F=1.7243, p<0.1607). The number of reversals significantly increased in \textit{p1p2vab} and \textit{vab} cotyledons versus the wild type and \textit{p1p2} (Fig. 4A) (DF=648, F=414.64, p<0.0001, Tukey HSD, \(\alpha<0.05\)) as would be expected due to the decrease in distal connections characteristic of \textit{vab} mutants (Hou et al., 2010; Naramoto et al., 2009; Steynen and Schultz, 2003). However, there was no significant difference between the \textit{p1p2vab} and \textit{vab} mutants, suggesting that the increase in reversals in the \textit{p1p2vab} triple mutant can be entirely attributed to the \textit{vab} mutation. Therefore, there do not appear to be any interactions between the \textit{p1p2} and \textit{vab} mutations that increase the frequency of vascular defects in the cotyledon.
Table 2. Average number of vascular defects, aeroles, free ends, and branch points in the $p1p2vab$ triple mutant. The number of gaps, reversals, vascular islands, areoles, free ends and branch points were scored in seven-day-old wild type, $p1p2$, $vab$, and $p1p2vab$ cotyledons. The average values ± one standard deviation were calculated.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Wild Type</th>
<th>$p1p2$</th>
<th>$vab$</th>
<th>$p1p2vab$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample Size</td>
<td>190</td>
<td>98</td>
<td>177</td>
<td>184</td>
</tr>
<tr>
<td>Gaps</td>
<td>0.07±0.3</td>
<td>0.06±0.2</td>
<td>0.09±0.3</td>
<td>0.05±0.3</td>
</tr>
<tr>
<td>Reversals</td>
<td>0.10±0.3</td>
<td>0.07±0.3</td>
<td>2.33±1.1</td>
<td>2.34±1.0</td>
</tr>
<tr>
<td>Vascular Islands</td>
<td>0.0±0</td>
<td>0.0±0</td>
<td>0.02±0.1</td>
<td>0.01±0.1</td>
</tr>
<tr>
<td>Areoles</td>
<td>2.87±0.7</td>
<td>2.79±0.7</td>
<td>1.19±0.9</td>
<td>1.0±0.8</td>
</tr>
<tr>
<td>Free Ends</td>
<td>1.10±0.9</td>
<td>1.07±0.8</td>
<td>2.79±1.4</td>
<td>2.79±1.3</td>
</tr>
<tr>
<td>Branch Points</td>
<td>5.66±1.0</td>
<td>5.39±1.1</td>
<td>4.54±0.9</td>
<td>3.70±0.9</td>
</tr>
<tr>
<td>Secondary Veins</td>
<td>3.90±0.4</td>
<td>3.79±0.5</td>
<td>3.67±0.6</td>
<td>3.45±0.7</td>
</tr>
</tbody>
</table>
Figure 4. *p1p2vab* mutants show subtle differences from *p1p2* and *vab* mutants. Seven-day-old *p1p2vab* (n=184), *vab* (n=177), *p1p2* (n=98), and wild type (n=190) seedlings were fixed and their cotyledons were cleared in 9:3:1 (w/v/v) chloral hydrate: water: glycerol. The cleared cotyledons were scored and the means per cotyledon ± one standard deviation are shown for vascular characteristics with different letters indicating significant differences between genotypes as indicated by one-way ANOVA analysis and Tukey HSD pairwise comparisons (\(\alpha<0.05\)). (A) Reversals: DF=648, F=414.64, \(p<0.0001\). (B) Closed Areoles: DF=648, F=254.86, \(p<0.0001\). (C) Free Ends: DF=648, F=114.90, \(p<0.0001\). (D) Branch Points: DF=648, F=146.51, \(p<0.0001\). (E) Secondary Veins: DF=648, F=22.185, \(p<0.0001\).
Similarly, there were significantly fewer areoles and significantly more free ends in \textit{p1p2vab} and \textit{vab} mutants when compared to \textit{p1p2} and wild type, but there were no significant differences between \textit{p1p2vab} and \textit{vab} (Fig. 4B-C) (Areoles: one-way ANOVA, DF=648, F=254.86, p<0.0001, Tukey HSD test, $\alpha <0.05$; Free ends: one-way ANOVA, DF=648, F=114.90, p<0.0001, Tukey HSD test, $\alpha <0.05$). This is consistent with the lack of distal connections characteristic of \textit{vab} mutants (Hou et al., 2010; Naramoto et al., 2009). Since the variation in areole formation within a sample is large (Fig. 4B), the frequency of cotyledons with a given number of areoles was also evaluated in order to assess the distribution within each genotype. The frequencies of closed areoles show that \textit{p1p2vab} and \textit{vab} are more likely than WT or \textit{p1p2} to form 0 or 1 areoles, and they have nearly identical distributions of areoles (Fig. 5). This similarity supports the finding that the number of areoles in \textit{p1p2vab} and \textit{vab} mutants are not different (Fig. 4B) by showing that there is no difference in the intra-sample variation that was lost when taking averages, which confirms the similarities between the two genotypes.
Figure 5. *p1p2vab* cotyledon venation forms slightly fewer areoles than *vab*. Seven-day-old *p1p2vab* (n=184), *vab* (n=177), *p1p2* (n=98), and wild type (n=190) seedlings were fixed and their cotyledons were cleared in 9:3:1 (w/v/v) chloral hydrate: water: glycerol. The number of fully closed areoles was scored for all genotypes and the proportion of the sample with 0, 1, 2, 3 and 4 areoles were calculated.
Despite these apparent similarities between $p1p2vab$ and $vab$ mutants, small but significant decreases in the number of branch points and secondary veins were observed in $p1p2vab$ compared to $vab$ mutants and both of these mutants showed a decrease relative to $p1p2$ and wild type (Fig. 4D-E) (Branch points: one-way ANOVA, DF=648, $F=146.51$, $p<0.0001$, Tukey HSD test, $\alpha <0.05$; Secondary veins: one-way ANOVA, DF=648, $F=22.185$, $p<0.0001$, Tukey HSD test, $\alpha <0.05$). The decrease in branch points is a consequence of the decrease in secondary veins because there are fewer veins to make connections. Since a decrease in secondary veins and branch points suggests a decrease in vascular complexity in $p1p2vab$ mutants, the vascular complexity within each genotype was evaluated by using areoles: free ending secondary veins scores (Fig. 6A). Previous use of this scoring system to evaluate vascular complexity showed that about 45% of wild type cotyledons are 3-1, 25% are 4-0 and 20% are 2-2 (Roschztardtz et al., 2014). Our wild type distributions resulted in fewer 3-1 cotyledons, but the overall pattern of 3-1 being the most common phenotype followed by 4-0 and 2-2 was consistent with previous reports (Fig. 6B). Therefore, this classification system appears to be a reliable way to assess vascular complexity.

In agreement with the decrease in branch points and secondary veins, $vab$ and $p1p2vab$ mutants were noticeably less complex than wild type and $p1p2$ (Fig. 6B). The wild type and $p1p2$ had high proportions of cotyledons with 3-1 and 4-0 patterns, which were present only in very low proportions in the $vab$ mutant, and were not found in any $p1p2vab$ mutants (Fig. 6B).
Figure 6. *p1p2vab* cotyledons have subtly reduced vascular complexity compared to *vab* (A) The number of areoles and the number of free ends were scored and a score of areoles: free ending secondary veins was assigned to each cotyledon. The orientation of vein growth did not factor into this scoring. Representative images for each potential score are shown. Any vascular patterns that did not fit into these categories, such as those with gaps, were scored as other. All scale bars represent 1mm. (B) The frequency of each complexity score was calculated for all genotypes. The complexity vascular pattern gets more complex as you move to the right.
On the other end, \( plp2vab \) and \( vab \) mutants had high frequencies of phenotypes with zero or only one closed areole, which is characteristic of the forked phenotype of \( vab \) mutants previously described (Hou et al., 2010; Naramoto et al., 2009; Steynen and Schultz, 2003), and these vascular patterns were not seen in the wild type or \( plp2 \) (Fig. 6B). In addition to these phenotypes, the \( plp2vab \) triple was slightly shifted toward less complex vascular patterns compared to \( vab \) mutants. The \( plp2vab \) triple mutant had a higher frequency of 0-2, 1-1 and 0-3 patterns, but had no 3-1 or 4-0 cotyledons, which was seen in the \( vab \) mutants at a low frequency (Fig. 6B). This decrease in vascular complexity suggests there may be interactions between \( plp2 \) and \( vab \), but the effects of this interaction are subtle. Therefore, it appears that there are very weak interactions between \( PATL1/2 \) and \( VAB \).

\( PATL1/2 \) and \( CVL1 \) interact weakly

Given the role of \( CVL1 \) and \( CVP2 \) in regulating PtdIns(4,5)P\(_2\) levels during PIN1 trafficking (Ischebeck et al., 2013; Tejos et al., 2014), potential interactions between \( PATL1/2 \) and \( CVL1 \) and \( CVP2 \) were evaluated to try to better understand the roles of \( PATL1/2 \) in vascular development. In order to evaluate these genetic interactions, \( plp2 \) and \( cvl1-1 \) mutants were crossed and their progeny self-fertilized to produce an F2 generation that would segregate out \( plp2cvl1 \) mutants. These triple mutants were identified through PCR genotyping using a T-DNA specific primer and two gene-specific primers for \( PATL1 \), \( PATL2 \) or \( CVL1 \) (data not shown). Once this triple mutant was identified, it was re-potted and self-fertilized to produce \( plp2cvl1 \) triple mutants for analysis.
To determine if combinations of \( p1p2 \) and \( cvl1 \) mutations resulted in a more severe phenotype, seedlings of \( p1p2cvl1 \) (n=194), \( p1p2 \) (n=192), \( cvl1 \) (n=193) and wild type (n=192) were grown for seven days before their cotyledons were cleared for analysis and scored for vascular defects as previously described (Rackaityte, 2013; Roschzttardtz et al., 2014; Steynen and Schultz, 2003). No significant difference in gaps or reversals was seen in any of the genotypes (Table 3) (Gaps: one-way ANOVA, DF=770, F=0.940, p=0.421; Reversals: one-way ANOVA, DF=770, F=0.433, p=0.730). This lack of vascular defects is consistent with the wild type-like phenotype of \( cvl1 \) mutants previously reported (Carland and Nelson, 2009) and suggests that \( PATL1/2 \) and \( CVL1 \) are not necessary to produce a continuous vascular pattern. Given that there was no significant difference observed in vascular defects, the remainder of the analysis focused on evaluating areoles and free ends.

**Table 3. Initial analysis of vascular defects in the \( p1p2cvl1 \) triple mutant.** The number of gaps and reversals were scored in seven-day-old wild type, \( p1p2 \), \( cvl1 \), and \( p1p2cvl1 \) cotyledons. The average values ± one standard deviation were calculated.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Wild Type</th>
<th>( p1p2 )</th>
<th>( cvl1 )</th>
<th>( p1p2cvl1 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample Size</td>
<td>192</td>
<td>192</td>
<td>193</td>
<td>194</td>
</tr>
<tr>
<td>Gaps</td>
<td>0.08±0.3</td>
<td>0.05±0.2</td>
<td>0.07±0.3</td>
<td>0.05±0.2</td>
</tr>
<tr>
<td>Reversals</td>
<td>0.07±0.3</td>
<td>0.06±0.2</td>
<td>0.08±0.3</td>
<td>0.05±0.2</td>
</tr>
</tbody>
</table>
In a subsequent analysis, additional cotyledons were grown and the pooled data from \( p1p2cvl1 \) (n=386), \( p1p2 \) (n=364), \( cvl1 \) (n=385) and wild type (n=382) cotyledons were analyzed. \( p1p2 \) mutants had significantly fewer areoles than the wild type (Fig. 7A) (one-way ANOVA, DF=770, \( F=21.81, p<0.0001 \), Tukey HSD test, \( \alpha <0.05 \)). Given the much larger sample size used (Fig. 7), it is possible that \( p1p2 \) mutants have a slight decrease in areole formation, but these results are not consistent from experiment to experiment. Additionally, \( p1p2cvl1 \) mutants had a small, but significant decrease in areoles and a corresponding increase in free ends compared to \( p1p2 \) (Fig. 7A-B) (Areoles: one-way ANOVA, DF=770, \( F=21.81, p<0.0001 \), Tukey HSD test, \( \alpha <0.05 \); Free ends: one-way ANOVA, DF=770, \( F=8.21, p<0.0001 \), Tukey HSD test, \( \alpha <0.05 \)).

Given the high variability within a genotype, the frequencies of different numbers of areoles and vascular complexity scores were evaluated to ascertain if these differences in average areoles and free ends in the pooled experiment corresponded to shifts in the distribution of vascular complexity. The distribution of closed areoles show that a larger proportion of \( p1p2cvl1 \) cotyledons have only 2 closed areoles than \( cvl1, p1p2, \) or wild type cotyledons (Fig. 7C). This distribution agrees with the decrease in areoles and shows a slight decrease in vascular complexity. Similarly, when vascular complexity scores were analyzed, \( p1p2cvl1 \) had more 2-2 scores and fewer 4-0 scores than the other genotypes, suggesting a slight decrease in complexity (Fig. 8). Collectively, these results suggest weak interactions between \( PATL1/2 \) and \( CVL1 \) that contribute to cotyledon vascular complexity and areole formation.
Figure 7. *p1p2cvl1* triple mutants show a slight decrease in vascular complexity compared to *cvl1* mutants. Seven-day-old *p1p2cvl1* (*n*=386), *p1p2* (*n*=364), *cvl1* (*n*=385), and wild type (*n*=382) seedlings were fixed and their cotyledons were cleared in 9:3:1 (w/v/v) chloral hydrate: water: glycerol. The cleared cotyledons were scored and the means per cotyledon ± one standard deviation are shown with different letters indicating significant differences between genotypes as indicated by one-way ANOVA analysis and Tukey HSD pairwise comparisons (α<0.05). (A) Areoles: DF=770, F=21.81, p<0.0001 (B) Free ends: DF=770, F=8.21, p<0.0001. (C) The number of fully closed areoles was scored for all genotypes and the frequencies of 0, 1, 2, 3, 4 and 5 areoles were calculated.
**Figure 8.** *p1p2cvl1* triple mutants have a slightly less complex vascular pattern than *cvl1* mutants. Seven-day-old *p1p2cvl1* (n=386), *p1p2* (n=364), *cvl1* (n=385), and wild type (n=385) seedlings were fixed and their cotyledons were cleared in 9:3:1 (w/v/v) chloral hydrate: water: glycerol. (A) The number of areoles and the number of free ends were scored and a score of areoles: free ending secondary veins was assigned to each cotyledon. Representative images for each potential score are shown. Any vascular patterns that did not fit into these categories, such as those with gaps or more than 4 secondary veins were scored as other. All scale bars represent 1mm. (B) The frequency of each complexity score was calculated for all genotypes. The complexity vascular pattern gets more complex as you move to the right.
PATL1/2 and CVP2 interact

The phenotypic changes in p1p2cvl1 mutants were subtle, but this is not surprising given the functional redundancy between CVL1 and CVP2 and the low in vitro activity of CVL1 (Carland and Nelson, 2009; Naramoto et al., 2009). Therefore, we tested for interaction between PATL1/2 and CVP2 to assess the potential role of PATL1/2 in this pathway. In order to identify any potential interactions, p1p2 and cvp2 mutants were crossed and their progeny self-fertilized to produce p1p2cvp2 mutants. These progeny were genotyped for PATL1/2 by PCR amplification with one T-DNA specific primer and two gene-specific primers and for CVP2 by sequence analysis (data not shown). Once a triple mutant was identified, it was self-fertilized to produce a generation of p1p2cvp2 mutants.

Seedlings of p1p2cvp2 (n=122), cvp2 (n=30), p1p2 (n=119), and wild type (n=113) were grown for seven days before cotyledons were fixed and cleared for analysis of their vascular patterns. A qualitative analysis of this sample showed that p1p2cvp2 mutants have noticeably more reversals and gaps (Fig. 9) than p1p2cvl1 mutants, which is consistent with the stronger phenotype of cvp2 single mutants compared to cvl1 mutants (Carland and Nelson, 2009; Naramoto et al., 2009). A quantitative analysis revealed that p1p2cvp2 has significantly more gaps and reversals than the cvp2 single mutant, which had significantly more of these defects than p1p2 and wild type cotyledons (Fig. 10A-B) (Gaps: one-way ANOVA, DF=384, F=182.67, p<0.0001, Tukey HSD, α<0.05; Reversals: one-way ANOVA, DF=384, F=84.78, p<0.0001, Tukey HSD, α<0.05). This increase in vascular defects in the p1p2cvp2 triple mutant suggests that there may be genetic interactions between PATL1/2 and CVP2.
Figure 9. Characterization of *p1p2cvp2* and *p1p2cvl1cvp2* mutants. A cross between *p1p2* and *cvp2* mutants and between *p1p2* and *cvl1cvp2* mutants produced triple or quadruple heterozygous progeny respectively. These progeny were allowed to self fertilize to generate F2 generations. Once identified, seven-day-old wild type, *cvp2*, *p1p2cvp2*, *cvl1cvp2*, and *p1p2cvl1cvp2* mutants were fixed and their cotyledons were cleared in 9:3:1 (w/v/v) chloral hydrate: water: glycerol. Two representative images are shown for each genotype, with wild type and *p1p2* images representing phenotypes typical for both genotypes.
Figure 10. *p1p2cvp2* triple mutants have an increase in vascular defects and a decrease in vascular complexity compared to *cvp2*. Seven-day-old *p1p2cvp2* (n=122), *cvp2* (n=30), *p1p2* (n=119), and wild type (n=113) seedlings were fixed and their cotyledons were cleared in 9:3:1 (w/v/v) chloral hydrate: water: glycerol. The cleared cotyledons were scored. The means per cotyledon ± one standard deviation are shown with different letters indicating significant differences between genotypes as indicated by one-way ANOVA analysis and Tukey HSD pairwise comparisons (α<0.05). (A) Gaps: DF=384, F=182.67, p<0.0001 (B) Reversals: DF=384, F=84.78, p<0.0001 (C) Free Ends: DF=383, F=245.81, p<0.0001 (D) Branch Points: DF=384, F=22.19, p<0.0001 (E) Secondary Veins: DF=382, F=36.93, p<0.0001
Specifically, the increase in reversals resulted in a forked, vab-like phenotype (Fig. 9), suggesting that the loss of genetic interactions between \( PATL1/2 \) and \( CVP2 \) may disrupt the same pathway as the vab mutation. However, this disruption likely occurs at a distinct point in the pathway from \( VAB \) given that only very weak interactions were observed between \( PATL1/2 \) and \( VAB \).

On the other hand, \( cvp2 \) and \( p1p2cvp2 \) mutants had significantly more vascular islands than wild type and \( p1p2 \) mutants, but they were not significantly different from each other (Table 4) (one-way ANOVA, DF=384, \( F=11.33 \), \( p<0.0001 \), Tukey HSD, \( \alpha<0.05 \)). Therefore, the increase in vascular islands in \( p1p2cvp2 \) mutants is likely a result of the \( cvp2 \) mutation alone.

In addition to these changes in vascular defects, the vascular complexity was assessed by scoring areoles, free ends, branch points and number of secondary veins per cotyledon. The frequency of vascular islands that were found in the \( cvp2 \) mutant, 0.17±0.5 (Table 4), agrees with 0.2±0.5 vascular islands reported previously (Carland and Nelson, 2009). Our finding that \( cvp2 \) mutants had 1.57±1.0 areoles was higher than the previously reported 0.7±0.7 areoles (Carland and Nelson, 2009), but the high standard deviation values in both studies suggests that this difference may just be due to variability. The \( cvp2 \) plants in this study had fewer branch points and secondary veins than previously reported, but similar differences were seen in the branch point values reported for the wild type (Carland and Nelson, 2009) and there may have been differences in how secondary veins that are interrupted by vascular defects were scored, suggesting that these differences stem from differences in scoring techniques rather than true phenotypic differences. Therefore, the \( cvp2 \) sample used in this study seems to be representative of
Table 4. Average number of vascular defects, areoles, free ends, branch points and secondary veins in \( p1p2cvp2 \) and \( p1p2cvl1cvp2 \) mutants.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>WT</th>
<th>( p1p2 )</th>
<th>( cvp2 )</th>
<th>( cv1cvp2 )</th>
<th>( p1p2cvp2 )</th>
<th>( p1p2cvl1cvp2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample Size</td>
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<td>119</td>
<td>30</td>
<td>100</td>
<td>122</td>
<td>118</td>
</tr>
<tr>
<td>Gaps</td>
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<td>0.08±0.3</td>
<td>0.47±0.6</td>
<td>6.22±3.0</td>
<td>1.83±1.1</td>
<td>5.12±2.2</td>
</tr>
<tr>
<td>Reversals</td>
<td>0.06±0.2</td>
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<td>0.37±0.6</td>
<td>0.74±0.9</td>
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<td>0.75±0.8</td>
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<td>Vascular Islands</td>
<td>0.0±0</td>
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<td>0.17±0.5</td>
<td>4.67±2.7</td>
<td>0.18±0.5</td>
<td>4.87±1.9</td>
</tr>
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<td>Areoles</td>
<td>2.81±0.7</td>
<td>2.80±0.8</td>
<td>1.57±1.0</td>
<td>0.03±0.2</td>
<td>0.96±0.8</td>
<td>0.08±0.3</td>
</tr>
<tr>
<td>Free Ends</td>
<td>1.11±0.7</td>
<td>1.17±1.0</td>
<td>1.90±1.9</td>
<td>13.20±4.5</td>
<td>5.08±1.7</td>
<td>13.42±3.9</td>
</tr>
<tr>
<td>Branch Points</td>
<td>5.52±1.1</td>
<td>5.62±0.9</td>
<td>4.17±1.5</td>
<td>2.64±0.9</td>
<td>6.03±1.3</td>
<td>3.07±0.9</td>
</tr>
<tr>
<td>Secondary Veins</td>
<td>3.86±0.5</td>
<td>2.89±0.4</td>
<td>2.83±0.9</td>
<td>-</td>
<td>4.11±0.7</td>
<td>-</td>
</tr>
</tbody>
</table>
the cvp2 phenotype despite the limited sample size, allowing us to evaluate our $p1p2cvp2$ mutant for increased phenotypic severity. The $p1p2cvp2$ mutant had significantly fewer areoles than the wild type, $p1p2$, or cvp2 mutant (Fig. 11A) (one-way ANOVA, $DF=383, F=165.95, p<0.0001$, Tukey HSD, $\alpha<0.05$). This decrease in areoles relative to the cvp2 single mutant is accompanied by a increase in free ends (Fig. 10C) (one-way ANOVA, Free Ends: $DF=383, F=245.81, p<0.0001$, Tukey HSD, $\alpha<0.05$), branch points (Fig. 10D) and secondary veins (Fig. 10E) (Branch points: one-way ANOVA, $DF=384, F=22.19, p<0.0001$, Tukey HSD, $\alpha<0.05$; Secondary veins, one-way ANOVA, $DF=383, F=36.93, p<0.0001$, Tukey HSD, $\alpha<0.05$). In some cases, these additional secondary veins would form small areoles outside of the normal vascular pattern, but most branched out of the normal vascular pattern and ended freely.

Due to the increase in gaps, the distribution of vascular complexity in these samples was more difficult to evaluate using the vascular complexity scoring mentioned previously so only the distributions of the numbers of areoles were compared. These comparisons showed that the distributions of $p1p2cvp2$ and cvp2 mutants are shifted toward fewer areoles per cotyledon than wild type or $p1p2$ (Fig. 11B). Additionally, $p1p2cvp2$ mutants had fewer cotyledons with 2 areoles and almost 5 times as many cotyledons with zero areoles than cvp2 mutants. Overall, these results suggest that $p1p2cvp2$ mutants have a decrease in vascular complexity compared to cvp2 mutants. Therefore, the combined results suggest that there are genetic interactions between PATL1/2 and CVP2 that help regulate secondary vein differentiation and the formation of their distal connections.
Figure 11. The p1p2cvp2 cotyledon vascular pattern is less complex than cvp2. Seven-day-old p1p2cvp2 (n=122), p1p2 (n=119), cvp2 (n=30), and wild type (n=113) seedlings were fixed and their cotyledons were cleared in 9:3:1 (w/v/v) chloral hydrate: water: glycerol. (A) The cleared cotyledons were scored for the average number of areoles (DF=383, F=165.95, p<0.0001). The means ± one standard deviation are shown with different letters indicating significant differences between genotypes as indicated by one-way ANOVA analysis and Tukey HSD pairwise comparisons (α<0.05). (B) The number of fully closed areoles was scored for all genotypes and the frequencies of 0, 1, 2, 3, and 4 areoles were calculated for comparisons between genotypes.
p1p2cvl1cvp2 mutants do not show an enhanced phenotype

Given the presence of slightly enhanced phenotypes in the p1p2cvl1 and p1p2cvp2 triple mutants and the redundancy of CVL1 and CVP2 (Carland and Nelson, 2009; Naramoto et al., 2009), p1p2cvl1cvp2 quadruple mutants were studied to see if a more severe genetic interaction could be identified. These p1p2cvl1cvp2 quadruple mutants were produced by crossing p1p2 and cvl1cvp2 mutants, and allowing heterozygous progeny to self fertilize. This F2 generation did not produce any quadruple mutants, so progeny that were mutant for at least two genes and heterozygous for the rest were self-fertilized to produce progeny that would segregate out the quadruple mutant at a higher rate. Members of this F3 generation were genotyped for PATL1, PATL2, and CVL1 using a T-DNA specific primer and two gene specific primers per gene, and for CVP2 through sequencing to identify a p1p2cvl1cvp2 quadruple mutant (data not shown).

This p1p2cvl1cvp2 individual was self-fertilized to produce p1p2cvl1cvp2 mutants. These p1p2cvl1cvp2 mutants (n=118), as well as cvl1cvp2 (n=100), p1p2 (n=119), and wild type (n=113) plants were grown for seven days before their cotyledons were cleared for analysis. A qualitative phenotypic evaluation of this quadruple mutant showed a large frequency of vascular islands compared to wild type and p1p2, which resembles the cvl1cvp2 double mutant phenotype (Fig. 9) (Carland and Nelson, 2009; Naramoto et al., 2009). In order to identify if there were any significant differences between cvl1cvp2 and p1p2cvl1cvp2, vascular defects and complexity characteristics were scored for comparison. The cvl1cvp2 double mutant had 0.03±0.2 areoles and 4.67±2.7 vascular islands (Table 4), which agree well with the previously reported values of 0.02 areoles and 6.3±2.0 vascular islands given the high intra-sample variation (Carland and Nelson, 2009).
Therefore, the *cvl1cvp2* line in this study accurately represents the phenotype of this double mutant, allowing us to make comparison between *p1p2cvl1cvp2* and *cvl1cvp2*.

The *cvl1cvp2* and *p1p2cvl1cvp2* mutants showed significant increases in gaps, reversals, and vascular islands relative to the wild type and *p1p2*, but they were not significantly different from each other (Fig. 12A-C) (Gaps: one-way ANOVA, DF=450, F=502.39, p<0.0001, Tukey HSD α<0.05; Reversals: one-way ANOVA, DF=450, F=47.36, p<0.0001, Tukey HSD α<0.05; Vascular islands: one-way ANOVA, DF=450, F=451.11, p<0.0001, Tukey HSD α<0.05). Similarly, the number of areoles is significantly lower and the number of free ends significantly higher in *p1p2cvl1cvp2* and *cvl1cvp2* mutants compared to wild type and *p1p2*, but there was no difference between *p1p2cvl1cvp2* and *cvl1cvp2* (Fig 12D-E) (Areoles: one-way ANOVA, DF=449, F=899.88, p<0.0001, Tukey HSD α<0.05; Free ends: one-way ANOVA, DF=449, F=615.04, p<0.0001, Tukey HSD α<0.05). When the distribution of closed areoles in the sample was assessed, almost all *cvl1cvp2* and *p1p2cvl1cvp2* mutants had no areoles (Fig. 13), suggesting that the decrease in areoles and increase in free ends can be attributed to the disruption of areoles by gaps and the high number of vascular islands.
Figure 12. *p1p2cvl1cvp2* quadruple mutants do not have more severe phenotypes than *cvl1cvp2* mutants. Seven-day-old *p1p2cvl1cvp2* (*n*=118), *cvl1cvp2* (*n*=100), *p1p2* (*n*=119), and wild type (*n*=113) seedlings were fixed and their cotyledons were cleared in 9:3:1 (w/v/v) chloral hydrate: water: glycerol. The cleared cotyledons were scored and the means per cotyledon ± one standard deviation are shown with different letters indicating significant differences between genotypes as indicated by one-way ANOVA analysis and Tukey HSD pairwise comparisons (α<0.05). (A) Gaps: DF=450, F=502.39, p<0.0001 (B) Reversals: DF=450, F=47.36, p<0.0001 (C) Vascular islands: DF=450, F=451.11, p<0.0001 (D) Aerolcs: DF=449, F=899.88, p<0.0001 (E) Free ends: DF=449, F=615.04, p<0.0001 (F) Branch points: DF=450, F=314.65, p<0.0001
Figure 13. *p1p2cvl1cvp2* and *cvl1cvp2* show similar decreases in areoles. Seven-day-old *p1p2cvl1cvp2* (n=118), *p1p2* (n=119), *cvl1cvp2* (n=100), and wild type (n=113) seedlings were fixed and their cotyledons were cleared in 9:3:1 (w/v/v) chloral hydrate: water: glycerol. The number of fully closed areoles was scored for all genotypes and the frequencies of 0, 1, 2, 3, and 4 areoles were calculated for comparisons between genotypes.
Given the lack of significant differences between \( plp2cvllcvp2 \) and \( cvllcvp2 \), the increase in vascular defects can be attributed to the \( cvllcvp2 \) mutations alone. This lack of an enhanced phenotype in the \( plp2cvllcvp2 \) mutant is surprising considering the weak interactions we observed between \( PATL1/2 \) and \( CVL1 \) or \( CVP2 \) in the triple mutants (Fig. 7, Fig. 8, Fig. 10, Fig. 11). However, the \( cvllcvp2 \) cotyledon vasculature is so fragmented that there is not much room for additional gaps, and the extent of discontinuity made it difficult to identify the vascular pattern (Fig. 9), so any small changes to vascular complexity would be difficult to identify. The one significant difference between \( plp2cvllcvp2 \) and \( cvllcvp2 \) that could be identified was an increase in branch points (Fig. 12F) (one-way ANOVA, DF=450, F=314.65, p<0.0001). The number of secondary veins could not be determined because the vascular pattern was so fragmented, but this increase in branch points is likely due to alterations in the regulation of secondary vein formation, as was seen in the \( plp2cvp2 \) triple (Fig. 10). Therefore, it appears that \( PATL1/2 \) interact with \( CVL1/CVP2 \) to control secondary vein formation and vascular complexity in subtle ways.

\( p6 \) mutants have decreased complexity

These potential interactions with \( CVL1/CVP2 \) suggest that \( PATL1/2 \) are involved in regulating cotyledon vascular development, but the \( p1p2 \) double mutant phenotype largely resembled wild type throughout these experiments, which strongly suggests that there is more functional redundancy in the patellin family that has yet to be identified. In order to test if \( PATL1/2 \) are functionally redundant with \( PATL6 \), another member of the patellin family that is expressed in the vascular tissue, \( patl1patl2patl6 (p1p2p6) \) triple mutants were analyzed. These triple mutants were produced by crossing \( p1p2 \) and \( p6 \)
mutants, and allowing the progeny to self-fertilize. Members of the resulting F2 generation were genotyped by PCR amplification using one T-DNA specific primer and two gene specific primers for each gene (data not shown).

Once $p1p2p6$ mutants were identified, they were self-fertilized to produce $p1p2p6$ mutants. These triple mutants (n=114), $p1p2$ (n=109), $p6$ (n=107) and wild type (n=107) were grown for seven days before cotyledons were cleared for analysis. Initial qualitative analysis of $p6$ and $p1p2p6$ mutants revealed that there was an apparent decrease in vascular complexity in these mutants (Fig. 14). In order to determine if these differences were significant and to determine if the $p1p2p6$ and $p6$ phenotypes were distinct, vascular defects and vascular complexity characteristics were scored.

Analysis of vascular defects showed that $p6$ mutants have significantly more gaps than $p1p2$, but neither of these mutants are significantly different than the wild type or the $p1p2p6$ mutant (Fig. 15A) (one-way ANOVA, DF=436, F=3.38, p=0.018, Tukey HSD, $\alpha<0.05$). This lack of significant differences compared to the wild type and $p1p2p6$ suggests that $p1p2$ and $p6$ cotyledons have gap frequencies that are skewed toward opposite ends of the wild type range, and that combination of these mutations increases the variability across this range. Since none of these mutants are significantly different from wild type in the number of gaps observed, $p1p2$ and $p6$ do not appear to enhance the frequency of gaps.
Figure 14. Characterization of patl-6 and p1p2p6 mutants. Seven-day-old wild type, p1p2, patl-6, and p1p2p6 mutants were fixed and their cotyledons were cleared in 9:3:1 (w/v/v) chloral hydrate: water: glycerol. Representative images are shown for each genotype. Scale bars represent 1mm.
Similarly, the number of reversals and vascular islands were not significantly different between these genotypes (Table 5) (Reversals: one-way ANOVA, DF=436, F=1.51, p=0.21, Tukey HSD, \( \alpha < 0.05 \); Vascular islands, DF=436, F=0.22, p=0.80, Tukey HSD, \( \alpha < 0.05 \)). Therefore, \( PATL1/2 \) and \( PATL6 \) are not necessary for vascular continuity or determining the directionality of vein formation.

Despite this lack of effect on the production of vascular defects, \( p1p2p6 \) and \( p6 \) mutants had significantly fewer areoles, branch points, and secondary veins than wild type and \( p1p2 \) mutants, suggesting a decrease in vascular complexity (Fig. 15B-D) (Areoles: one-way ANOVA, DF=436, F=14.41, p<0.0001, Tukey HSD, \( \alpha < 0.05 \); Branch points: one-way ANOVA, DF=436, F=19.84, p<0.0001, Tukey HSD, \( \alpha < 0.05 \); Secondary veins, one-way ANOVA, DF=436, F=21.97, p<0.0001, Tukey HSD, \( \alpha < 0.05 \)). There were no significant differences in free ends (Table 5) (one-way ANOVA, DF=436, F=1.14, p=0.33), so the secondary veins “missing” in these mutants likely corresponded to closed areoles in the wild type. However, there were no significant differences between \( p1p2p6 \) and \( p6 \), suggesting that these decreases in areoles, branch points and secondary veins are due to the \( patl6 \) mutation alone (Fig. 15B-D).
Table 5 Average number of vascular defects, aeroles, free ends, branch points and secondary veins in p6 and p1p2p6 mutants.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Wild Type</th>
<th>p1p2</th>
<th>p6</th>
<th>p1p2p6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample Size</td>
<td>114</td>
<td>109</td>
<td>107</td>
<td>107</td>
</tr>
<tr>
<td>Gaps</td>
<td>0.04±0.2</td>
<td>0.03±0.2</td>
<td>0.13±0.4</td>
<td>0.11±0.3</td>
</tr>
<tr>
<td>Reversals</td>
<td>0.06±0.2</td>
<td>0.10±0.3</td>
<td>0.15±0.4</td>
<td>0.09±0.3</td>
</tr>
<tr>
<td>Vascular Islands</td>
<td>0.01±0</td>
<td>0.0±0</td>
<td>0.01±0.1</td>
<td>0.01±0.1</td>
</tr>
<tr>
<td>Areoles</td>
<td>2.90±0.8</td>
<td>2.76±0.7</td>
<td>2.35±0.6</td>
<td>2.48±0.6</td>
</tr>
<tr>
<td>Free Ends</td>
<td>1.06±0.9</td>
<td>1.11±0.8</td>
<td>1.26±0.9</td>
<td>1.11±0.8</td>
</tr>
<tr>
<td>Branch Points</td>
<td>5.75±1.0</td>
<td>5.60±0.9</td>
<td>4.84±1.2</td>
<td>5.03±1.1</td>
</tr>
<tr>
<td>Secondary Veins</td>
<td>3.92±0.4</td>
<td>3.84±0.5</td>
<td>3.45±0.6</td>
<td>3.47±0.6</td>
</tr>
</tbody>
</table>
Figure 15. *p1p2p6* triple mutants do not show enhanced vascular defects or delays compared to *p6*. Seven-day-old *p1p2p6* (n=114), *p6* (n=107), *p1p2* (n=109), and wild type (n=107) seedlings were fixed and their cotyledons were cleared in 9:3:1 (w/v/v) chloral hydrate: water: glycerol. The cleared cotyledons were scored for the average number of vascular characteristics per cotyledon. The means ± one standard deviation are shown with different letters indicating significant differences between genotypes as indicated by one-way ANOVA analysis and Tukey HSD pairwise comparisons (α<0.05). (A) Gaps: DF=436, F=3.38, p=0.018 (B) Areoles: DF=436, F=14.41, p<0.0001 (C) Branch points: DF=436, F=19.84, p<0.0001 (D) Secondary veins: DF=436, F=21.97, p<0.0001
In addition to this analysis, the distributions of vascular complexity throughout the $p1p2p6$ and $p6$ samples were compared in order to assess if there are subtle differences between the two genotypes that are not captured through averages. Both the distribution of the number of areoles and of the vascular complexity score areoles: free ending secondary veins showed that $p1p2p6$ and $p6$ are skewed toward less complex vascular patterns, but their distributions are largely equivalent (Fig. 16). Therefore, $PATL6$ appears to be involved in regulating vascular complexity, but there is no evidence of any interactions between $PATL1/2$ and $PATL6$. Overall, these results suggest that $PATL16$ functions in a separate pathway from $PATL1/2$ and $CVL1/CVP2$ to regulate vascular development.
Figure 16. *p6* causes a decrease in the complexity of the cotyledon vascular pattern compared to wild type. Seven-day-old *p1p2p6* (n=114), *p6* (n=107), *p1p2* (n=109), and wild type (n=107) seedlings were fixed and their cotyledons were cleared in 9:3:1 (w/v/v) chloral hydrate: water: glycerol. (A) The number of fully closed areoles was scored for all genotypes and frequencies of 1, 2, 3, and 4 areoles were calculated. (B) The number of areoles and the number of free ends were scored and a score of areoles: free ending secondary veins was assigned to each cotyledon. Representative images for each potential score are shown. Any vascular patterns that did not fit into these categories, such as those with gaps or more than 4 secondary veins were scored as other. All scale bars represent 1mm. (C) The frequency of each complexity score was calculated for all genotypes. The complexity of the vascular pattern increases as you move to the right.
DISCUSSION

*PATL1/2* are expressed in developing and mature vascular tissues, and they encode Sec14-like proteins, which suggests they help regulate phosphoinositide metabolism and membrane trafficking events during vascular development (Peterman et al., 2004; Rackaityte, 2013). Given the importance of phosphoinositide metabolism in PIN1 trafficking (Heilmann, 2016a), it was hypothesized that PATL1/2 may play a role in regulating the membrane trafficking events that maintain the PIN1 polarity necessary for proper vascular patterning. Several players involved in this trafficking pathway are known to bind to phosphoinositide species, so if our hypothesis is correct, PATL1/2 would be expected to interact with some of these proteins. Therefore, potential genetic interactions between *PATL1/2* and well-characterized vascular genes that interact with phosphoinositides in this pathway were assessed to gain better insight into the role of PATL1/2 in vascular development.

*PATL1/2* interact only weakly with *VAB*

VAB binds to PtdIns(4)P (Hou et al., 2010; Naramoto et al., 2009) and is believed to help regulate vesicle formation off of the TGN by improving VAN3 localization (Naramoto et al., 2009; Sawa et al., 2005). In order to assess if *PATL1/2* play a role in this process, *p1p2vab* triple mutants were evaluated for evidence of genetic interactions between *PATL1/2* and *VAB*. These triple mutants did not show a more severe phenotype, with no significant increases in vascular defects (Fig. 4, Table 2) or dramatic changes to vascular complexity (Fig. 5, Fig. 6). The combination of these mutations did result in a slight decrease in secondary veins and branch points (Fig. 4D-E), which resulted in a slight decrease in vascular complexity (Fig. 5, Fig. 6), but these changes were very subtle.
Therefore, there is some evidence that \textit{PATL1/2} interact with \textit{VAB}, but these interactions are very weak.

This lack of a strong genetic interaction between \textit{PATL1/2} and \textit{VAB} suggests that \textit{PATL1/2} aren’t directly involved in production or regulation of PtdIns(4)P pools at the TGN, which is surprising given the role of Sec14-like proteins in PtdIns(4)P production (Bankaitis et al., 2010; Mousley et al., 2012; Schaaf et al., 2008). VAN3 normally relies on PtdIns(4)P binding (Koizumi et al., 2005) and interactions with VAB (Naramoto et al., 2009) to properly localize to the TGN and regulate vesicle formation. Therefore, if PtdIns(4)P pools at the TGN were disrupted and VAB was absent in \textit{p1p2vab} mutants, VAN3 localization would be hindered more than in the \textit{vab} mutant alone. With lower levels of proper VAN3 localization, PIN1 trafficking at the TGN would be further disrupted (Naramoto et al., 2009) and we would expect a more severe phenotype that was reminiscent of \textit{van3} mutants to arise. Since the \textit{p1p2vab} phenotype was only very mildly more severe, and did not show any increase in vascular discontinuities that are characteristic of \textit{van3} mutants (Deyholos et al., 2000; Koizumi et al., 2005; Sieburth et al., 2006), the PtdIns(4)P pools at the TGN are likely minimally affected in these \textit{p1p2vab} mutants.

Therefore, \textit{PATL1/2} and \textit{VAB} are likely acting in different pathways in vascular development that have only indirect and/or minor regulatory impacts on one another. Given that \textit{VAB} (Hou et al., 2010) and \textit{PATL1/2} (Rackaityte, 2013) are both expressed during procambial development, the weakness of their interaction cannot be attributed to temporal differences. Therefore, our data suggest that \textit{PATL1/2} and \textit{VAB} must be spatially and/or functionally distinct in their regulation of vascular development. One
possibility is that PATL1/2 are not involved in PtdIns(4)P production, as their Sec14-like homology would suggest, but rather function to regulate the levels of another phosphoinositide species. The most likely alternative candidates for PATL1/2 regulation are PtdIns(4,5)P₂, PtdIns(3)P and PtdIns(5)P given that PATL1/2 bind to these species in vitro (Peterman et al., 2004). These phosphoinositide species are concentrated at different membranes than PtdIns(4)P (Heilmann and Heilmann, 2015), so PATL1/2 would not be expected to localize to the TGN if it acted on one of these phosphoinositides in vivo. Therefore, PATL1/2 would be spatially separated from VAB and regulate membrane trafficking or signaling pathways associated with PtdIns(3)P, PtdIns(5)P or PtdIns(4,5)P₂ during vascular development.

However, it is also possible that PATL1/2 are involved in helping present PI to PI4-kinases as their Sec14-like nature would suggest, and produce a distinct pool of PtdIns(4)P that doesn’t bind to VAB and VAN3. High levels of PtdIns(4)P have been localized at the plasma membrane of plant cells (Simon et al., 2014), so PATL1/2 could play a role in producing this PtdIns(4)P, creating the negative electrostatic field that localizes important effector proteins such as PI4OID to the plasma membrane (Simon et al., 2016). Alterations to this pool of PtdIns(4)P due to p1p2 mutation would be expected to interfere with PIN1 trafficking, but at a different stage of the pathway than would be impacted by a vab mutation. Therefore, there would not be synergistic effects on PIN1 trafficking and vascular patterning in this situation, as was seen in the p1p2vab triple mutant (Fig. 4-6). Alternatively, PATL1/2 could be regulating PtdIns(4)P levels at the TGN, but creating a microdomain that is distinct from the VAB and VAN3 binding sites. Small structural differences, such as the level of unsaturation in the fatty acid tail of PIs
can help create these microdomains (Heilmann, 2016b). Therefore, if PATL1/2 favored binding to PI with one of these specific structural features, it could create a distinct microdomain of PtdIns(4)P that acts independently of the VAB/VAN3 regulated vesicle formation at the TGN to help regulate vascular development. In any of these scenarios, the phosphoinositide pool regulated by PATL1/2 would be distinct from the PtdIns(4)P that directs VAB localization, causing a lack of strong genetic interactions. However, the PI-phosphatase and kinase activity in the cell works to maintain relatively consistent levels of PIs, so alterations in the levels of one of these phosphoinositide species could have a small impact on the levels of PtdIns(4)P that binds to VAB, explaining the weak interactions seen.

**PATL1/2 interact with CVL1/CVP2**

A role of PATL1/2 in the PIN1 trafficking pathway could also lead to genetic interactions between PATL1/2 and the functionally redundant CVL1 and CVP2 (Carland and Nelson, 2009). Therefore, these potential genetic interactions were investigated by evaluating *p1p2cvl1*, *p1p2cvp2* and *p1p2cvl1cvp2* mutants for enhanced phenotypic severity. *p1p2cvl1* triple mutants had a small decrease in areoles and a corresponding increase in free ends (Fig. 7), which resulted in a slight shift toward a less complex cotyledon vascular pattern (Fig. 8). These phenotypic changes were subtle, but the functional redundancy and the low activity of CVL1 (Carland and Nelson, 2009; Naramoto et al., 2009) suggests that this subtly is due to the continued activity of CVP2 in these triple mutants. Combined with the increase in vascular defects (Fig. 10A-B), particularly the increase in reversals (Fig. 10B) and the decreases in vascular complexity (Fig. 11) that resulted in a more forked, *vab*-like phenotype in the *p1p2cvp2* triple mutant,
these results suggest that \textit{PATL1/2} act in the same pathway as these functionally redundant 5PTases (Carland and Nelson, 2009; Carland and Nelson, 2004). Given the resemblance of some \textit{p1p2cvp2} triple mutants to \textit{vab} mutants, these genes likely act in the same PIN1 trafficking process as \textit{VAB}, but at a different point in the pathway, preventing genetic interactions between \textit{PATL1/2} and \textit{VAB}.

\textit{CVL1} and \textit{CVP2} dephosphorylate PtdIns(4,5)P\textsubscript{2} and, by acting in opposition to PIP-kinases (Carland and Nelson, 2009; Carland and Nelson, 2004), help tightly regulate the levels of PtdIns(4,5)P\textsubscript{2} necessary for initiation of endocytosis during PIN1 trafficking (Rodriguez-Villalon et al., 2015; Tejos et al., 2014). This regulation of PtdIns(4,5)P\textsubscript{2} levels is necessary for proper procambial establishment (Rodriguez-Villalon et al., 2015), which aligns with the timing of \textit{PATL1/2} expression (Rackaityte, 2013), suggesting that \textit{CVL1/CVP2} and \textit{PATL1/2} act during the same developmental stage. In order for the observed genetic interactions to occur, this temporal similarity must be accompanied by interactions with the same substrates or complexes (Perez-Perez et al., 2009). Given these interactions and the known roles of \textit{CVL1/CVP2}, our evidence suggests that \textit{PATL1/2} may play a role in directly or indirectly regulating PtdIns(4,5)P\textsubscript{2} levels. If this interaction is direct, \textit{PATL1/2} could play a role in making PtdIns(4,5)P\textsubscript{2} in the plasma membrane more accessible to \textit{CVL1/CVP2} for dephosphorylation. This hypothesis is supported by the binding of \textit{PATL1/2} to PtdIns(4,5)P\textsubscript{2} \textit{in vitro} (Peterman et al., 2004). Therefore, in the \textit{p1p2cvl1} or \textit{p1p2cvp2} mutants, one of the 5PTases would be missing and the loss of \textit{PATL1/2} would make the remaining 5PTase less efficient at dephosphorylating PtdIns(4,5)P\textsubscript{2} in this scenario. In this case, both the \textit{cvl1} or \textit{cvp2} and the \textit{p1p2} mutations would contribute to deregulation of PtdIns(4,5)P\textsubscript{2} levels, and so a more severe phenotype
would be expected in these triple mutants due to the increased disruption of clathrin-mediated endocytosis of PIN1 (Tejos et al., 2014).

However, the inositol head of PtdIns(4,5)P₂ is more accessible than PI in the membrane so “presentation” of PtdIns(4,5)P₂ to catalytic enzymes may not be relevant in a cellular context. Therefore, another possibility is that PATL1/2 indirectly regulate PtdIns(4,5)P₂ levels by helping to form the PtdIns(4)P that will act as a substrate in PtdIns(4,5)P₂ formation. Given the lack of interaction with VAB, PATL1/2 could help generate PtdIns(4)P at the plasma membrane (Simon et al., 2014), creating the substrate for PIP kinases where they are localized (Tejos et al., 2014). In this case, we would expect plasma membrane localization of PATL1/2, causing their products to be spatially separated from VAB. However, recent studies in animal cells have shown transport of phosphoinositides between membranes (Sohn et al., 2016) so PATL1/2 could generate a specific pool of PtdIns(4)P at another membrane that is destined for transport to the plasma membrane. In this instance, loss of PATL1/2 would be expected to impair PtdIns(4)P production and consequently reduce the levels PtdIns(4,5)P₂ at the plasma membrane.

A reduction in PtdIns(4,5)P₂ caused by this plp2 mutation could balance the increase in PtdIns(4,5)P₂ expected from a loss of phosphatase activity in cvl1 or a cvp2 mutants. However, it is possible that increased PIP kinase activity or utilization of another PtdIns(4)P pool at the plasma membrane could occur in an attempt to compensate for this mutation. In that case, any compensatory increases in or changes to PIP kinases activity could not be balanced as well without CVL1 or CVP2, which could cause a further misregulation of PtdIns(4,5)P₂. These two possibilities could be evaluated by investigating
the subcellular localization of PATL1/2 or quantifying PtdIns(4,5)P₂ levels in the p1p2 double mutant.

Since any further increase or decrease in PtdIns(4,5)P₂ could cause increased defects in PIN1 endocytosis, regardless of the mechanism, because tight regulation of PtdIns(4,5)P₂ levels is required to maintain proper PIN1 localization (Rodriguez-Villalon et al., 2015; Tejos et al., 2014), both of these scenarios would result in further disruption of PIN1 trafficking in p1p2cvl1 or p1p2cvp2 mutants. Due to this disruption of PIN1 endocytosis, altered levels of PtdIns(4,5)P₂ would cause the loss of the apical-basal polarity of PIN1 (Tejos et al., 2014). Without proper dynamic localization of PIN1 (Geldner et al., 2003; Jaillais and Gaude, 2007), auxin cannot be properly directed to the growing end of the vein, causing the developing vascular tissue to fail to connect into a continuous pattern. This mechanism would help explain the increase in vascular defects in p1p2cvp2 mutants (Fig. 10) and the decreased vascular complexity in p1p2cvl1 mutants (Fig. 7, Fig. 8). Similarly, the loss of apical-basal distribution would cause improper lateral localization of PIN1, which could cause auxin to flow laterally when it would normally be restricted to apical-basal flow. Aberrant lateral flow of auxin would cause new vascular determination and differentiation to occur outside of the normal vascular pattern. This effect of PIN1 mislocalization could explain the increase in secondary veins in the p1p2cvp2 mutants (Table 4). Therefore, a role of PATL1/2 in regulating PtdIns(4,5)P₂ levels can explain the phenotypic alterations seen in the p1p2cvl1 and p1p2cvp2 triple mutants.

Alternatively, PATL1/2 may interact with CVL1 and CVP2 in non-redundant mechanisms. Although both of the 5PTases have been shown to play redundant roles in
regulating PI(4,5)P2 levels during PIN1 trafficking, CVL1 has also been shown to play a role in phosphoinositide signaling during stress response (Kaye et al., 2011). cvl1 single mutants have severely increased sensitivity to salt stress due to a reduction in reactive oxygen species production (Kaye et al., 2011). The severity of this phenotype suggests that the role of CVL1 in stress response is not redundant with CVP2 (Kaye et al., 2011). These results combined with the low activity of CVL1 against PI(4,5)P2 could suggest that phosphoinositide signaling during stress response is the primary role of CVL1 when CVP2 is present. Therefore, CVL1 may only play a major role in regulating PIN1 endocytosis when CVP2 is missing. If this is true and PATL1/2 are involved in the phosphoinositide signaling responsible for reactive oxygen species production as well as the PI(4,5)P2 regulation during endocytosis, distinctions between these roles of PATL1/2 may cause them to impact CVL1 differently than CVP2, explaining the phenotypic differences between the p1p2cvl1 and p1p2cvp2 triple mutants.

Genetic interactions were not seen in the p1p2cvl1cvp2 mutant (Fig. 12, Fig. 13), which initially seems to contradict the evidence of genetic interactions between PATL1/2 and CVL1/CVP2. However, the severity of the cvl1cvp2 mutant (Fig. 9) (Carland and Nelson, 2009; Naramoto et al., 2009) could be masking the evidence of genetic interactions between the two sets of functionally redundant genes. The severity of the cvl1cvp2 double mutant suggests that CVL1 and CVP2 are the only 5PTases regulating the levels of PtdIns(4,5)P2 that are necessary for PIN1 trafficking during the development of the secondary veins (Carland and Nelson, 2009; Naramoto et al., 2009). If PAT1/2 were responsible for directly helping CVL1/CVP2 access their substrates, this function of PATL1/2 would effectively be lost in cvl1cvp2 mutants and so loss of PATL1/2 would not
cause any further mechanistic disruptions. Alternatively, if PATL1/2 were indirectly responsible for regulation of PtdIns(4,5)P$_2$ levels by producing PtdIns(4)P, the reduction in PtdIns(4,5)P$_2$ caused by a loss of PATL1/2 may be too small to counteract the increase in PtdIns(4,5)P$_2$ caused by the total loss of CVL1 and CVP2 5PTase activity (Tejos et al., 2014). Therefore, our evidence suggests that PAT1/2 play a role in the tight regulation of PtdIns(4,5)P$_2$ levels necessary for PIN1 trafficking, but their role is small when compared to the combined effect of CVL1 and CVP2.

Interestingly, public protein interaction data shows that PATL1 and At5PTase11 both physically interact with AtEXO70E2, a component of the exocyst (BioGRID interaction data set; http://thebiogrid.org). This potential interaction with another 5PTase during exocytosis suggests that PATL1/2 may play a larger and more complex role in phosphoinositide metabolism by interacting with more members of the 5PTase family than was originally expected.

*Arabidopsis thaliana* has 15 5PTases that play diverse roles in membrane trafficking and signaling (Kaye et al., 2011). One of these 5PTases, At5PTase13, is expressed during vascular development in young seedlings (Lin et al., 2005). At5PTase13 has been shown to dephosphorylate Ins(1,4,5)P$_3$ *in vitro* and induces the expression of auxin biosynthesis and transport genes, such as PIN4 (Lin et al., 2005). Therefore, At5PTase13 activity in the cell is distinct from that of CVL1 and CVP2, but *at5ptase13* mutation results in an increase in the number of secondary veins and branch points, causing new small loops to form (Lin et al., 2005), which is reminiscent of one of the *p1p2cvp2* phenotypic outcomes reported here. These phenotypic similarities suggest there is some overlap in the developmental processes regulated by the interactions of PATL1/2.
with CVL1 and CVP2, and those regulated by At5PTase13. Therefore, it would be interesting to investigate potential interactions between PATL1/2 and At5PTase13 to better understand the role of PATL1/2 in determining how different 5PTases work together to regulate vascular development.

Further functional redundancy with PATL1/2 has yet to be identified

The evidence that the role of PATL1/2 is relatively minor in PATL1/2 and CVL1/CVP2 interactions is consistent with the lack of a severe phenotype seen in the p1p2 mutants throughout this study. However, the absence of a significant difference between p1p2 double mutants and wild type throughout most of our experiments and the presence of only 0.05 to 0.08 gaps (Tables 2-4) directly contradicts previous characterizations of the double mutant phenotype (Rackaityte, 2013). Since the genotypes of our p1p2 mutants were confirmed, the phenotypic differences between the two studies were likely the result of differences in clearing and scoring techniques. In these studies, small gaps were assumed to be breaks in the vasculature rather than true gaps, potentially making our reported numbers an underestimate. Alternatively, the clearing techniques used by Rackaityte (2013) may have been harsher on the vascular tissue, causing an increase in breaks in the vascular tissue during mounting procedures. If this is the case, the significant increase in gaps that was previously reported compared to the wild type (Rackaityte, 2013) may represent an increased fragility of the vascular tissue in p1p2 mutants, which suggest that p1p2 mutants resemble the fra3 mutant.

FRA3 is another 5PTase that has been shown to play some role in vascular development, and which dephosphorylates PI(4,5)P2 and the soluble Ins(1,4,5)P3 (Zhong et al., 2004). Mutation of this 5PTase causes disorganization of the actin cytoskeleton and
formation of thinner secondary cell walls in the fiber cells (Zhong et al., 2004). These fiber cells form bundles between the vascular bundles and are largely responsible for producing the mechanical strength of the stem (Zhong et al., 2001), causing the fra3 mutants to have a more fragile stem (Zhong et al., 2004). Since we believe that similar fragility of the vascular tissue is responsible for the increase in “apparent” gaps previously reported (Rackaityte, 2013), these results would indicate that p1p2 mutations increase the fragility of the vascular tissue. This increased fragility in p1p2 mutants would suggest that PATL1/2 may play a role in regulating secondary wall formation as well as vesicle trafficking. Therefore, investigation of potential interactions between PATL1/2 and FRA3 could provide further insight into the regulatory roles of PATL1/2 in the vascular tissue.

In either case, our results suggest that an increase in discontinuities in the vasculature due to patterning defects may not be the true phenotype of the p1p2 double mutant. The only significant difference that we observed in the p1p2 mutants was a slight decrease in areoles (Fig. 7), corresponding to a slight decrease in vascular complexity (Fig. 8) with a very large sample size of p1p2. Therefore, our results suggest that the p1p2 mutant has a much more subtle phenotype than was originally thought.

Despite this lack of a drastic phenotype in the p1p2 double mutants, our evidence of genetic interactions between PATL1/2 and CVL1/CVP2, as well as the expression of PATL1/2 in the procambium and mature vascular tissue (Rackaityte, 2013) supports that PATL1/2 play a role in vascular development. Therefore, the lack of a significant phenotype in the vasculature of p1p2 double mutants suggests that there is additional functional redundancy that is compensating for the loss of PATL1/2. If these functionally redundant gene(s) could be identified, higher order mutants with these genes and PATL1/2
knocked out would have a more severe phenotype. This phenotype would be easier to characterize and to distinguish from the wild type, making it easier to characterize the effects of the loss of PATL1/2 function.

*PATL6 plays a distinct role in vascular development*

Due to similarities in domain structures within the patellin family (Peterman et al., 2006), the other patellin proteins are the most likely sources of this redundancy. Therefore, *p1p2p6* triple mutants were evaluated for increased phenotypic severity that would suggest redundancy. Although *p6* mutants showed a decrease in vascular complexity, there were no significant differences between the phenotypes of the *p6* and *p1p2p6* mutants (Fig. 15, Fig. 16), suggesting *PATL6* and *PATL1/2* are not redundant. Therefore, additional genes, such as the other members of the patellin family should be screened for redundancy with *PATL1/2* to help create a more distinct mutant phenotype. *PATL4* is the most likely candidate for this redundancy given that it has been shown to colocalize with PIN1 (Tejos Ulloa, 2012).

This lack of redundancy also shows that the patellin family likely plays several diverse roles during vascular development. Since *PATL6* is not redundant with *PATL1/2*, our results suggest that it does not interact with *CVL1* and *CVP2* to regulate PtdIns(4,5)P₂ levels. However, the Sec14-like nature of *PATL6* (Peterman et al., 2006) indicates that it likely plays a role in regulating phosphoinositide metabolism or signaling. Therefore, *PATL6* may generate a distinct pool of PtdIns(4)P or possibly another phosphoinositide species, to help regulate proper PIN1 trafficking during vascular development. Disruption of this regulatory function would result in PIN1 mislocalization, which could interrupt proper auxin flow and prevent formation of the complete vascular pattern, as is seen.
However, we can only speculate on the specific role of PATL6 until further studies are performed. *In vitro* analysis of the activity of PATL6 could help identify the phosphoinositides that this Sec14-like protein is most likely to interact with. Similarly, studies to identify potential genetic interactions with known vascular genes, for example between *PATL6* and *VAB* since both genes do not interact strongly with *PATL1/2*, could help provide more insight into the role of PATL6.

In conclusion, our results suggest that *PATL1/2* and other redundant genes that have yet to be identified may interact with *CVL1* and *CVP2* to regulate the PtdIns(4,5)P$_2$ levels necessary for proper endocytosis and trafficking of PIN1 during vascular development. On the other hand, PATL6 has a distinct function in vascular development and its mutation leads to phenotypic outcomes that are similar to characterized vascular mutants with mutations in the PIN1 trafficking pathway. Therefore, the present study indicates that the family of PATL proteins may play diverse roles in the lipid metabolism that regulates PIN1 trafficking during vascular development.
REFERENCES


