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Regulation of Auxin Transport in Arabidopsis Leaf Vascular Development

by

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LIST OF ABBREVIATIONS

AGC - eukaryotic protein kinase A (PKA), cyclic GMP-dependent protein kinase (PKG), and protein kinase C (PKC)
ANOVA – Analysis of Variance
ASK – ARABIDOPSIS Skp1-LIKE1
ATHB8 – ARABIDOPSIS THALIANA HOMEOBOX 8
AUX – AUXIN-RESISTANT
AUX/IAA – AUXIN/INDOLE-3-ACETIC ACID
AuxRE – Auxin Response Element
ARF- AUXIN RESPONSE FACTOR
AXR6 – AUXIN RESISTANT 6

CAND1 – CULLIN-ASSOCIATED AND NEDDYLATION-DISSOCIATED
CFP- Cyan Fluorescent Protein
CRY-CRYPTOCHROME

DAG – Days After Germination

EAR – Ethylene (Response Factor)-associated Ampiphilic Repression
EYFP – Enhanced Yellow Fluorescent Protein

HVE – HEMIVENATA

LAX – LIKE-AUX1
LUT-Look-Up Table

MP – MONOPTEROS

PED – PIN1 Expression Domain
PHOT- PHOTOTROPIN
PHY-PHYTOCHROME
PIN – PIN-FORMED

SHR- SHORT-ROOT

TIR1/AFB – TRANSPORT INHIBITOR RESISTANT1/AUXIN SIGNALLING F-BOX PROTEIN
TPL – TOPLESS

CHAPTER 1: GENERAL INTRODUCTION

The plant vascular system is a network of continuous vascular bundles that extend throughout the plant and transport water, nutrients, and regulatory molecules (Esau, 1965; Nelson & Dengler, 1997; Taiz & Zeiger, 2010). Vascular bundles are cylinders of radially juxtaposed xylem and phloem—the two vascular tissues. Xylem transports water from root to shoot, and phloem transports photosynthates from source to sink tissues (Taiz & Zeiger, 2010). The continuity of vascular tissues—a requirement for transport function—is achieved by the end-to-end alignment of cells in files that extend the length of the vascular bundles (Taiz & Zeiger, 2010). During plant growth by lengthening—primary growth—xylem and phloem differentiate from within bundles of files of continually dividing, vascular precursor procambial cells (Beck, 2005). In some plants, a layer of mitotically inactive procambial cells remains in each vascular bundle, between the xylem and phloem formed during primary growth (Beck, 2005). During plant growth by thickening—secondary growth—this procambial cell layer resumes cell division to give rise to the vascular cambium, from which secondary xylem and phloem differentiate.

Auxin Transport in Vascular Development

A number of substances can trigger vascular cell differentiation (Clouse & Sasse, 1998; Dettmer et al., 2009; Werner & Schmulling, 2009), but auxin is the only molecule that promotes the formation of continuous vascular strands. Exogenous application of auxin to hypocotyl or root tissues induces the differentiation of parenchyma cells into vascular cell files that connect the applied auxin with the pre-existing vasculature (Sachs, 1981). The auxin-induced vascular differentiation response is characterized by five defining properties (Sachs, 1981; Berleth et al., 2000). First, the response is local: the vascular strand is initiated at the specific site of auxin application. Second, the response is polar: vascular strand formation proceeds from the site of auxin application towards the basal pole of the plant. Third, the response is continuous: the differentiation process generates uninterrupted files of vascular cells. Fourth, the response is radially constrained: vascular cell files are organized in narrow bundles. Fifth, the response is dependent on auxin transport: the response is obstructed by polar auxin transport inhibitors, suggesting that it is the transport of auxin, and not auxin *per se*, that is required for vascular strand formation. These considerations form the basis of the 'Canalization Hypothesis' (Sachs, 1981; Sachs, 1991). The hypothesis proposes that auxin flow through a cell gradually increases that cell's capability to transport auxin, thus increasing auxin transport efficiency as the cell is able to transport higher volumes of auxin. Canalization occurs because broad areas of auxin-transport-competent cells, with a net apolarity of transport, gradually restrict to narrow cell files of robust, polar auxin transport. It is these cell files ("canals") that go on to form vascular strands (Sachs, 1991).

The apical-basal polarity of auxin-induced vascular strand formation suggests that the underlying mechanism recruits polar cues already present in the organism. The sensitivity of auxin-induced vascular strand formation to polar auxin transport inhibitors suggest that those polar cues may be provided by the polar transport of auxin occurring in plant tissues. Auxin is in fact synthesized primarily in young leaves and floral buds and is transported, mainly through vascular tissues, towards the roots (Lomax et al., 1995). The 'Chemiosmotic Hypothesis'

proposes a cellular mechanism by which long-distance, polar transport of auxin could occur (Rubery & Sheldrake, 1974; Raven 1975). The hypothesis is based on the observation that Indole-3-Acetic Acid (IAA), the most prevalent auxin in higher plants, is a weak acid (Rubery & Sheldrake, 1974), and is therefore protonated to IAAH in the acidic extracellular space; the non-polar IAAH could enter the cell by passive diffusion across the plasma membrane. Once inside the cell, the neutral pH of the cytosol would cause IAAH to deprotonate to the anionic form, IAA^-. To exit the cell, the polar IAA^- would require the action of efflux carrier proteins. Importantly, the hypothesis predicts that these auxin efflux carriers are localized to the basal plasma membrane only, and that this polar localization of auxin efflux carriers is the driving force of polar auxin transport (Rubery & Sheldrake, 1974; Raven 1975).

The Arabidopsis *PIN-FORMED* (*PIN*) gene family has eight members (*PIN1-PIN8*) (Papanov et al., 2005), five of which encode proteins that localize to the plasma membrane (Galweiller et al. 1998; Muller et al., 1998; Friml et al., 2002; Friml et al., 2002b; Vieten et al., 2005). The plasma-membrane-localized PIN1 protein has 12 transmembrane alpha-helical segments, a structure that is common to a superfamily of bacterial and eukaryotic transport proteins, whose substrates are as diverse as drugs, sugars, and oligosaccharides (Marger & Saier Jr., 1993; Galweiller et al., 1998). The PIN1 protein had originally been implicated as a crucial component of the auxin transport machinery because *pin1* mutants have reduced auxin transport and *pin1* mutant phenotypes can be mimicked by growing wild-type plants on polar auxin transport inhibitors (Okada et al., 1991; Galweiller et al. 1998; Mattson et al., 1999). Consistent with the predictions made by the Chemiosmotic Hypothesis for auxin efflux carriers, PIN proteins localize polarly to the plasma membrane of mature cells (Galweiller et al., 1998; Muller et al., 1998; Friml et al., 2002b; Friml et al., 2002; Vieten et al., 2005), and are sufficient to promote auxin efflux in both Arabidopsis and heterologous cell systems (Petrasek et al., 2006).

While the Chemiosmotic Hypothesis does not predict the existence, or requirement, of auxin influx carriers, *AUXIN-RESISTANT1* (*AUX1*) and *LIKE-AUX1* (*LAX*) *1*, *LAX2* and *LAX3*, which encode proteins similar to plant and fungal amino acid permeases, encode putative auxin influx proteins (Bennett et al., 1996; Parry et al., 2001). To date, however, only AUX1 and LAX3 have been biochemically characterized as auxin influx proteins (Yang et al., 2006; Swarup et al., 2008). AUX1 is localized polarly to the apical and lateral plasma membrane of leaf epidermal cells (Reinhardt et al., 2003) and the apical plasma membrane of root phloem cells (Swarup et al., 2001), while LAX3 is apolarly localized to the plasma membrane of root phloem cells (Swarup et al., 2008). Chromatographic measurements of auxin concentrations indicate that *aux1* mutants over-accumulate auxin in leaves, but under-accumulate auxin in roots (Marchant et al., 2002). As such, AUX1 may be involved in loading auxin in source organs (i.e. leaf) and unloading it in sink organs (i.e. root) (Swarup et al., 2001; Marchant et al., 2002).

Auxin Signal Transduction in Vascular Development

If proper auxin transport is required for vascular strand formation, auxin signal transduction should be required to trigger vascular cell differentiation. The auxin signal transduction cascade is initiated with the binding of auxin to an auxin receptor of the TRANSPORT INHIBITOR RESISTANT1/AUXIN SIGNALLING F-BOX PROTEIN (TIR1/AFB) family (Dharmasiri et al., 2005; Kepenski & Leyser, 2005). TIR1 is the F-box protein subunit of the SCF^{TIR1} (Skp1-Cul1-F-box protein) E3 ubiquitin ligase complex (Gray et al., 1999), which also comprises

CULLIN1 (CUL1) [encoded by *AUXIN RESISTANT6* (*AXR6*) (Hellmann et al., 2003)] and two proteins related to yeast Skp1, ASK1 and ASK2 (ARABIDOPSIS Skp1-LIKE1 and 2) (Gray et al., 1999). Upon binding to auxin, TIR1 recruits proteins of the AUXIN/INDOLE-3-ACETIC ACID (AUX/IAA) family into SCF^{TIR1}, where ubiquitination targets them for degradation by the 26S proteasome (Dharmsiri et al., 2005). The *HEMIVENATA* (*HVE*) gene encodes a CAND1 (CULLIN-ASSOCIATED AND NEDDYLATION-DISSOCIATED) protein that interacts with CUL1/AXR6 to regulate the formation of the SCF^{TIR1} complex (Alonso-Peral et al., 2006).

When not bound by TIR1, AUX/IAA proteins interact with transcription factors of the Auxin Response Factor (ARF) family. ARF proteins bind Auxin Response Elements (AuxREs) in the promoter of auxin-responsive genes (Liu et al., 1994; Kim et al., 1997; Guilfoyle et al., 1998) through an amino-terminal, plant-specific DNA-binding domain (Liu et al., 1994). Domains III and IV in the carboxy-terminal region allow ARF proteins to form homo- or hetero-dimers with other ARFs, or with AUX/IAA proteins, which also contain domains III and IV in their carboxy-terminal regions (Kim et al., 1997). ARF-mediated transcription is repressed by the formation of a complex between ARFs, AUX/IAAs, and the global transcriptional co-repressor TOPLESS (TPL), which binds to domain I of AUX/IAAs through an EAR ([Ethylene Response Factor]-associated Ampiphilic Repression) motif (Szemenyei et al., 2008). TPL functions as a transcriptional co-repressor through its histone deacteylase activity, which by chromatin remodelling inhibits spatial availability of DNA to transcriptional machinery at the location of the ARF-AUX/IAA-TPL complex on the chromosome (Long et al., 2006).

AuxREs are cis-acting elements that confer auxin responsiveness to the promoter of auxin-responsive genes (Ballas et al., 1993; Li et al., 1994; Liu et al., 1994; Ulmasov et al., 1995). Naturally occurring AuxREs contain a TGTCTC element, or an element with related sequence (Donner et al., 2009; Walcher & Nemhauser, 2011), and an adjacent or overlapping coupling element (Ulmasov et al., 1995). The TGTCTC element confers auxin responsiveness to the promoter, while the coupling element confers constitutive expression to the gene (Ulmasov et al., 1997). The TGTCTC element represses activity of the coupling element when auxin levels are low; at high auxin levels, repression of the coupling element is released and the auxin-inducible gene is transcribed (Ulmasov et al., 1995). Synthetic AuxREs have been created that employ multimers of the TGTCTC element with appropriate spacing and orientation (Ulmasov et al., 1997). The synthetic AuxRE DR5 contains tandem repeats of the TGTCTC element and a TATA box (Ulmasov et al., 1997). DR5 has found widespread use among plant biologists as a means to visualize auxin distribution. Fused to a reporter gene, DR5 confers auxin-responsive reporter gene expression that provides a snapshot of auxin distribution at the cellular level in wild-type or mutant backgrounds (e.g., Sabatini et al., 1999; Mattsson et al., 2003; Friml et al., 2003).

The role of auxin signal transduction in vascular development is reflected in the high levels of auxin signalling—visualized through DR5-driven reporter gene expression—that defines prospective vascular cells (Friml et al., 2003; Mattson et al., 2003; Aida et al., 2004; Ibanes et al., 2009), and in the vascular defects of mutants in components of auxin-dependent gene regulation, such as *tir1* (Ruegger et al., 1998), *axr6* (Hobbie et al., 2000), and *hve* (Alonso-Peral et al., 2006). However, the most dramatic examples of impaired auxin signal transduction and associated vascular defects are found in mutants of *ARF5/MONOPTEROS (MP)* (Berleth & Jurgens, 1993; Przemek et al., 1996; Hardtke & Berleth, 1998; Mattsson et al., 2003). *mp* mutants have fewer vascular strands, and within these vascular strands cells are misaligned

(Berleth & Jurgens, 1993; Przemek et al., 1996). Because mutation in *MP* greatly reduces or abolishes levels of an integral component of the auxin signal transduction machinery, vascular defects in *mp* have been interpreted as failure to properly express auxin-responsive genes that regulate vascular development (Mattson et al., 2003; Donner et al., 2009).

Leaf Vascular Development

The leaf is a convenient organ in which to study vascular patterning because leaf vascular strands ('veins') are formed *de novo* during the development of every new leaf. A mature leaf is characterized by a number of vascular pattern hallmarks: a prominent, central, primary midvein that extends the entire length of the leaf; secondary vein loops that branch off the midvein, and connect distally to the midvein or other vein loops; and tertiary, higher-order veins that branch from the midvein or loops, and can either connect two veins or end freely in the leaf (Kang & Dengler, 2002).

At early stages of leaf development, the subepidermal tissue of the leaf primordium is composed of morphologically-identical, isodiametric ground cells. Ground cells differentiate into one of two mutually-exclusive cell types: mesophyll cells and procambial cells. Procambial cells, which are characteristically elongated cells with dense cytoplasms, are the precursors of all mature vascular cell types (Esau, 1965; Nelson & Dengler, 1997). Ground cells that will differentiate into procambial cells initiate expression of the genes *ATHB8* (*ARABIDOPSIS THALIANA HOMEOBOX 8*) and *SHR* (*SHORT-ROOT*) (Kang & Dengler, 2002; Scarpella et al., 2004; Gardiner et al., 2011). Direct downstream targets of *ATHB8* have not yet been reported, but available evidence suggests possible functions for *ATHB8* in procambial development (Donner et al., 2009). While the function of *SHR* in leaf vascular development is not known, *SHR* is required for the correct differentiation of vascular tissues in the root (Yu et al., 2010).

Auxin Transport in Leaf Vascular Development

Consistent with the critical role of auxin transport in the development of other organs, polar auxin transport is crucial to the development of the leaf and its veins. Because expression of plasma-membrane-localized PIN proteins marks auxin's exit site from the cell (Wisniewska et al., 2006), auxin transport paths in leaf development can be visualized by imaging PIN protein localization. PIN1 is the only plasma-membrane-localized PIN to be expressed at early stages of leaf development (Scarpella et al., 2006), stages at which PIN1 localizes to the apical plasma membrane of all epidermal cells, suggesting auxin transport towards the apex of the leaf. Coordinated apical localization of PIN1 in epidermal cells results in formation of a convergence point of opposing PIN1 polarities at the leaf tip (Scarpella et al., 2006; Wenzel et al., 2007), and successive appearance of a subepidermal domain of PIN1 expression connected with the epidermal convergence point suggest that auxin enters subepidermal tissues at epidermal convergence points. The subepidermal PIN1 expression domain is initially broad but eventually narrows to the site of midvein formation (Scarpella et al., 2006; Wenzel et al., 2007). This dynamic seems to be reiterated during formation of the lateral (i.e. lower) component of each vein loop: formation of a PIN1 convergence point in the marginal epidermis at the side of the leaf; formation of a broad subepidermal domain of PIN1 expression that connects the epidermal convergence point with the developing midvein; and restriction of the broad subepidermal

domain of PIN1 expression to the future site of formation of the lower component of the vein loop. Consistent with these dynamics, epidermal expression of PIN1 is sufficient to direct normal patterning of midvein and vein loops (Bilsborough et al., 2011). However, unlike the midvein and lateral components of vein loops, marginal (i.e. upper) components of vein loops and higher-order veins both originate from subepidermal PIN1 expression domains that branch off of pre-existing PIN1 expression domains with no correlation to any epidermal points of convergent PIN1 polarity (Scarpella et al., 2006; Wenzel et al., 2007). Marginal and higher-order PIN1 expression domains are initially freely ending but can connect to pre-existing PIN1 expression domains on both sides, the latter being usually the case with marginal PIN1 expression domains. In all narrow PIN1 expression domains, PIN1 polarity is always directed to pre-existing veins: freely ending PIN1 expression domains display a single uniform PIN1 polarity, while in bilaterally-connected PIN1 expression domains the two opposite polarities at the extremities are integrated by a bipolar cell along the course of the connected PIN1 expression domain. Consistent with expression of PIN1 at stages critical for vein patterning, *pin1* mutant leaves show characteristic defects: bifurcation of the midvein towards the leaf tip, and lateral veins often fail to connect to the midvein, and instead run parallel to it to form a wide midvein. (Mattsson et al., 1999; Bilsborough et al., 2011). More severely affected leaves in *pin1* show separation defects ('fused leaves') (Okada et al., 1991; Bilsborough et al., 2011).

Disruption of auxin transport by treatments with auxin transport inhibitors provides an additional means to analyze the contributions of auxin transport to leaf vein patterning. Obstruction of auxin transport in wild-type leaves causes major alterations in auxin distribution that are associated with defects in vascular patterning similar to, yet more severe than, those in *pin1* (Mattsson et al., 1999; 2003; Sieburth, 1999). Enhanced sensitivity of *athb8* mutant leaves to auxin transport inhibitors suggest that *ATHB8* is required to stabilize developing veins against disruptions in auxin transport (Donner et al., 2009).

Auxin Signalling in Leaf Vascular Development

Visualization of auxin response in developing leaves through the activity of the DR5 promoter has implicated auxin signalling in leaf vein development: DR5 expression is the strongest in ground cells just prior to their elongation into procambial cells, suggesting that vascular differentiation occurs at sites of maximum auxin response (Mattsson et al., 2003). DR5 expression fades during differentiation of procambial cells into vascular cells, and is very low or absent in mature veins (Mattsson et al., 2003).

The necessity of auxin signal transduction to proper leaf vein development can be observed in leaf vascular phenotypes of mutants in components of auxin-dependent gene regulation, such as *axr6* (Steynen & Schultz, 2003) and *hve* (Alonso-Peral et al., 2006)**.** *MP* mutant leaves show the most severe examples of impaired auxin signal transduction phenotypes. Leaves of strong *mp* alleles have an extremely reduced and simplified vascular pattern, which often consists of only a bifurcated midvein and scattered vein fragments (Przemek et al., 1996; Donner et al., 2009). Veins in *mp* leaves are often interrupted and composed of few, improperly aligned vascular cells (Przemek et al., 1996; Donner et al., 2009). Leaves of weak *mp* alleles show a spectrum of vascular phenotypes, ranging from a complexity similar to that of wild type to a more simplified vascular pattern with a bifurcated midvein (Donner et al., 2009). Additionally, leaves of weak *mp* alleles lack vascular tissues at the leaf margins and therefore

show a more "centralized" vasculature (Donner et al., 2009). The spatial association between location of auxin maxima and sites of vein formation in the leaf appear to be disrupted in *mp* mutants, and this may be responsible for the severely reduced vascular complexity of *mp* leaves (Wenzel et al., 2007). Consistent with a role in leaf vein patterning, *MP* transcripts initially accumulate in broad, subepidermal domains of the leaf that become restricted during leaf development to sites of incipient vein formation (Hardtke & Berleth, 1998; Wenzel et al., 2007). Restriction of transcript accumulation seems to coincide with increase in transcript abundance at sites that prelude vein formation (Wenzel et al., 2007). *MP* transcript accumulation is highest in files of procambial cell precursors, intermediate in procambial strands, and very low or absent in fully differentiated, mature veins (Wenzel et al., 2007). Expression of the MP protein during leaf development follows a similar dynamic: initial expression in broad domains that resolve into narrow sites of vein formation before subsiding to undetectable levels (Donner et al., 2009). MP is a direct, positive regulator of *ATHB8*, and this interaction is essential for early (vein and auxin responsive expression of *ATHB8* (Donner et al., 2009), suggesting that activation of *ATHB8* expression is one of the direct inputs of MP-mediated auxin signal transduction in leaf vascular development.

Scope of the Work

This research will investigate the contributions of light perception and polarity alterations in leaf vein development. Although light provides a crucial energy source for plants, its role in regulating plant development through signal transduction has only recently been elucidated, and any possible role that light perception plays in tissue patterning has been largely uninvestigated. Similarly, although changes in PIN1 polarity occur throughout leaf vein patterning (Scarpella et al., 2006; Wenzel et al., 2007), the expression and function of the AGCVIIIa serine-threone kinases—proteins that regulate PIN1 polarity—during early stages of leaf and vein development remains unknown. My research will investigate these two areas, and provide important insights into previously unexplored facts of auxin-dependent vein formation.

CHAPTER 2: THE ROLE OF LIGHT SIGNALLING IN ARABIDOPSIS LEAF VEIN DEVELOPMENT

Introduction

The vascular system of plant leaves is a continuous network of vascular strands ("veins") that transport water, nutrients, and regulatory molecules (Esau, 1965; Nelson & Dengler, 1997; Taiz & Zeiger, 2010). From a developmental standpoint, the formation of the vein network is particularly intriguing because it seems to occur *de novo* during the development of each new leaf (Foster, 1952; Pray, 1955). At early stages of leaf development, the subepidermal tissue of the leaf primordium is composed of ground cells—isodiametric, morphologically identical cells—that will differentiate into either photosynthetic mesophyll cells or vascular-precursor procambial cells (Scarpella et al., 2004). Files of ground cells that will differentiate into procambium can be recognized because they selectively express the homeobox gene *ATHB8* (*ARABIDOPSIS THALIANA HOMEBOX GENE 8*) and *SHR* (*SHORT-ROOT*) (Baima et al., 2001; Scarpella et al., 2004, Gardiner et al., 2011).

While the molecular details are not entirely clear, a role for the plant signalling molecule auxin and its polar transport in vein formation is supported by varied evidence: auxin application induces formation of new veins (Sachs, 1989); the inductive effect of applied auxin on vein formation is suppressed by auxin transport inhibitors (Gersani, 1987); and auxin transport inhibitors induce defined and reproducible defects in vein patterns (Mattsson et al., 1999; Sieburth, 1999). Auxin is primarily synthesized in young apical organs, and is transported polarly to the roots (Ljung et al., 2002). This polar auxin transport is accomplished at the cellular level by the asymmetric localization to the plasma membrane of vascular cells of auxin efflux proteins of the PIN-FORMED (PIN) family (Galweiller et al., 1998; Muller et al., 1998; Friml et al., 2002; Friml et al., 2002b; Vieten et al., 2005; Petrasek et al., 2006). The *pin1* mutant of Arabidopsis shows a range of leaf and vascular defects, which can include leaf fusion, midvein bifurcation, and improper vein connection (Okada et al., 1991, Mattsson et al., 1999; M. Sawchuk & E. Scarpella, unpublished), which suggests that *PIN1* is a critical component of auxin transport during leaf and vein development.

While auxin transport is required for vein formation, auxin signalling is required for vascular cell differentiation (Friml et al., 2003; Mattson et al., 2003; Aida et al., 2004; Ibanes et al., 2009). Auxin promotes vascular cell differentiation through complex transcriptional cascades that are mediated by the ARF (AUXIN RESPONSE FACTOR) family of transcription factors (Ulmasov et al., 1999). ARFs bind auxin-response elements in the promoters of auxin-responsive genes, but in the absence of auxin transcription is repressed by formation of a complex between ARFs and repressor proteins of the AUX-IAA (AUXIN/INDOLE-3-ACETIC ACID) family (Ulmasov et al., 1997). Auxin promotes gene transcription by binding to an auxin receptor of the TIR1/AFB (TRANSPORT INHIBITOR RESISTANT1/AUXIN SIGNALLING F-BOX PROTEIN) family and to AUX/IAAs, thus targeting AUX/IAAs for degradation and releasing ARFs from inhibition (Dharmasiri et al., 2005; Kepinski & Leyser, 2005). The *arf5*/*monopteros* (*mp*) mutant of Arabidopsis has fewer vascular strands, and in which vascular cells are frequently misaligned, suggesting that *ARF5/MP* is a crucial component of auxin signal transduction in vein development (Berleth & Jurgens, 1993; Przemek et al., 1996).

Light is the primary source of energy to plants and the main signal plants use to acquire information on their surrounding environment. Plants have developed sophisticated methods to sense the quality, quantity, direction, and duration of light through light-sensing proteins called photoreceptors, which initiate transduction cascades to trigger complex developmental and physiological responses (Smith & Whitelam, 1997; Devlin et al., 1999; Quail, 2002; Devlin et al., 2003). There are three main families of photoreceptors in Arabidopsis: CRYPTOCHROMES (CRY1, CRY2) and PHOTOTROPINS (PHOT1, PHOT2), which perceive UV-A and blue light (Cashmore et al., 1999; Briggs & Huala, 1999), and PHYTOCHROMES (PHYA, PHYB, PHYC, PHYD, PHYE), which perceive red/far-red light (Sharrock & Quail, 1989; Clack et al., 1994). Available evidence has associated photoreceptor-mediated light signal transduction with mostly quantitative responses in plant development (e.g., hypocotyl elongation, cotyledon expansion, and transition to flowering) (e.g., Neff & Chory, 1998; Mockler et al., 1999), and very few studies have investigated the role of light in patterning of plant features (e.g., Yoshida et al., 2011). Here I have explored a possible role for light signalling in vein patterning. I found that specific photoreceptors perceiving both UV-A/blue and red/far-red light are expressed at early stages of vein development. The photoreceptors *CRY1*, *CRY2*, *PHOT1*, and *PHOT2*, as well as *PHYA, B, D,* and *E*, are redundantly required to negatively control vein network formation. I also found that *PHYC* antagonizes function of *PHYA, PHYB*, *PHYD*, and *PHYE* in vein network formation. Additionally, PHYB-mediated red/far-red light perception antagonizes the function of PIN1-dependent polar auxin transport in vein formation.

MATERIALS AND METHODS

Vector construction

To generate CRY::YFP, PHOT::YFP and PHY::YFP transcriptional fusions, the respective genes' upstream regions were amplified from *Arabidopsis thaliana* ecotype Col-0 genomic DNA using Finnzymes Phusion high-fidelity DNA Polymerase (New England Biolabs Inc., Ipswich, MA, USA) and gene-specific primers (Table 1). To generate CRY1::YFP, the 2313-bp region from -2587 to -274 (with respect to the start codon) of *CRY1* (AT4G08920) was used. To generate CRY2::YFP, the 1119-bp region from -1303 to -184 of *CRY2* (AT1G04400) was used. To generate PHOT1::YFP, the 4631-bp region from -4636 to -5 of *PHOT1* (AT3G45780) was used. To generate PHOT2::YFP, the 864-bp region from -898 to -34 of *PHOT2* (AT5G58140) was used. To generate PHYA::YFP, the 2603-bp region from -2609 to -6 of *PHYA* (AT1G09570) was used. To generate PHYB::YFP, the 1677-bp region from -1809 to -132 of *PHYB* (AT2G18790) was used. To generate PHYC::YFP, the 5488-bp region from -5542 to -54 of *PHYC* (AT5G35840) was used. To generate PHYD::YFP, the 722-bp region from -760 to -38 of *PHYD* (AT4G16250) was used. To generate PHYE::YFP, the 756-bp region from -763 to -7 of *PHYE* (AT4G18130) was used. The amplified regions were integrated into pDONR221 (Invitrogen, Carlsbad, CA, USA) with BP Clonase II (Invitrogen), sequence checked, and recombined into the Gateway-adapted pFYTAG binary vector, which contains a translational

fusion between the coding region of histone 2A (HTA6; AT5G598700) and that of the enhanced YFP (EYFP) (Zhang et al., 2005), with LR Clonase II (Invitrogen).

Table 1 – Oligonucleotides used to amplify photoreceptor genes' upstream regions

Gene	Oligonucleotides
CRY1	CRY1 FOR: GGG GAC AAG TTT GTA CAA AAA AGC AGG CTC AAA TAT AAA ATA GTT GGA G
	CRY1 REV: GGG GAC CAC TTT GTA CAA GAA AGC TGG GTG GAC AAA GTA GTC TCT AAG
CRY2	CRY2 PROM FOR 2: GGG GAC AAG TTT GTA CAA AAA AGC AGG CTG GTG TGA GAT CAG TTA AAG
	CRY2 PROM REV 2: GGG GAC CAC TTT GTA CAA GAA AGC TGG GTC ATA ACA GAG AGA GAT TCG
PHOT1	PHOT1 FORW: GGG GAC AAG TTT GTA CAA AAA AGC AGG CTG ATA CCA TAA AGG AAT ACA G
	PHOT 1 REV: GGG GAC CAC TTT GTA CAA GAA AGC TGG GTC TCT CTC TAT ACA CGA AAC
PHOT2	PHOT2 FORW: GGG GAC AAG TTT GTA CAA AAA AGC AGG CTG CGG GAA AAT AAT AAG GC
	PHOT2 REV : GGG GAC CAC TTT GTA CAA GAA AGC TGG GTA GAG TAA TGA ATA GTC TGC
PHYA	PHYA FORW: GGG GAC AAG TTT GTA CAA AAA AGC AGG CTC AAA CTG AAG AAG AAG ATG
	PHYA REV: GGG GAC CAC TTT GTA CAA GAA AGC TGG GTT CCT GAC ACA GAG ACA AGA C
PHYB	PHYB FORW: GGG GAC AAG TTT GTA CAA AGC AGG CTG ATT ATG AGA GAA CGA ACA C
	PHYB REV: GGG GAC CAC TTT GTA CAA GAA AGC TGG GTG AGG AAG AAG AAA ATG GGG
PHYC	PHYC FORW: GGG GAC AAG TTT GTA CAA AAA AGC AGG CTG AGC ACG CAA GAT GAT GAA AG
	PHYC REV: GGG GAC CAC TTT GTA CAA GAA AGC TGG GTC AGA AAT GGA GGT GAG AG
PHYD	PHYD FORW: GGG GAC AAG TTT GTA CAA AAA AGC AGG CTG AGA ATC AAA AGA GTC CTG AG
	PHYD REV: GGG GAC CAC TTT GTA CAA GAA AGC TGG GTG AAT TAG TGC GAG AGA CGA AG
PHYE	PHYE FORW: GGG GAC AAG TTT GTA CAA AAA AGC AGG CTG CGT AAG GAA GTG ACC TGA C
	PHYE REV : GGG GAC CAC TTT GTA CAA GAA AGC TGG GTG GGG AAA GTG TGG AGT GAG

Plant material, transformation, and growth conditions

Origins of lines used in this study are in Table 2. Mutants were genotyped with gene-specific primers and 'LBb1.3' or 'SAIL LB3' T-DNA primer (*phyA*, *phyC*, *phyD*, *phyE*, *cry1*, *phot1*, *phot2*), with gene-specific primers followed by restriction-enzyme digestion (*phyB-9*, *pin1-1*, *mpB4149*), or with gene-specific primers (*cry2-1*). Arabidopsis seeds were surface-sterilized, synchronized, and germinated on growth medium — half strength Murashige and Skoog (MS) Salts (Caisson Labs, North Logan, UT, USA), 15 g l^{-1} sucrose (Fisher Scientific, Fair Lawn, NJ, USA), 0.5 mg l^{-1} MES (BioShop Canada Inc., Burlington, ON, Canada), 0.8% (w/v) agar (Phytotechnology Laboratories, Shawnee Mission, KS, USA), pH 5.7— at the approximate density of 1 seed cm^{-2} as previously described (Scarpella et al., 2004). Sealed plates were incubated at 25ºC under continuous fluorescent light (100 µmol m^{-2} s^{-1}). 'Days after germination' (DAG) are defined as days following exposure of imbibed seeds to light. Four-DAG seedlings were transferred to Promix BX soil (Evergro/Westgro) in 7- x 7- x 8-cm pots at a density of 0.1 seedlings cm^{-2}. Seedlings were grown at 22°C under a 16 h/8 h light:dark photoperiod of 130-140 µmol m^{-2} s^{-1} (vein density analyses) or 100-150 µmol m^{-2} s^{-1} (all other studies). Arabidopsis plants (ecotype Col-0) were transformed with *Agrobacterium tumefaciens* strain GV3101::pMP90 (Koncz & Schell, 1986) harbouring single photoreceptor transcriptional fusions. Primary transformants were selected on growth medium supplemented with 200 ug ml^{-1} carbenicillin (Teknova, Hollister, CA, USA), 10 ug ml^{-1} glufonsinate ammonium (Sigma-Aldrich Canada Ltd., Oakville, ON, Canada), and 50 ug ml^{-1} nystatin (Bioshop Canada, Burlington, ON, Canada). Ten single-insertion lines per construct were analyzed to determine the most representative expression pattern of the gene. Detailed analyses were performed on at least 3 lines per construct that were chosen because of strong HTA6:EYFP expression that was representative of the expression pattern of the gene.

Table 2 – Plant lines and their origins

Line	Origin
ATHB8::CFP	(Sawchuk et al., 2007)
cry1	SALK_069292 (ABRC; Alonso et al., 2003; Ruckle et al., 2007) containing a single T-DNA at position -1521 of *CRY1* (AT4G08920)
cry2-1	Guo et al., 1998
phot1	SAIL_147_B12 (ABRC; McElver et al., 2001) containing a single T-DNA at position -1880 of *PHOT1* (AT3G45780)
phot2	SALK_051046 (ABRC; Alonso et al., 2003) containing a single T-DNA at position -71 of PHOT2 (AT5G58140)
phyA-T	SALK_014575 (ABRC; Alonso et al., 2003; Folta, 2004) containing a single T-DNA at position -1164 of *PHYA* (AT1G09570)
phyB-9	Reed et al., 1993
phyC-2	SALK_057517 (Monte et al., 2003)
phyD	SALK_126326 (ABRC; Alonso et al., 2003) containing a single T-DNA at position -55 of *PHYD* (AT4G16250)
phyE	SALK_092529 (ABRC; Alonso et al., 2003) containing a single T-DNA at position -882 of *PHYE* (AT4G18130)
pin1-1	Goto et al. 1987; Gälweiler et al. 1998; WT at the TTG1 (AT5G24520) locus (M. Sawchuk and E. Scarpella, unpublished)
mp^{B4149}	Weijers et al., 2006
arf5-2	Donner et al., 2009

Table 3 – Oligonucleotides and enzymes used to genotype mutants

Mutant	Oligonucleotides and enzymes
phyA-T	phyA-LP: CCA GTC AGC TCA GCA ATT TTC phyA-RP: AAT GCA AAA CAT GCT AGG GTG LBb1.3: ATT TTG CCG ATT TCG GAA C
phyB-9	phyB9-1: AGC TAG TGG AAG AAG CTC GAT GAG GCC TTG phyB9-2 2: ACC GTC ACA TTT CAC TAA GTC CAT GAT ACT StyI digestion
phyC-2	PHYC 057517 RP: GAT GGA GCT GAG CAT AGA ACG PHYC 057517 LP: TTA GGC TTA CGT AGC TTG CCC LBb1.3
phyD	PHYD-RP2: ATC GGT TAC AGT GAA AAT GCG PHYD LP2 : AAC CCG GTA GAA TCA GAA TGG LBb1.3
phyE	phyE-RP: TAT CAG TGG TTA AAC CCG TCG phyE-LP: TTT GAT TGC TGT CGA AGA ACC LBb1.3
phot1	PHOT1 RP: TCA CGA TTG CTC CCA TTA AAG PHOT1 LP: TAT CGG GAA GCC TAG GAT CAG SAIL LB3: TAG CAT CTG AAT TTC ATA ACC AAT CTC GAT ACA C
phot2	PHOT2 RP2b: AGT GTC ATT GCT CAC GGA TTC PHOT2 LP: ATG GCG CAT GTT CTG TTC TAC LBb1.3
cry1	CRY1-RP: TCC CGA CAG ACT GGA TAC ATC CRY1-LP: TTC ATG CCA CTT GGT TAG ACC LBb1.3
cry2-1	CRY2 U/S FOR: CAG TCT CCA TTG CGA CAA G CRY2 U/S REV: CAA CCT GTT ATC CTC CAC ATG
pin1-1	PIN1-1 FOR: ATG ATT ACG GCG GCG GAC TTC TA PIN1-1 REV: TTC CGA CCA CCA CCA GAA GCC TatI digestion
mp^{B4149}	MP1498S: CTC TCA GCG GAT AGT ATG CAC ATC GG MP 2082 AS: ATG GAT GGA GCT GAC GTT TGA GTT C MseI digestion
arf5-2	mp SALK 021319 RP: CCT TCT TCA CTC ATC TGC TGG mp SALK 021319 LP: CCT GGA AAC TGA TGA GCT GAC LBb1.3

Microtechniques and microscopy
Dissected leaves were mounted in water and observed with a 20x Planaprochromat (NA, 0.8) objective on a Zeiss Axiovert 100M microscope equipped with a Zeiss LSM510 laser module confocal unit (Carl Zeiss, Oberkochen, Germany). YFP was excited with the 514 nm line of an Argon laser at 55% of output (equivalent to approximately 6 A) and 5% transmission and emission detected with a BP565-615 filter. For colocalization analysis, leaves were observed with a 40x C-Apochromat water (NA, 1.2) objective. CFP was excited with the 458 nm line of an Argon laser at 55% of output and 85% to 100% transmission and emission detected with a BP480-520 filter, while YFP was excited with the 514-nm line of an Argon laser at 1% to 45% transmission and emission detected with a BP565-615 filter. Mature (14-21 DAG) first leaves were fixed in 3:1 (v/v) ethanol:acetic acid and stored in 70% (v/v) ethanol. Fixed leaves were rehydrated in distilled water prior to mounting in chloral hydrate:glycerol:water 8:3:1 (w/v/v), and viewed under dark-field illumination with an Olympus SZ61TR stereomicroscope (Olympus Optical Co., Tokyo, Japan). Images were captured with an AxioCam HR camera (Carl Zeiss).

Image analysis and processing
Brightness and contrast of 8-bit images were adjusted through linear stretching of the histogram in ImageJ (National Institute of Health, http://rsb.info.nih.gov/ij). Images were colour-coded in ImageJ by applying a look-up table (LUT) in which black was used to encode global background, blue to encode local background, and cyan, green, yellow, orange, and red to encode increasing signal intensities (Sawchuk et al., 2007). Signal colocalization was visualized with an extended dual-channel LUT from cyan to magenta through green, yellow, and red (Demandolx and Davoust, 1997): fluorescence in each detection channel was displayed in either cyan or magenta, and then merged using the differential operator in Adobe Photoshop 7.0 (Adobe Systems Inc., San Jose, CA, USA). As a result, excess of cyan signal over colocalized magenta signal is encoded in green; opposite in red; and colocalized cyan and magenta signals of equal intensity in yellow. Images were cropped using Adobe Photoshop 7.0 (Adobe Systems Inc. San Jose, CA, USA), and assembled into figures in Canvas 8 (ACD Systems International, Victoria, BC, Canada).

RESULTS

Expression of Arabidopsis photoreceptor genes at early stages of vein development
Expression data at the organ level are available for most Arabidopsis photoreceptor genes (Clack et al., 1994; Kupper et al., 2007; Kang et al., 2009;). In Arabidopsis, photoreceptor gene expression is controlled mainly at the transcriptional level (Sharrock & Quail., 1989; Clack et al., 1994). Therefore, to visualize expression of Arabidopsis photoreceptor genes at cellular resolution, I used transcriptional reporter gene fusions.

The 3-kb sequence upstream of the translation start codon is sufficient to recapitulate the endogenous mRNA expression pattern for 35 of 44 tested Arabidopsis transcription factors (Lee et al., 2006). Therefore, to construct transcriptional fusions of photoreceptor genes, I used either ~3 kb of upstream non-coding sequence or the entire upstream non-coding region, whichever was shorter (see Materials and Methods).

To improve sensitivity of gene expression detection, all upstream non-coding sequences were fused to a nuclear yellow fluorescent protein (YFP) consisting of a translational fusion between the coding regions of histone 2A (HTA6; At5g59870) and that of the enhanced YFP (EYFP; Zhang et al., 2005). Since YFP is approximately 50% brighter than GFP, and four times brighter than the cyan fluorescent protein (CFP; Dobbie et al., 2008), I reasoned that YFP should allow detecting transcriptional fusions at much lower expression levels than GFP or CFP. Furthermore, by targeting all the YFP produced in an individual cell to the nucleus, it is possible to locally increase the concentration of YFP by 30-50%, which results in an enhanced sensitivity of signal detection (Joly, 2007). Finally, YFP signals can be easily separated from both CFP and GFP fluorescence in plants (e.g. Kato et al., 2002; Sawchuk et al., 2007), and therefore YFP fusions can reliably be used in colocalization studies (see below).

Most photoreceptor genes are expressed in leaves (Clack et al., 1994), but whether photoreceptor genes are expressed at early stages of vein development remains unknown. To address this, I visualized expression of transcriptional fusions of photoreceptor genes in first leaves of seedlings 4 days after germination (DAG) because their veins are predominantly at preprocambial and procambial stages of development (Sawchuk et al., 2007; Donner et al., 2009). I found that fusions of *CRY1*, *CRY2*, *PHYC*, and *PHYE* are expressed near-ubiquitously and evenly throughout the leaf (Fig. 1A,B,G,I). Expression of the *PHOT1* fusion seemed to be excluded from veins (Fig. 1C). The fusion of *PHOT2* was expressed exclusively in trichomes (Fig. 1D), and fusions of *PHYA* and *PHYB* were expressed more strongly at sites of vein formation (Fig. 1E,F). Finally, the *PHYD* fusion was expressed strongly at the leaf margin and weakly in inner regions of the leaf (Fig. 1H).

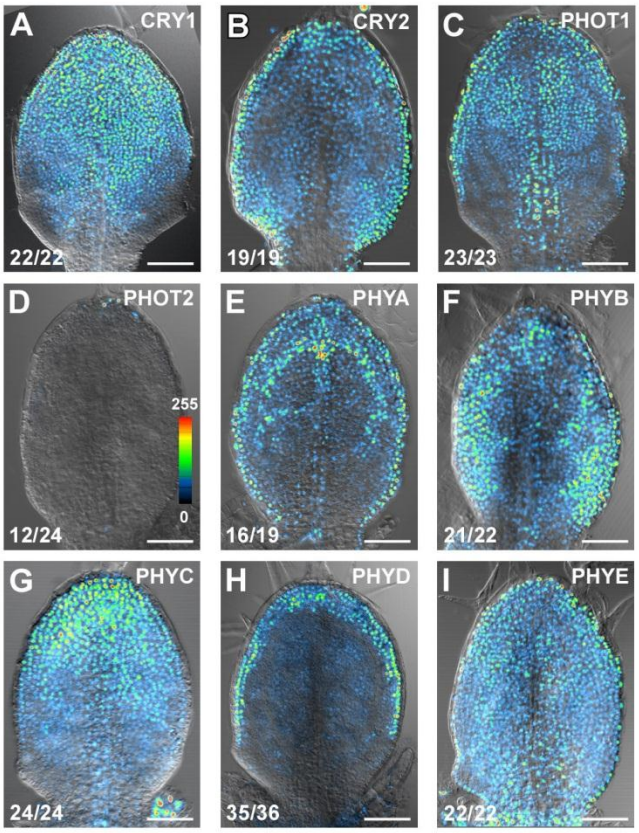


Fig. 1. Photoreceptor gene expression in Arabidopsis leaves. A-I: First leaves 4 days after germination (DAG). Overlay of confocal laser scanning and differential interference microscopy images, subepidermal focal plane. A look-up table (LUT; displayed in D), in which black was used to encode background, and cyan, green, yellow, orange, and red to encode increasing signal intensities (Sawchuk et al., 2008), was applied to eight-bit gray scaled images to generate colour coded images. Top right, gene identity. Bottom left, fraction of samples showing the displayed features. Scale bars, 50 µM.

Stage-specific photoreceptor gene expression in vein development

Transcriptional fusions of specific photoreceptor genes seem to be expressed at sites of vein formation (Fig. 1), but a stringent criterion to test this hypothesis would be to visualize the expression of photoreceptor genes relative to that of the early vascular marker gene *ATHB8* (Baima et al., 1995). I therefore visualized the degree of colocalization between expression of transcriptional fusions of photoreceptor genes to HTA6:EYFP and expression of a transcriptional fusion of *ATHB8* to a nuclear CFP (ATHB8::ECFP-Nuc; Sawchuk et al., 2007) in second vein loops of 4-DAG first leaves.

The human eye has a differential sensitivity to light perception across the visible spectrum (Russ, 2002). Therefore, in order to improve the discrimination of fluorescence signals in double-labeling images—and thus accurately visualize signal colocalization—I used an extended dual-channel look-up table (LUT). The LUT ranges from cyan to magenta through green, yellow, and red (Demandolx & Davoust, 1997). Fluorescence in each detection channel was displayed in either cyan (e.g. Fig. 2A) or magenta (e.g. Fig. 2B), and single fluorophore images were then merged using a differential operator (see Materials and Methods). As a result, higher levels of cyan signal over colocalized magenta signal is encoded in green, and the opposite scenario is encoded in red. Colocalized cyan signals and magenta signals of equal intensity are encoded in yellow (e.g. Fig. 2C).

As shown in Figure 2, transcriptional fusions of *CRY2*, *PHYA*, *PHYB*, and *PHYC* (Fig. 2D-F), (Fig. 2M-O), (Fig. 2P-R), (Fig. 2S-U), but not of all other photoreceptor genes, were expressed in cells expressing ATHB8::ECFP-Nuc, suggesting that *CRY2*, *PHYA*, *PHYB*, and *PHYC* are expressed at early stages of vein development.

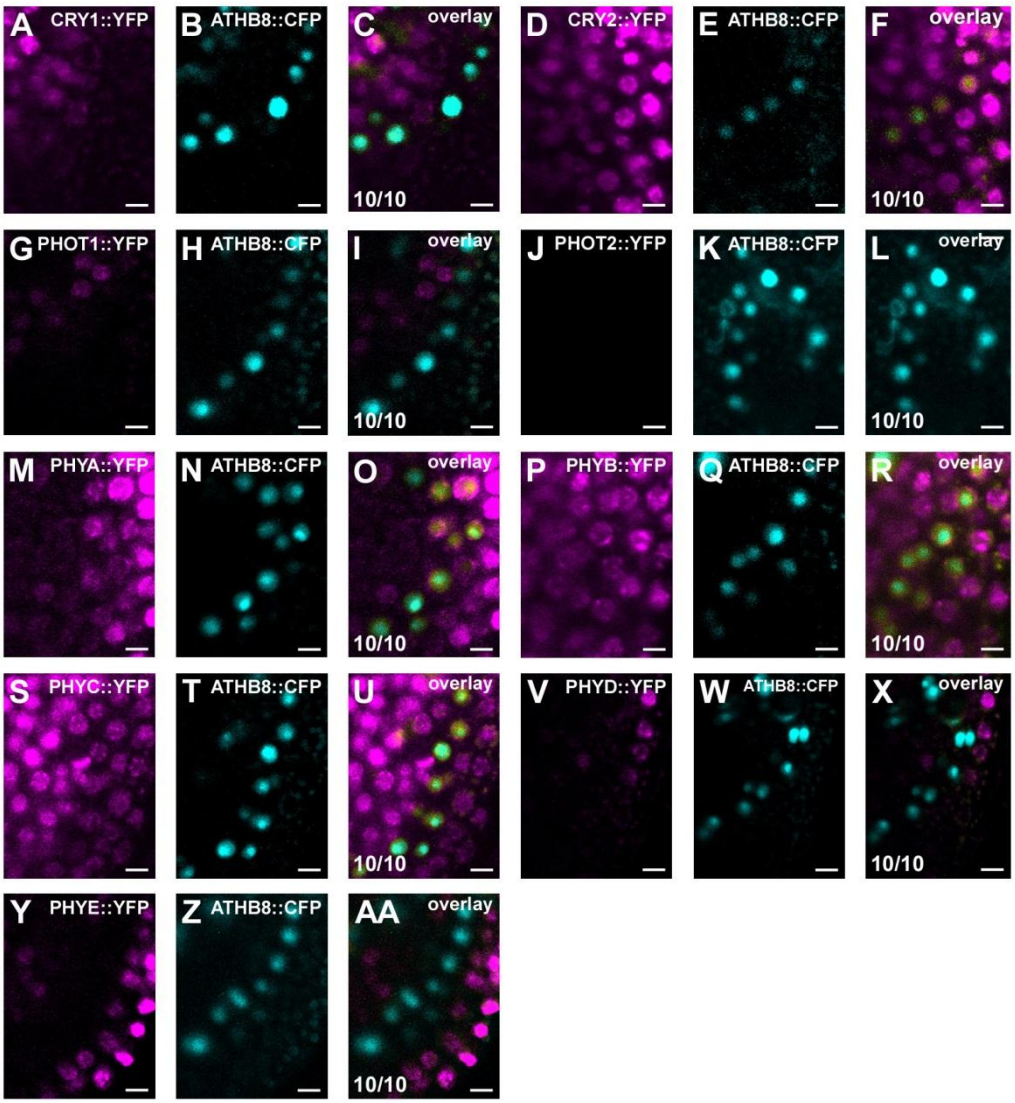


Fig. 2. Stage-specific photoreceptor gene expression in vein development. A–AA: Details of second loops of 4-DAG first leaves. Confocal laser scanning microscopy images, subepidermal focal plane. Top right, gene identity. Bottom left, fraction of samples showing the displayed features. A,C: Magenta, CRY1::HTA6:EYFP expression. B,C,E,F,H,I,K,L,N,O,Q,R,T,U,W,X,Z,AA: Cyan, ATHB8::ECFP-Nuc expression. D,F: Magenta, CRY2::HTA6:EYFP expression. G,I: Magenta, PHOT1::HTA6:EYFP expression. J,L: Magenta, PHOT2::HTA6:EYFP expression. M,O: Magenta, PHYA::HTA6:EYFP expression. P,R: Magenta, PHYB::HTA6:EYFP expression. S,U: Magenta, PHYC::HTA6:EYFP expression. V,X: Magenta, PHYD::HTA6:EYFP expression. Y,AA: Magenta, PHYE::HTA6:EYFP expression. C,F,I,L,O,R,U,X,AA: Merge of images in A and B, D and E, G and H, J and K, M and N, P and Q, S and T, V and W, and Y and Z, respectively. Images are color-coded with a dual-channel LUT from cyan to magenta through green, yellow and red (Demandolx and Davoust, 1997). Fluorescence in each detection channel was displayed in either magenta or cyan. Single-fluorophore images were then merged using a differential operator. As a result, preponderance of cyan signal over colocalized magenta signal is encoded in green, opposite in red, and colocalized cyan and magenta signals of equal intensity in yellow. Scale bars, 10 µM.

Function of photoreceptor genes in vein network formation

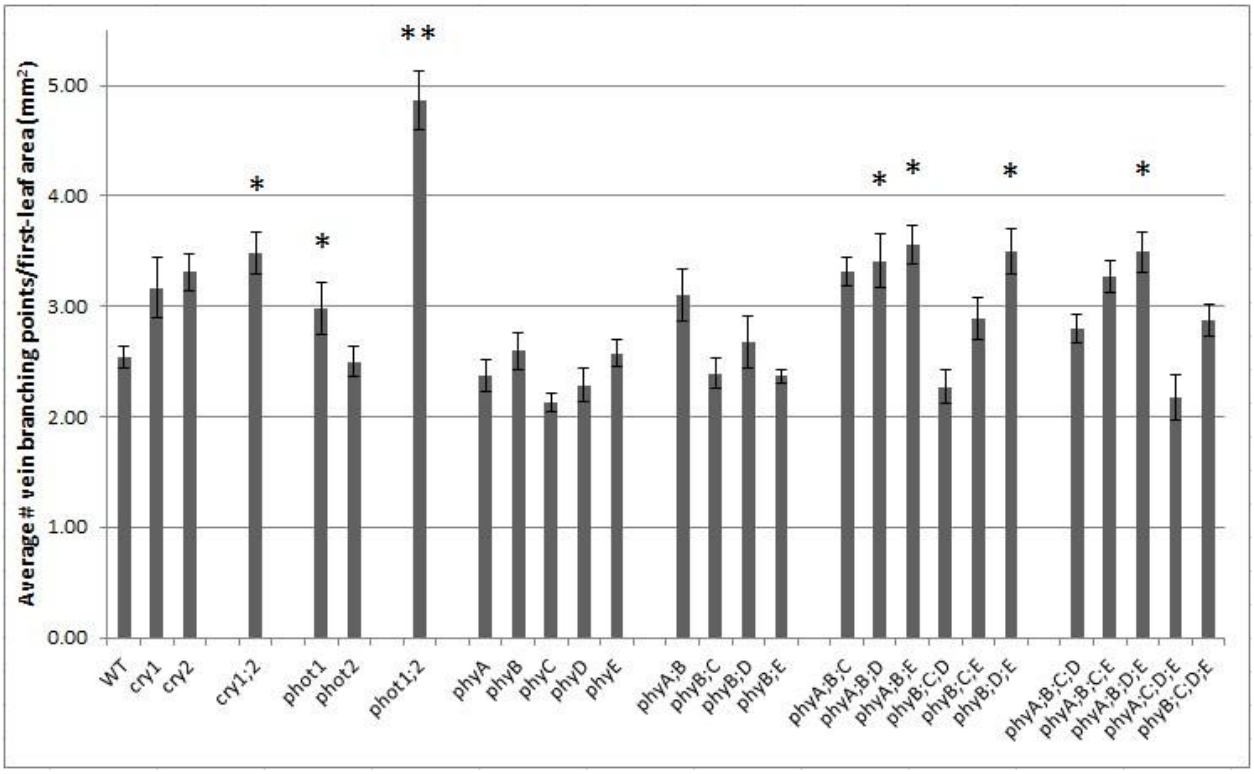
Because genes encoding photoreceptors that detect both blue light (*CRY2*) and red/far-red light (*PHYA*, *PHYB* and *PHYC*) are expressed at early stages of vein development (Fig. 2), I asked whether mutation of photoreceptor genes may result in defects in vein development. To address this question, I created single, double, triple, and quadruple intra-family photoreceptor mutants (e.g. *cry1;2, phot1;2, phyA;B;C;D*), and analyzed vein patterns of first leaves. Leaves of all photoreceptor mutant combination had wild-type (WT) vein patterns (data not shown), but leaves of *cry1;2*, *phot1*, *phot1;2*, and of triple and quadruple-mutant combinations of *phyA*, *phyB*, *phyD*, and *phyE* had greater numbers of vein branching points per leaf area unit (Fig. 3)—a proxy for complexity of vein networks (Candela et al., 1999)—suggesting that *CRY1*, *CRY2*, *PHOT1*, *PHOT2*, and *PHYA*, *B*, *D*, and *E* are redundantly required to negatively control vein network formation. Mutation of *PHYC* in *phyA;B;D*, *phyA;B;E*, and *phyB;D;E* backgrounds normalizes vein network complexity (Fig. 3), suggesting that *PHYC* antagonizes function of *PHYA, PHYB, PHYD*, and *PHYE* in vein network formation.

Greater numbers of vein branching points per leaf area unit could result from smaller leaves with vein networks of WT complexity, from normal-size leaves with more complex vein networks, or from combinations of the two. Leaves of *cry1*, *cry2* and *cry1;2* are smaller than WT (Fig. 4), but only the double-mutant leaves have greater numbers of vein branching points per leaf area unit, suggesting that the effect of *cry1*and *cry2* on vein network complexity is, at least in part, independent of the effects of *cry1* and *cry2* on leaf growth. Size of *phot1* leaves is comparable to that of WT, while *phot2* leaves are larger and *phot1;2* leaves are smaller than WT (Fig. 4). However, only leaves of *phot1* and *phot1;2* have greater numbers of vein branching points per leaf area unit, suggesting that the effects of *phot1* on vein network complexity are, at least in part, independent of the effects of *phot1* on leaf growth; the effects of *phot2* on vein network complexity may be attributable, at least in part, to increased leaf size.

Separating the effects of mutation of phytochrome genes on leaf growth and vein network complexity is more difficult because of opposing functions of PHYA, on one side, and PHYB, PHYC, PHYD, and PHYE on the other, in leaf growth; however, for example, *phyB* leaves are as small *phyA;B;D* leaves (Fig. 3), but only the triple-mutant leaves have greater numbers of vein branching points per leaf area unit (Fig. 4), which suggests that the effects of *phyA*, *phyB*, and *phyD* on vein network complexity are, at least in part, independent of the effects of *phyA*, *phyB*, and *phyD* on leaf growth.

In summary, CRY1/CRY2- and PHOT1/PHOT2-mediated blue light perception negatively regulates vein network formation. PHYA/PHYB/PHYD/PHYE-mediated red/far-red light perception also negatively regulates vein network formation, and the function of the PHYA/PHYB/PHYD/PHYE-dependent pathway in vein network formation is antagonized by PHYC-mediated red/far-red light perception.

A.



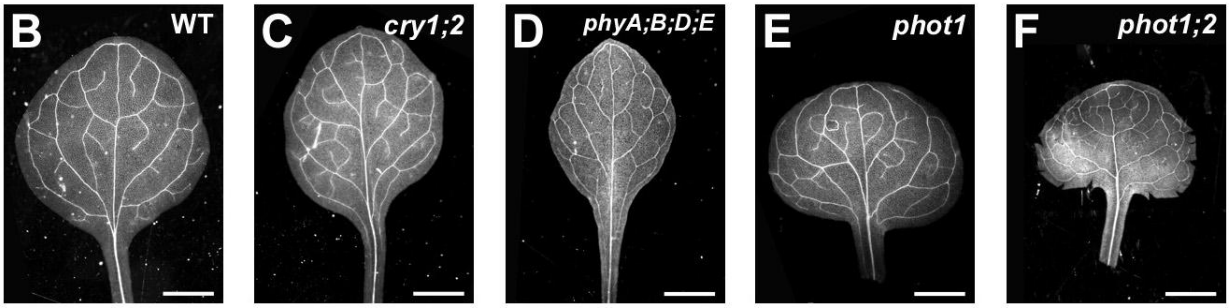


Fig. 3. A, Vein network complexity of photoreceptor mutants. Data indicate mean ± SE number of vein branching points per first-leaf area in mm^2 (Candela et al., 1999). Difference between sample and positive control was significant at $P<0.05$ (*) or $P<0.01$ (**) by one-way ANOVA and Tukey's test. Sample population size: 10 leaves per genotype. B-F, Dark-field illumination of cleared, mature first leaves of WT and of genotypes with significantly different vein densities. B: WT; C: *cry1;2*; D: *phyA;B;D;E*; E: *phot1*; F: *phot1;2*. Scale bars, 0.46 mm.

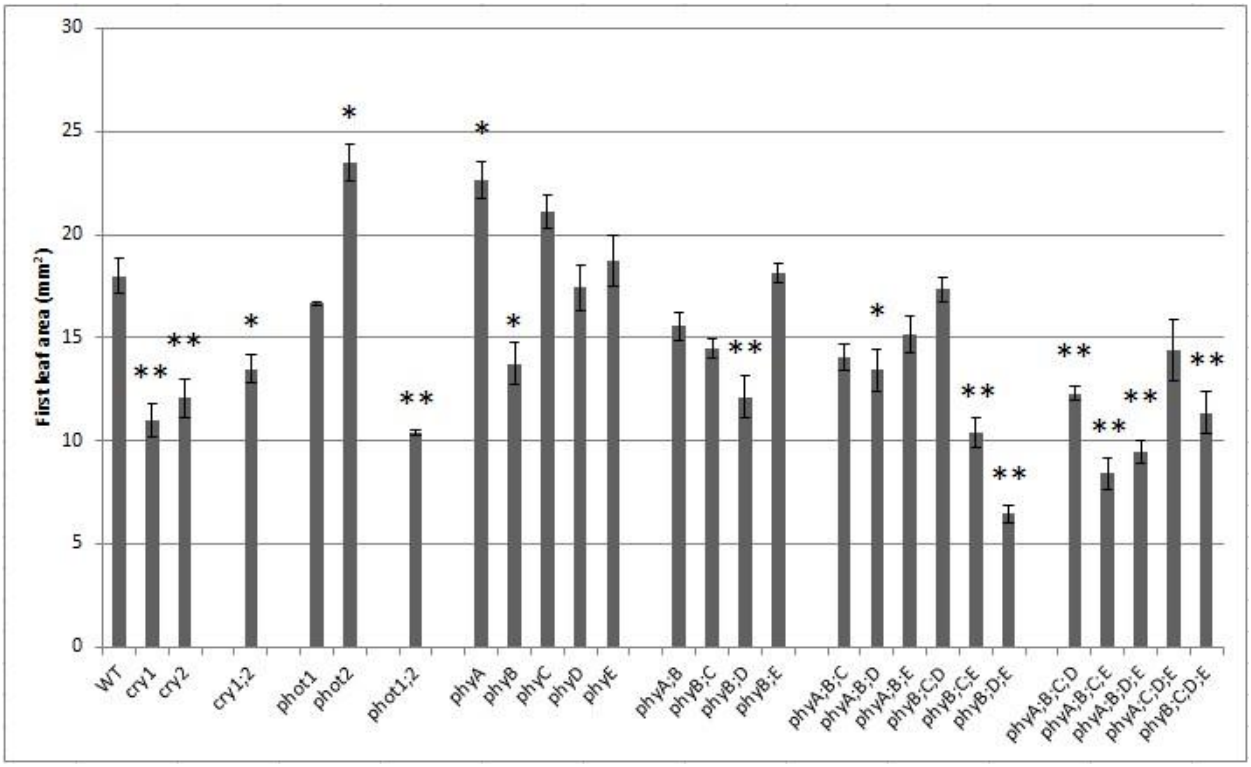
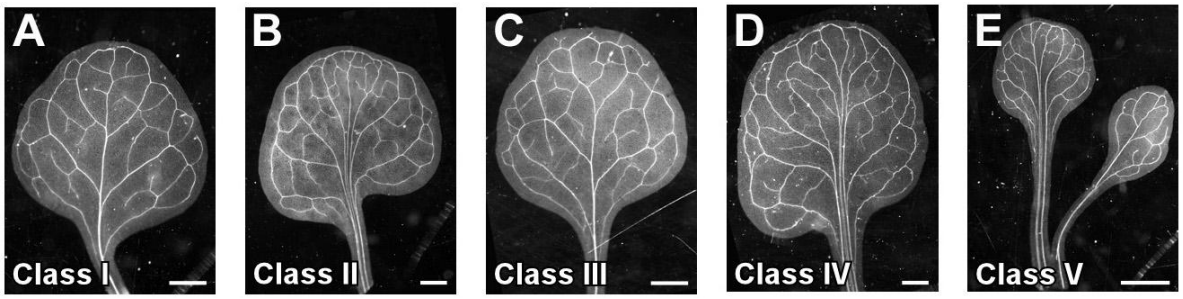


Fig. 4. Area of photoreceptor mutant first leaves. Data bars indicate average leaf area (mm^2). Difference between sample and positive control was significant at $P<0.05$ (*), or $P<0.01$ (**) by one-way ANOVA and Tukey's test. Population sample size is 10 leaves for each genotype.

Genetic interaction between red/far-red light perception, auxin transport and auxin signalling

Red/far-red light perception controls vein network formation (Figure 3), and auxin transport and signalling provide crucial cues for vein formation (Sachs, 1991; Mattsson et al., 1999; Sieburth, 1999; Mattsson et al., 2003; Chapter 1). I therefore asked whether red/far-red light perception interacted with auxin transport or signalling in control of vein patterning. *PHYB* is strongly expressed at sites of vein formation (Fig. 1F) and is the least redundant photoreceptor in red/far-red light perception (Vandenbussche et al., 2003). Similarly, the PIN-FORMED1 (PIN1) auxin transporter and the MONOPTEROS (MP) auxin response transcription factor are strongly expressed at sites of vein formation and are the least redundant components of the auxin transport and signalling machinery, respectively (Okada et al., 1991; Mattsson et al., 1999; Scarpella et al., 2006; Wenzel et al., 2007; Donner et al., 2009; Chapter 1). Therefore, to test for interaction between red/far-red light perception, auxin transport and auxin signalling, I analyzed vein patterns of double mutants between *phyB* and *pin1* or *mp*.

WT Arabidopsis grown under normal conditions forms separate leaves, whose vein patterns are characterized by a single, narrow midvein that runs the length of the leaf ("unbranched, narrow midvein"; Fig. 5A) and by lateral veins that branch from the midvein and join distal veins to form loops (Nelson & Dengler, 1997; Candela et al., 1999). *pin1* mutation causes leaf fusion ("fused leaves" Fig. 5E), and *pin1* vein patterns differ from WT in at least two respects: the midvein may bifurcate near the leaf tip ("bifurcated midvein"; Fig. 5C,D), and lateral veins may fail to join the midvein and instead run parallel to it to form a wide midvein ("wide midvein"; Fig. 5B,D). *phyB* normalizes *pin1* vein defects (Fig. 5F). *mp* leaves have fewer and incompletely differentiated veins (Berleth & Jurgens, 1993; Przemek et al., 1996) (Fig. 6). *phyB;mp* leaves are indistinguishable from *mp* leaves (Fig. 6). In summary, *phyB* normalizes *pin1* vein pattern defects, while *phyB* has no effect on *mp* vein pattern defects.



F.

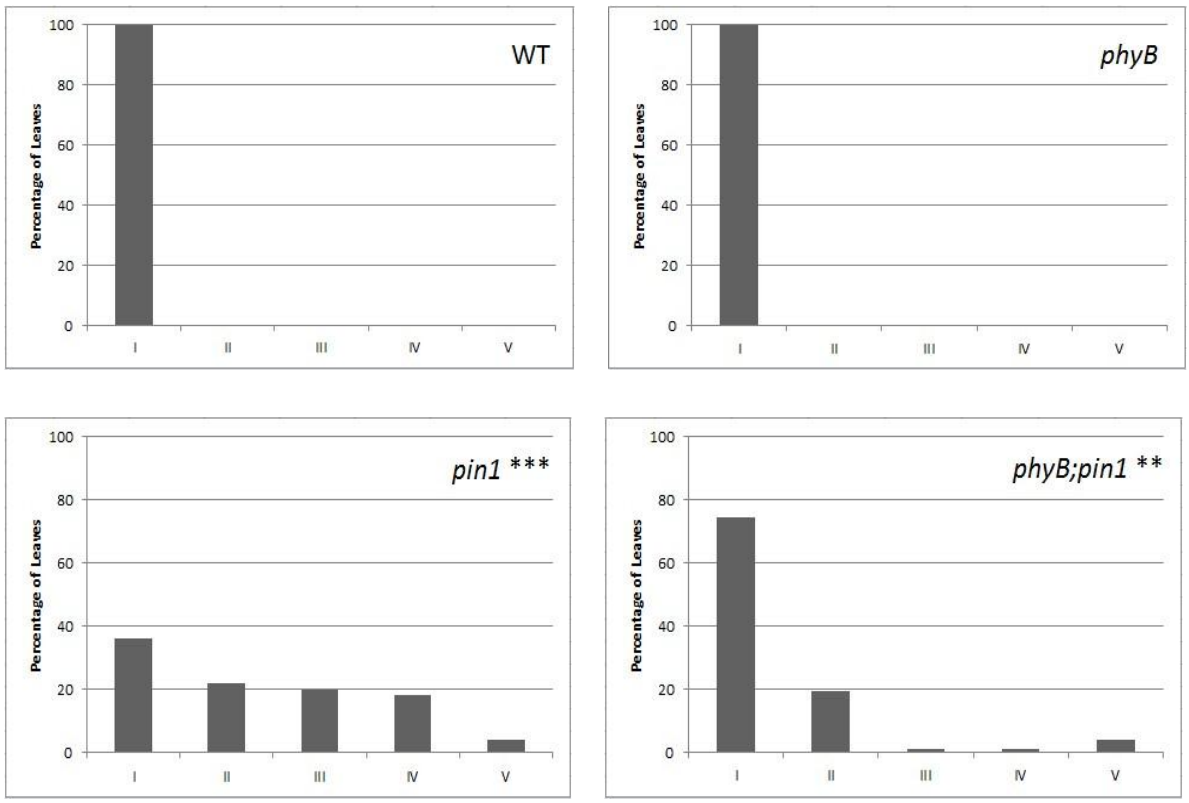


Fig. 5. Interaction between *phyB* and *pin1* in vein patterning. A-E, Dark-field illumination of cleared mature first leaves illustrating phenotype classes. A: unbranched, narrow midvein, B: unbranched, wide midvein; C: bifurcated, narrow midvein; D: bifurcated, wide midvein; E: fused leaves. Scale bars, 0.50 mm F: Percentages of phenotype classes. *pin1* is significantly different from WT at $P<0.001$ (***) and *phyB;pin1* is significantly different from *pin1* at $P<0.01$ (**) by Mann-Whitney test. Sample population sizes: WT, 25, *phyB*, 26*; pin1*, 91; *phyB;pin1*, 78.

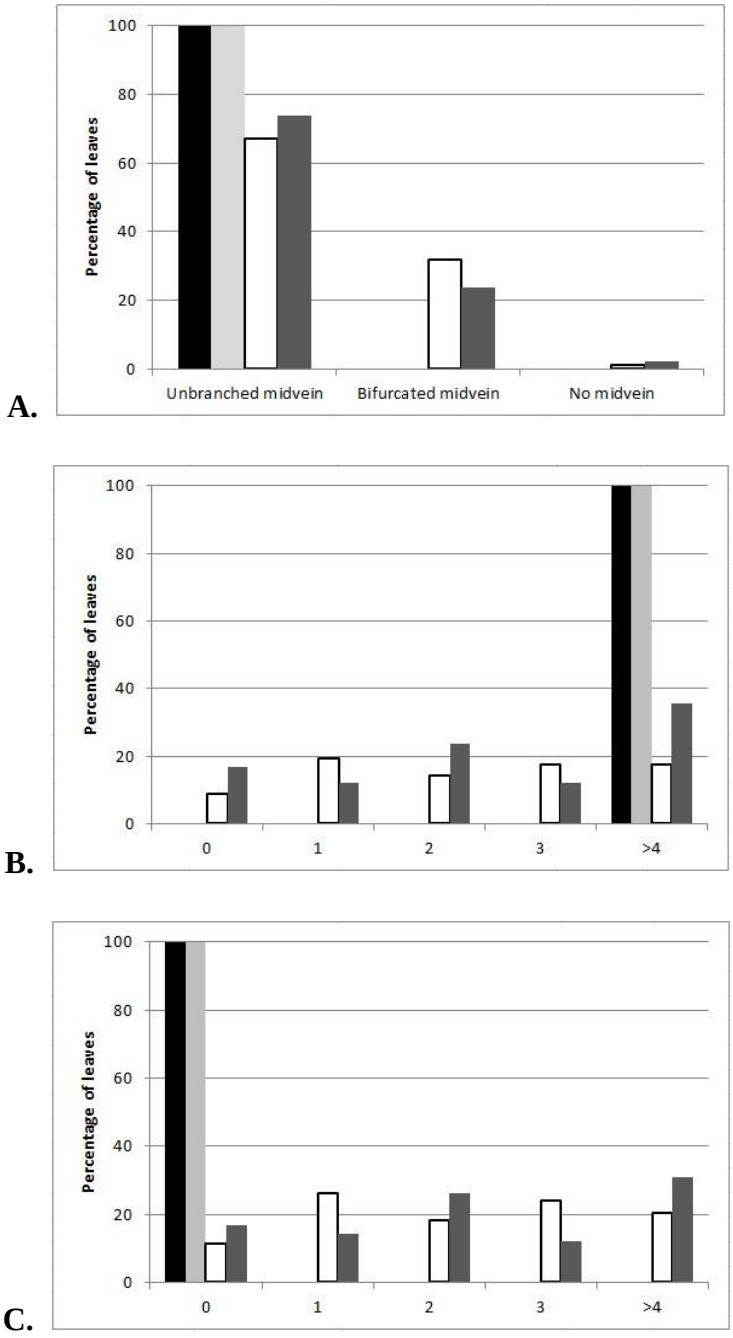


Fig. 6. Interaction between *phyB* and *mp* in vein patterning. A: Percentage of leaves with unbranched, bifurcated or no midvein. B: Percentage of leaves with zero, one, two, three, or more than four complete loops. C: Percentage of leaves with zero, one, two, three or more than four incomplete vein loops. Black, WT; light grey, *phyB*; white, *mp*; charcoal, *phyB;mp*. Sample population sizes: WT, 23; *phyB*, 27; *mp*, 88; *phyB;mp*, 42.

DISCUSSION

Leaf and vein expression of photoreceptor genes

My analysis of photoreceptor gene expression shows *PHYA, PHYB, PHYC,* and *CRY2* are expressed at early stages of vein development, while *CRY1*, *PHOT1*, *PHOT2*, *PHYD*, and *PHYE* are not. My results are in agreement with lower-resolution studies of the expression of *PHYA* and *PHYB* (Clack et al., 1994; Somers & Quail, 1995; Toth et al., 2001) and of *CRY2* (Toth et al., 2001, Endo et al., 2007) in the leaf. However, discrepancies exist between my data and previous reports of the expression of *PHYC, PHYD,* and *PHYE*, and *CRY1* (Goosey et al., 1997; Toth et al., 2001). *PHYC* expression was shown to be absent from the leaf (Toth et al., 2001), though I found expression to be ubiquitous and even at early stages of leaf development. *PHYD* expression was reported as even throughout the leaf (Goosey et al., 1997) or entirely absent from the leaf (Toth et al., 2001), whereas I found strong expression at the leaf margins and weak expression in subepidermal regions. *PHYE* expression was reported as strong in subepidermal and vascular tissues at the leaf margin (Goosey et al., 1997) or absent from the leaf altogether (Toth et al., 2001), whereas I found *PHYE* to be expressed strongly and evenly throughout the leaf. Reported *CRY1* expression is too low in resolution to verify (Toth et al., 2001), although I found *CRY1* to be expressed nearly ubiquitously throughout the leaf. Irrespective of similarities or differences between my findings and those in previous studies (Clack et al., 1994; Somers & Quail, 1995; Goosey et al., 1997; Toth et al., 2001), all analyses rely on transcriptional fusions as means to determine gene expression patterns. There are good reasons to use transcriptional fusions for this purpose: most regulation of photoreceptors gene expression occurs at the transcriptional level (Sharrock & Quail., 1989; Clack et al., 1994) and 80% of genes that have been tested in Arabidopsis show that the mRNA expression patterns can be recapitulated by transcriptional fusions (Lee et al., 2006). For these reasons, I feel that my data may represent the endogenous gene expression patterns for the *PHY* and *CRY* genes. However, it is possible that regulatory elements controlling *PHY* and *CRY* gene expression are located in regions outside the upstream sequences used in my study (Deyholos & Sieburth, 2000). This may not be the case for *PHOT1* and *PHOT2*, however; a functional translational PHOT1 fusion has been reported to be expressed in the leaf epidermis (Sakamoto & Briggs, 2002). Addtionally, the expression of a functional PHOT2::PHOT2:GFP construct has been reported in the epidermis of the cotyledons (Kong et al. 2006), which is somewhat in agreement with my expression data of *PHOT2* in the trichomes.

A role for light perception in vein network formation

Neither single nor multiple photoreceptor mutants show vein patterning defects; however, photoreceptor mutants show defects in vein network complexity.

Leaves of *cry1* and *cry2* do not show defects in vein network complexity, but *cry1;2* leaves show a more complex vein network than WT leaves. The increase in vein network complexity observed only upon loss of both *CRY1* and *CRY2* function shows that *CRY1* and *CRY2* function redundantly as negative regulators of vein network complexity. *CRY1* is not expressed at sites of vein formation, while *CRY2* is. It is therefore possible that *CRY2* is the primary gene involved in WT vein network formation, while *CRY1* compensates for the missing function of CRY2 in the *cry2* single mutant by becoming ectopically expressed at sites of vein

formation. However, it is also possible that *CRY1* or *CRY2* act in a non-cell-autonomous manner in vein network development.

Leaves of *phot1* and *phot1;2* have more complex vein networks than WT leaves, while *phot2* leaves have vein networks of WT complexity. This suggests that *PHOT1* and *PHOT2* function redundantly as negative regulators of vein network complexity, but that the redundancy is asymmetric: As it has a broader, nearly ubiquitous expression pattern, *PHOT1* is likely the primary partner in WT vein network formation and can be expressed ectopically in the absence of *PHOT2*. It is also possible that *PHOT1* and *PHOT2* regulate vein network complexity by acting non-cell-autonomously.

Leaves of triple and quadruple combinations of *phyA*, *phyB*, *phyD*, and *phyE* have more complex vein networks than WT. This suggests that *PHYA*, *PHYB*, *PHYD*, and *PHYE* act redundantly to negatively regulate vein network formation. Mutation of *PHYC* in *phyA;B;D*, *phyA;B;E*, and *phyB;D;E* normalizes vein complexity defects, suggesting that *PHYC* function antagonizes function of *PHYA*, *PHYB*, *PHYD*, and *PHYE* in vein network formation. This possibility is supported by expression data: *PHYC* is ubiquitously expressed at early stages of leaf and vein development and its expression domain is thus equal to the combined expression domains of *PHYA*, *PHYB*, *PHYD*, and *PHYE*, which would be expected if PHYC function were to antagonize the function of the other four PHY genes.

Genetic interference of light perception results in small leaves with more complex vein networks. Therefore, it is possible that the increased vascularization in leaves of photoreceptor mutants is a consequence of the leaves' reduced capacity for photosynthesis. There are a number of reasons why these leaves could have a lower photosynthetic output. First, a smaller leaf will have less area with which to capture light for photosynthesis. Second, loss of photoreceptor function may inhibit photosynthetic activity in the leaf; this is most notable in mutants of *PHOT* genes. Third, interference with light perception affects stomatal opening, which is necessary for photosynthesis to occur. Stomatal opening is mediated by UV-A/blue-light perception, and as such *cry* and *phot* mutants are impaired in stomatal regulation (Kang et al., 2009). The autotrophic nature of plants requires them to adapt to constant physiological stresses imposed by their environment in order to survive. Therefore, to compensate for possible reduction in photosynthetic capability, leaves of photoreceptor mutants may increase vascularization to penetrate deeper into the mesophyll to access even the smallest amount of photosynthetic product.

It is also possible that increased vascularization is a direct, negative response to reduced light perception. For example, UV-A/blue light perception by PHOTs develops mesophyll cells in the leaf (Briggs & Huala, 1999), and so obstructed blue light perception could result in an increase of vascular tissues, as the vascular and mesophyll cell types are mutually-exclusive. This hypothesis is consistent with my expression data, which show that *PHOT1* is expressed ubiquitously in subepidermal tissues, but is absent from developing veins, indicating that *PHOT1* could be expressed in ground cells prior to vein development. Additionally, Yoshida et al. (2011) have shown that light directly affects the establishment of efflux-dependent auxin gradients in the shoot apical meristem. In the absence of light, PIN1 became lost from the plasma membrane, which abolished auxin maxima. This interaction between light and auxin efflux could be present in the leaf as well, and would result in a decrease in vein development as light perception becomes impaired.

A role for red/far-red light perception in auxin-dependent vein formation

That *phyB* normalizes *pin1* vein defects suggests that the function of *PHYB* is antagonistic to that of *PIN1,* and that the two genes work in opposing pathways. Therefore, red/far-red light perception, mediated by PHYB, is antagonistic to PIN1-mediated polar auxin transport in leaf vein development. Conversely, *phyB* appears to have no effect on *mp* vein defects. This suggests at least two possible scenarios. First, *MP* and *PHYB* could work in the same pathway and *mp* would be epistatic to *phyB.* Alternatively, the two genes could work in independent pathways and, because *phyB* lacks vein defects, the phenotype of *phyB;mp*—though indistinguishable from that of *mp*—would actually represent an additive phenotype. However, because the pathways controlled by *MP* and *PIN1* overlap, at least in part (Schuetz et al., 2008), and because *PHYB* and *PIN1* work in separate, opposing pathways, it is less likely that *MP* and *PHYB* may work in the same pathway. Were that so, one would expect mutation of *MP* or *PHYB* to have the same effect—whether an enhancing or normalizing one—on *pin1* defects; instead, *mp* enhances (Schuetz et al., 2008) and *phyB* normalizes *pin1* defects. Therefore, it is likely that red/far-red light perception mediated by *PHYB* is independent of MP-mediated auxin signalling during vein development.

CHAPTER 3: THE ROLE OF AGCVIIIa SERINE-THREONINE KINASES IN VEIN DEVELOPMENT

Introduction

The vascular system of leaves is composed of a continuous network of vascular strands ("veins") that transport water, nutrients, and regulatory molecules (Esau, 1965; Nelson & Dengler, 1997; Taiz & Zeiger, 2010). Mature veins differentiate from continuous files of narrow, cytoplasm-dense procambial cells that form *de novo* from within a population of the morphologically identical subepidermal cells of the leaf primordium ("ground cells") (Foster, 1952; Pray, 1955). The mechanisms by which files of ground cells are selected to differentiate into procambial cell files is not entirely known, but available evidence supports a role for the plant signalling molecule auxin in this process (Sachs, 1981; Gersani, 1987). When applied to leaves, auxin induces the formation of new veins (Sachs, 1989). Auxin is synthesized primarily in young apical organs (i.e. leaves and shoots), and is transported polarly to the roots—a phenomenon known as polar auxin transport. At the cellular level, polar auxin transport is accomplished by the asymmetric localization of certain PIN-FORMED (PIN) auxin efflux proteins in the plasma membrane of auxin transporting cells (Wisniewska et al., 2006). Obstructions of polar auxin transport block auxin's capability to induce vein formation (Gersani, 1987; Sachs, 1989), and wild-type leaves grown on polar auxin transport inhibitors show reproducible leaf vein defects (Mattsson et al., 1999; Sieburth, 1999), suggesting that the polar transport of auxin is the inductive signal in vein formation.

The *pin1* mutant of Arabidopsis is the only *pin* single mutant with vein pattern defects (Mattsson et al., 1999; M. Sawchuk & E. Scarpella, unpublished), suggesting that *PIN1* is the least redundant auxin efflux protein in vein formation. Consistent with this interpretation, PIN1 is the only *PIN* gene to be expressed at early stages of vein formation (Scarpella et al., 2006), and all veins seem to derive from two modules of PIN1 expression dynamics (PEDs, hereafter) (Scarpella et al., 2006; Wenzel et al., 2007). In both modules, PIN1 expression dynamically narrows down from broad to narrow areas of expression. In one module, vein-associated subepidermal PIN1 expression domains arise in association with convergence points of opposing PIN1 polarity in the epidermis of the leaf margin; the midvein and lateral veins are formed this way. In the other module, vein-associated subepidermal PEDs arise in association with pre-existing subepidermal PEDs; marginal and higher-order veins arise this way. Although both marginal and higher-order PEDs are initially freely-ending, marginal domains are usually connected to other domains on both sides. PIN1 polarity is not uniformly directed toward pre-existing veins across broad PIN1 domains, but it is so within narrow PIN1 domains: in free-ending domains, a single polarity exists, while in connected domains, two opposite polarities are bridged by a bipolar cell (Scarpella et al., 2006; Wenzel et al., 2007).

In epidermal cells of the shoot apical meristem, PINOID (PID), a plasma-membrane-associated serine-threonine kinase, phosphorylates PIN1 to target it to the apical plasma membrane. PIN1 phosphorylation by PID is reversed by the activity of PP2A protein phosphatases, which target PIN1 to the basal membrane (Friml et al., 2004; Michniewicz et al., 2007). PID is a member of a plant-specific subfamily of the AGCVIIIa serine-threonine kinases, named after eukaryotic protein kinase A (PKA), cyclic GMP-dependent protein kinases (PKG), and protein kinase C (PKC) (Zegzouti et al., 2006; Galvan-Ampudia & Offringa, 2007). The clade of the AGCVIIIa

kinases that contains *PID* has three other members, *WAG1*, *WAG2*, and *AGC3.4/PID2* (Galvan-Ampudia & Offringa, 2007). PID, WAG1, and WAG2 are localized to the plasma membrane of epidermal cells of the root (Dhonukshe et al., 2010) and phosphorylate plasma-membrane-associated PIN proteins to target them to the apical plasma membrane (Dhonukshe et al., 2010). A function has yet to be assigned to AGC3.4. Because changes in PIN1 polarity in the leaf epidermis coincide with the onset of vascular patterning, and because AGCVIIIa kinases regulate PIN1 polarity in epidermal cells of the root, it is possible that the AGCVIIIa kinases regulate vein development, as well. Here I have explored a possible role of AGCVIIIa serine-threonine kinases in vein patterning. I found that PID, WAG1, and WAG2 are apolarly localized to the plasma membrane of the leaf epidermis during early stages of leaf development, and that *PID* is also expressed ubiquitously in ground cells, but declines in expression quickly in presumptive veins at the prior to differentiation. *AGC3.4* is expressed in veins, but accumulates in the nucleus. A functional redundancy exists between *PID*, *WAG1*, and *WAG2* in vein patterning, and *PID* and *WAG2* genetically interact with *PIN1* in vein patterning.

MATERIALS & METHODS

Vector construction

To generate PID::PID:EYFP, AGC3.4::AGC3.4:EYFP, and WAG2::WAG2:EYFP, the respective genes' entire upstream regions and coding sequences were amplified from *Arabidopsis thaliana* ecotype Col-0 genomic DNA using Finnzymes Phusion high-fidelity DNA Polymerase (New England Biolabs Inc., Ipswich, MA, USA) and gene-specific primers (Table 1), and cloned upstream of the EYFP coding sequence (Clontech) using an Asp-Pro-Gly linker, as described in Gallagher et al., (2004). To generate PID::PID:EYFP the 5661-bp region from -4043 (relative to the start codon) to +1618 of *PID* (AT2G34650) was used. To generate AGC3.4::AGC3.4:EYFP, the 4988-bp region from -2654 to +2334 of *AGC3-4* (AT2G26700) was used. To generate WAG2::WAG2:EYFP, the 4923-bp region from -3484 to +1439 of *WAG2* (AT3G14370) was used.

Table 1 – Oligonucleotides used in the construction of kinase translational fusions

Gene	Oligonucleotides
PID	PID SALI FOR: AAT GTC GAC GTT TAT ATG AGT ATA GTA GAT AAC AC
	PID KPN1 REV: ATA GGT ACC AAA GTA ATC GAA CGC CGC TGG
AGC3.4	AGC3-4 SALI FOR: AAT GTC GAC CAA TTA AGG CAT CAA TG
	AGC3-4 KPNI REV: ATA GGT ACC AAA ATA ATC AAA ATA ATT AGA CAC ATG G
WAG2	WAG2 SALI FOR: CAG GTC GAC TTT ATT TAT CTC TTT TAA GTT GTA GCC
	WAG2 KPNI REV: ATA GGT ACC AAC GCG TTT GCG ACT CGC
EYFP	MCHERRY C KPN1 FOR: ATA GGT ACC GTG AGC AAG GGC GAG GAG
	MCHERRY C SAC1 REV: ATT GAG CTC TTA CT GTA CAG CTC GTC C

Plant material, transformation, and growth conditions

Origins of lines used in this study are in Table 2. Mutants were genotyped with gene-specific primers and 'LBb1.3' (*pid-14*, *wag2*), or 'JMLB1' (*wag1*) T-DNA primer, or with gene-specific primers followed by restriction-enzyme digestion (*pin1-1*) (Table 3). Arabidopsis seeds were surface-sterilized, synchronized, and germinated on growth medium—half-strength Murashige and Skoog (MS) Salts (Caisson Labs, North Logan, UT, USA), 15 g l^{-1} sucrose (Fisher Scientific, Fair Lawn, NJ, USA), 0.5 mg l^{-1} MES (BioShop Canada Inc., Burlington, ON, Canada), 0.8% (w/v) agar (Phytotechnology Laboratories, Shawnee Mission, KS, USA), pH 5.7—at the approximate density of 1 seed cm^{-2} as previously described (Scarpella et al., 2004). Sealed plates were incubated at 25ºC under continuous fluorescent light (100 µmol m^{-2} s^{-1}). 'Days after germination' (DAG) are defined as days following exposure of imbibed seeds to light. 4-DAG seedlings were transferred to Promix BX soil (Evergro/Westgro) in 7- x7- x 8-cm pots at the approximate density of 0.1 seedlings cm^{-2}. Seedlings were grown at 22°C under a 16 h:8 h light (100-150 µmol m^{-2} s^{-1}):dark photoperiod. Arabidopsis plants (ecotype Col-0) were transformed with *Agrobacterium tumefaciens* strain GV3101::pMP90 (Koncz & Schell, 1986) harbouring single AGCVIIIa serine-threonine kinase translational fusions. Primary transformants were selected on growth medium supplemented with 200 µg ml^{-1} carbenicillin (Teknova, Hollister, CA, USA), 15 µg ml^{-1} hygromycin (Bioshop Canada, Burlington, ON, Canada), and 50 µg ml^{-1} nystatin (Bioshop). A minimum of fifteen single-insertion lines per construct were analyzed to determine the most representative expression pattern of the gene. Detailed analyses were performed on at least 3 lines per construct that were chosen because of strong EYFP expression that was representative of the expression pattern of the gene.

Table 2 – Plant lines and their origins

Line	Origin
WAG1::WAG1:EGFP	Dhonukshe et al., 2010
pid-14	Huang et al., 2010
wag1	Dhonukshe et al., 2010
wag2-1	Santner & Watson, 2006
pin1-1	Goto et al. 1987; Gälweiler et al. 1998; WT at the TTG1 (AT5G24520) locus (M. Sawchuk and E. Scarpella, unpublished)

Table 3 – Oligonucleotides and enzymes used to genotype mutants

Mutant	Oligonucleotides and enzymes
pid-14	PID-14 RP: ATT TTG CGA TGA AAG TTG TGG PID-14 LP: CAG TCG GGA AAC TCA ACT GTC LBb1.3: ATT TTG CCG ATT TCG GAA C
wag1	WAG1 RP 3: GGA GAA ACA ACC GCC AGG ACG WAG1 LP 3: ATG GAA GAC GAC GGT TAT TAC C JMLB1: GGC AAT CAG CTG TTG CCC GTC TCA CTG GTG
wag2	WAG2 RP: CCA AAA CCC CCA AAC ATA AAC WAG2 LP: TAA AG GAA TAT TCC GAA CGC C LBb1.3
pin1-1	PIN1-1 FOR: ATG ATT ACG GCG GCG GAC TTC TA PIN1-1 REV: TTC CGA CCA CCA CCA GAA GCC TatI digestion

Microtechniques and microscopy

Dissected leaves were mounted in water and observed with a 20x Planaprochromat (NA, 0.8) or 40x C-Apochromat water (NA, 1.2) objective on a Zeiss Axiovert 100M microscope equipped with a Zeiss LSM510 laser module confocal unit (Carl Zeiss, Oberkochen, Germany). YFP was excited with the 514-nm line of an Argon laser at 55% of output (equivalent to approximately 6 A) and 5% transmission, and emission detected with a BP565-615 filter. Mature (14-21 DAG) first leaves were fixed in 3:1 (v/v) ethanol:acetic acid and stored in 70% (v/v) ethanol. Fixed leaves were rehydrated in distilled water prior to mounting in chloral hydrate:glycerol:water 8:3:1 (w/v/v), and viewed under dark-field illumination with an Olympus SZ61TR stereomicroscope (Olympus Optical Co., Tokyo, Japan). Images were captured with an AxioCam HR camera (Carl Zeiss).

Image analysis and processing

Brightness and contrast of 8-bit images were adjusted through linear stretching of the histogram in ImageJ (National Institute of Health, http://rsb.info.nih.gov/ij). All images were cropped in Adobe Photoshop 7.0 (Adobe Systems Inc. San Jose, CA, USA) and assembled into figures in Canvas 8 (ACD Systems International, Victoria, BC, Canada).

RESULTS

Expression of AGCVIIIa serine-threonine kinases in Arabidopsis leaf development

Changes in PIN1 polarity recur during vein formation (Scarpella et al., 2006). If those are changes necessary for vein formation, regulators of PIN1 polarization would be expressed at critical stages of vein formation. In the shoot apical meristem, PIN1 is localized to the apical plasma membrane of epidermal cells (Reinhardt et al., 2003), and this localization is regulated by phosphorylation of three serine residues catalyzed by PINOID (PID) (Huang et al., 2010). PID belongs to a small subfamily of serine-threonine kinases (the AGCVIIIa [PROTEIN KINASE **A**, CYCLIC **G**MP-DEPENDENT PROTEIN KINASES, PROTEIN KINASE **C**] subfamily) that in Arabidopsis includes *WAG1*, *WAG2* and *AGC3.4/PID2* (Galvan-Ampudia & Offringa, 2007). However, whether AGCVIIIa serine-threonine kinases are expressed during leaf development is unknown. To address this question, I obtained seeds of a translational fusion of WAG1 to GFP (WAG1::WAG1:EGFP) (Dhonukshe et al., 2010) and created translational fusions of PID, WAG2, and AGC3.4 to EYFP (PID::PID:EYFP, WAG2::WAG2:EYFP, AGC3.4::AGC3.4:EYFP), and visualized their expression in first leaf primordia at 1, 2, 3, and 4 days after germination (DAG).

Throughout leaf development, PID:EYFP was localized apolarly to the plasma membrane (Fig. 1A-F). At 1 DAG, PID::PID:EYFP was expressed weakly, yet ubiquitously, in epidermal and subepidermal cells (Fig. 1A). Ubiquitous PID::PID:EYFP expression was maintained in epidermal cells until 4 DAG (Fig. 1A-E), but already from 1.5 DAG expression in subepidermal cells had subsided in presumptive vascular cells: first in the developing midvein (Fig. 1B), then in developing loops (Fig. 1D) and higher-order veins (Fig. 1F).

Throughout leaf development, WAG1:EGFP was localized apolarly to the plasma membrane of epidermal cells, to which WAG1::WAG1:EGFP expression seemed to be restricted (Fig. 1G-L). WAG1::WAG1:EGFP was expressed ubiquitously in epidermal cells from 1.5 to 3 DAG (Fig. 1G-I), but expression had subsided at the tip of 4-DAG leaves (Fig. 1J-L).

As WAG1: EGFP, WAG2:EYFP was localized apolarly to the plasma membrane of epidermal cells, but WAG2::WAG2:EYFP expression seemed confined to epidermal cells of the leaf tip (Fig. 1M,N), petiole (Fig. 1O,P,Q), and hydatode (Fig. 1R).

Finally, throughout leaf development AGC3.4::AGC3.4:EYFP expression was restricted to presumptive vascular cells, in the nuclei of which AGC3.4:EYFP preferentially accumulated (Fig. 1S-V).

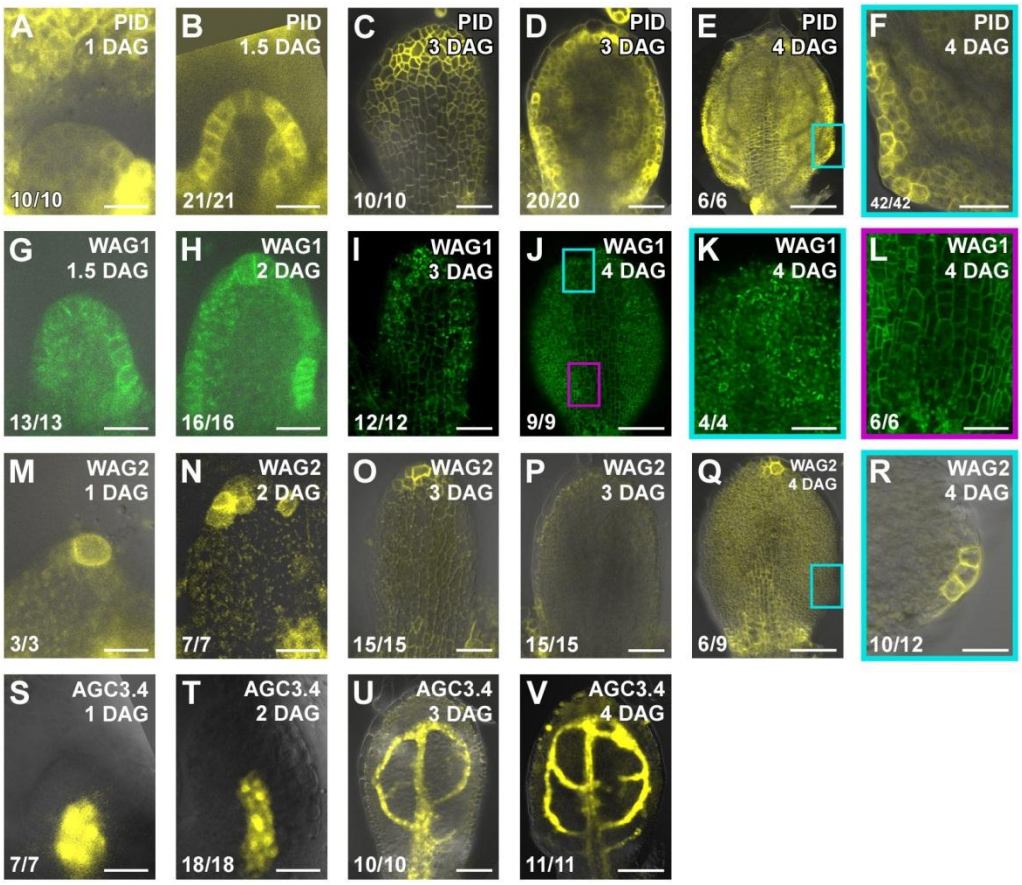


Fig. 1. Expression of AGCVIIIa serine-threonine kinases in Arabidopsis leaf development. Overlay of confocal laser scanning and differential interference microscopy images. Top right, time point (DAG). Bottom left, fraction of samples showing the displayed features. C,I,J,K,L,O,Q: epidermal focal plane. A,B,D,E,F,G,H,M,N,P,R,S,T,U,V: subepidermal focal plane. A-F: PID::PID:EYFP. G-L: WAG1::WAG1:EGFP. M-R: WAG2::WAG2:EYFP. S-V: AGC3.4::AGC3.4:EYFP. Scale bars:15 µM (A,B,G,H,L,M,N,S,T); 20 µM (C,D,I,J,O,P,Q,V); 50 µM (E,F,K,L,Q,R).

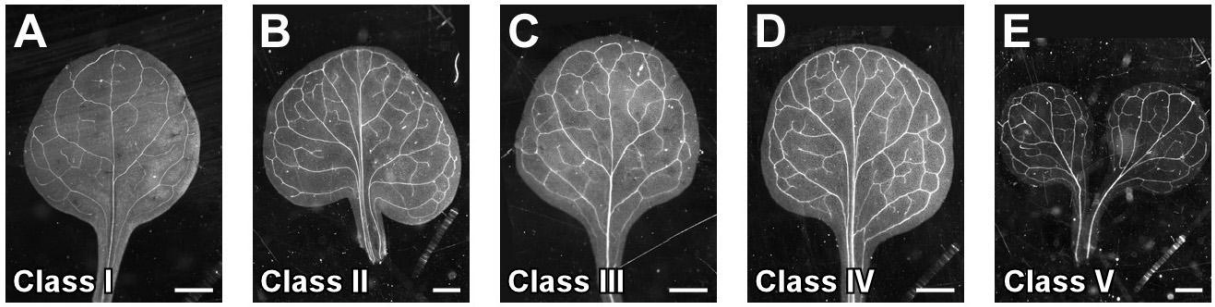
Genetic interaction between AGCVIIIa serine-threonine kinases in vein patterning

At least some AGCVIIIa serine-threonine kinases are expressed at early stages of vein formation (Fig. 1). Because PID, WAG1, and WAG2 regulate PIN1 polarity in cells of the root epidermis and cortex (Dhonukshe et al., 2010) and PIN1 polarity changes recur during vein formation, I therefore asked whether AGCVIIIa serine-threonine kinases control vein patterning. To test this, I analyzed the vein patterns of mature first leaves of *pid*, *wag1*, and *wag2* single mutants. Function of *AGC3.4* was not analyzed because localization of AGC3.4 to the nucleus makes *AGC3.4* an unlikely regulator of polarity of plasma-membrane-associated PIN proteins.

WT Arabidopsis leaf vein patterns are characterized by a single, unbranched narrow midvein that spans the length of the leaf ("unbranched, narrow midvein"; Fig. 2A); lateral veins branch from the midvein and by joining distal veins form loops (Nelson & Dengler, 1997; Candela et al., 1999). *pid* vein patterns differed from WT in at least two respects: the midvein could branch near the leaf tip ("bifurcated midvein"; Fig. 2C,D), and lateral veins could fail to connect to the midvein and could instead run parallel to it to form a wide midvein ("wide midvein"; Fig. 2B,D). Unlike *pid*, leaves of *wag1* and *wag2* were indistinguishable from WT (Fig. 2F).

To uncover possible redundant functions of *WAG1* or *WAG2* and *PID* in vein patterning, I analyzed vein patterns of mature first leaves of double mutants between *wag1* or *wag2* and *pid*—the only mutant among *pid*, *wag1*, and *wag2* with vein patterning defects. While *wag1* did not enhance *pid* vein defects, *wag2* did (Fig. 2F).

In summary, PID appears to be the least-redundant member of the AGCVIIIa serine-threonine kinases in vein patterning. In presence of WT *PID* function, function of *WAG1* and *WAG2* seem to be completely dispensable for vein patterning; however, in absence of WT *PID* function, *pid* vein pattern defects are enhanced by *wag2*.



F.

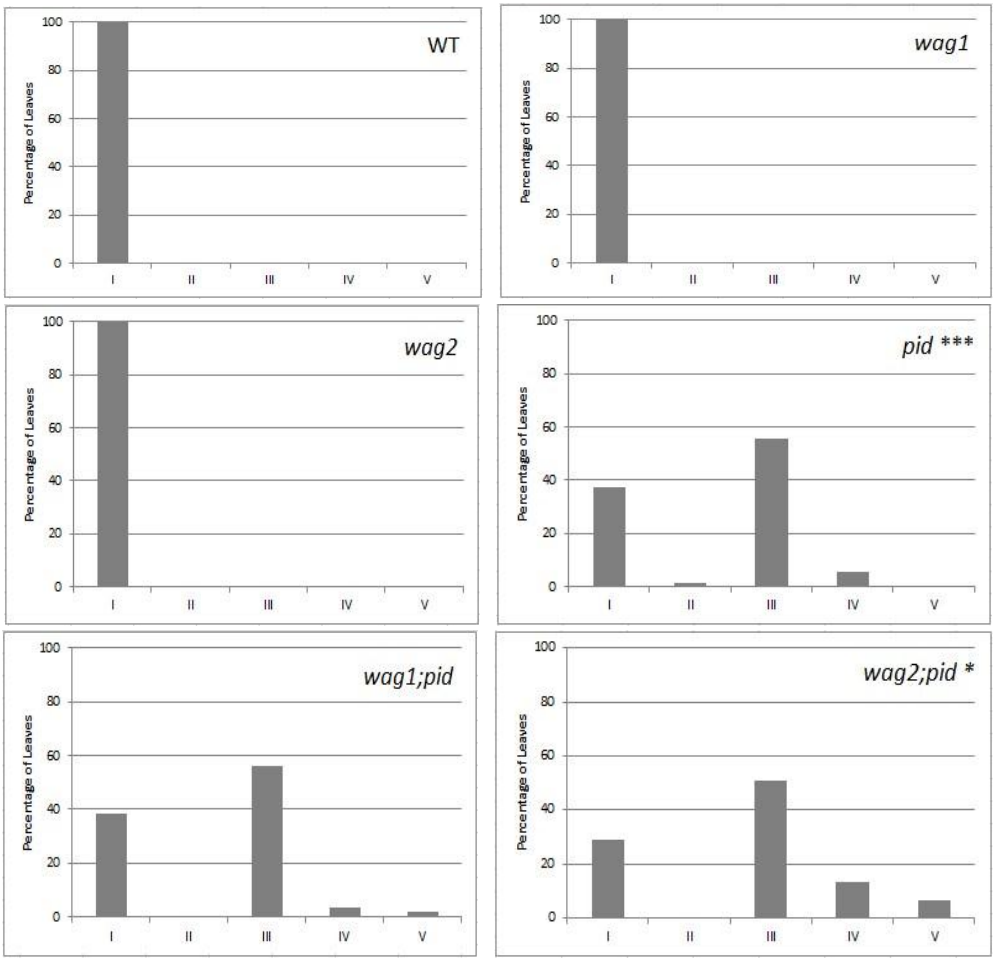


Fig. 2. Vein patterns of mutants in genes encoding AGCVIIIa serine-threonine kinases. (A-E) Dark-field illumination of Vein patterns of mutants in genes encoding AGCVIIIa serine-threonine kinases cleared, mature first leaves illustrating phenotype classes: (A) unbranched, narrow midvein; (B) bifurcated, narrow midvein; (C) unbranched, wide midvein; (D) bifurcated, wide midvein; (E) fused leaves. (F) Percentage of phenotype classes. Scale bars, 0.50 mm Difference between *pid* and WT was significant at $P<0.001$ (***) and difference between *pid* and *wag2;pid* was significant at $P<0.05$ (*) by Mann-

Whitney test. Sample population sizes: WT, 25; *wag1*, 24; *wag2*, 24; *pid*, 72; *wag1;pid*, 57; *wag2;pid*, 59.

Genetic interaction between *PIN1* and AGCVIIIa serine-threonine kinases in vein patterning

Should AGCVIIIa serine-threonine kinases control vein patterning by regulating plasma-membrane-associated PIN polarity, vein pattern defects of *pin1*—the only *pin* single mutant with vein patterning defects (M. Sawchuk and E. Scarpella, unpublished)— should be enhanced by mutations in *PID*, *WAG1*, or *WAG2*. To test this prediction, I analyzed vein patterns of mature first leaves of double mutant between *pin1* and *pid*, *wag1*, or *wag2*. While *wag1* did not affect *pin1* vein pattern defects, *wag2* enhanced them (Fig 3B). A new phenotypic class—never observed in either *pid* or *pin1* leaves—appeared in *pid;pin1* double-mutant leaves ("trumpet-shaped leaf", Fig. 3A), which is a very strong indication of synergism between *pid* and *pin1*(see Discussion).

In summary, in the absence of WT *PIN1* function, *pin1* vein pattern defects are enhanced by *wag2*, and most likely by *pid*, but *wag1* does not appear to enhance *pin1* defects.

A.

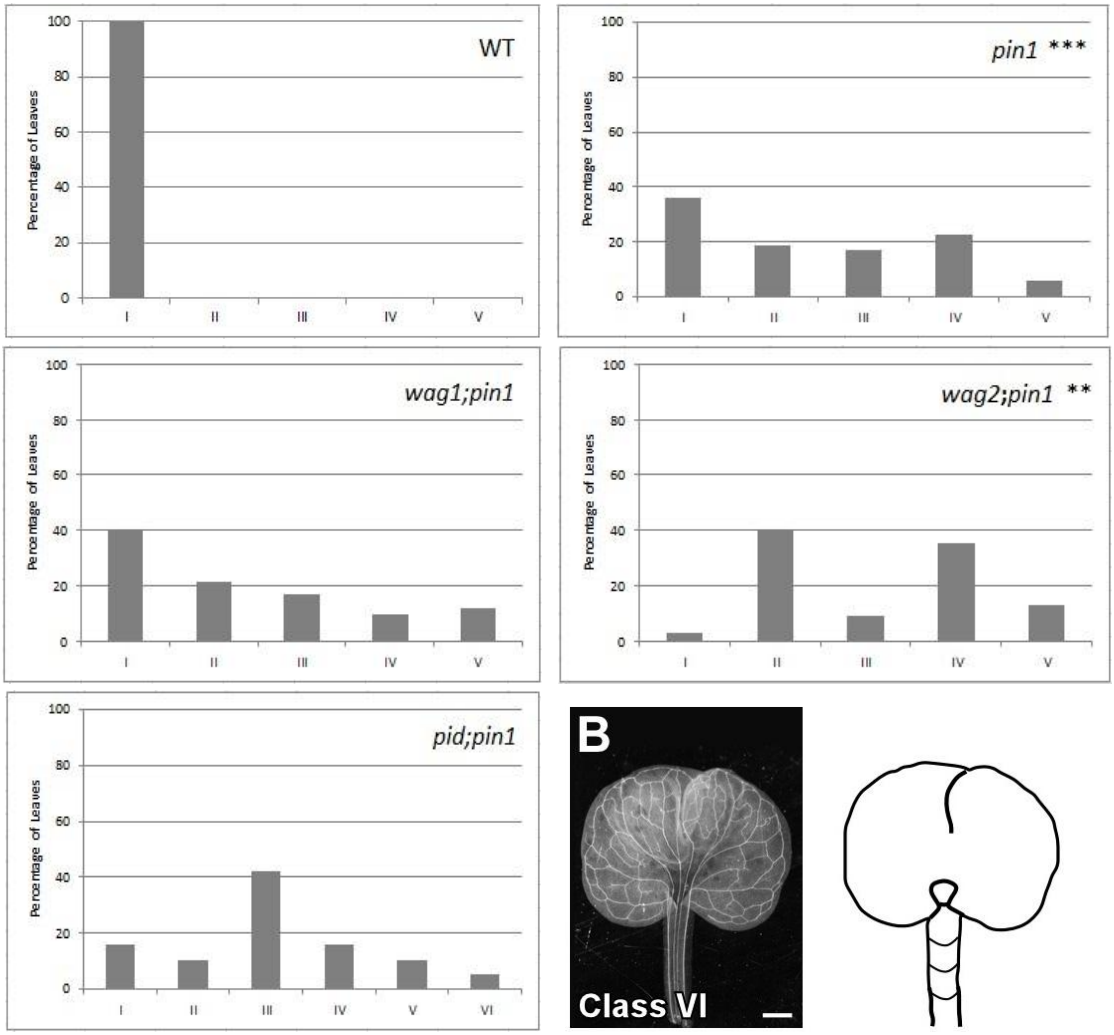


Fig. 3. Vein patterns of mutants of *PIN1* and of genes encoding AGCVIIIa serine-threonine kinases. (A) Percentage of phenotype classes. Difference between *pin1* and WT was significant at $P<0.001$ (***), and difference between *pin1* and *wag2;pin1* was significant at $P<0.01$ (**) by Mann-Whitney test. Sample population sizes: WT, 24; *wag1*, 24; *pin1*, 53; *wag; pin1*, 42; *wag2;pin1*; *pid;pin1*, 19. (B) Left: Dark-field illumination of cleared, mature trumpet-shaped first leaf illustrating the phenotype class. Scale bar, 0.25 mm. Right: Line drawing illustrating three-dimensional conformation of leaf fusion.

DISCUSSION

The AGCVIIIa serine-threonine kinases are expressed in leaf development

Analysis of expression of *PID*, *WAG1*, *WAG2*, and *AGC3.4* shows that these genes are expressed throughout leaf development. PID localizes apolarly to the plasma-membrane of PID-expressing cells and is expressed ubiquitously and evenly in the epidermis and subepidermal tissues; however, subepidermal expression is terminated in presumptive vascular cells. Both *WAG1* and *WAG2* are expressed in the epidermis but not in subepidermal tissues. However, *WAG1* is expressed evenly in the epidermis, while *WAG2* expression is confined to the epidermal cells of the leaf apex, hydathodes, and petioles. PID, WAG1 and WAG2 localize apolarly to the plasma membrane. Finally, unlike the other AGCVIIIa kinases, *AGC3.4* is expressed exclusively in developing veins and accumulates in the nucleus.

The subcellular localization of *PID*, *WAG1*, and *WAG2* in the leaf is in agreement with that in the root (Dhonukshe et al., 2010). Neither the published translational fusions of *PID*, *WAG1*, and *WAG2* (Dhonukshe et al., 2010) nor mine have been tested for functionality. Therefore, it is possible that my expression data do not represent the expression of these AGCVIIIa kinases in the leaf.

PID and *WAG2* function in vein patterning

I did not pursue functional characterization of *AGC3.4* because its nuclear localization makes it an unlikely candidate to regulate plasma-membrane-associated PIN proteins.

That PID is the only AGCVIIIa kinase with single-mutant vein defects suggests that *PID* performs a non-redundant function in vein patterning. *PID* is expressed ubiquitously and evenly in both epidermal and subepidermal tissues, suggesting that *PID* is required for formation of all veins. Formation of epidermal convergence points of PIN1 polarity at the leaf apex is associated with normal midvein formation (Scarpella et al., 2006; Wenzel et al., 2007). As such, midvein bifurcation in *pid* leaves may be a result of improper PIN1-convergence-point formation. Because PID is the only AGCVIIIa kinase to be expressed in the plasma membrane of subepidermal tissues, *PID* may also play a role in the formation of marginal and higher-order veins, which do not form in association with epidermal PIN1 convergent points (Scarpella et al., 2006). Furthermore, *PID* expression appears to be absent from incipient veins, which could suggest that PID plays a role in stabilizing PIN1 polarity during vein formation.

Because vein patterns of mature *wag1* leaves are indistinguishable from those of WT, several possibilities concerning the function of *WAG1* in vein development can be hypothesized. The first possibility is that *WAG1* has no function in leaf vein patterning. The second possibility is that *WAG1* does have a function in leaf vein patterning, but this function is not unique to *WAG1*. Therefore, the contribution of *WAG1* to vein patterning would be redundant and could be fully provided by other members of the AGCVIIIa subfamily (e.g., *PID* or *WAG2*). Leaves of *wag1;pid* resemble those of *pid*, which provides further scenarios of the possible function of *WAG1* in vein patterning and genetic interactions of *WAG1* with *PID*. The first possibility is that *WAG1* does not play a role in leaf vein patterning. The second possibility is that *WAG1* and *PID* function in the same pathway, with *PID* upstream of *WAG1*. This scenario is consistent with *wag1;pid* leaves being identical to those of mutants in the gene that is upstream in the pathway (*PID*); *pid* would therefore be epistatic to *wag1*. A third possibility is that *WAG1* and *PID*

function in separate pathways. This scenario would require *wag1;pid* to show defects that are the sum of the defects of *wag1* and *pid*. However, because *wag1* leaves do not have defects, it is possible that *wag1;pid* leaves have an additive phenotype in which *wag1*'s phenotypic contribution is not visible. Because *WAG1* expression is restricted to the epidermis, should *WAG1* function in vein patterning, this function would likely be limited to the control of midvein and lateral veins, that is the veins the formation of which is associated with epidermal PIN1 convergence points.

As *wag1*, vein patterns of *wag2* leaves are normal. Therefore, as *WAG1*, *WAG2* may not have a function in leaf vein patterning. Alternatively, *WAG2* does have a function in this process, but the function is redundant. However, unlike *wag1;pid*, *wag2;pid* leaves have more severe patterning defects than *pid* leaves, indicating that *wag2* enhances *pid* vein defects. This suggests that *WAG2* does, in fact, play a role in leaf vein patterning, and that the function of *WAG2* is redundant with that of *PID* in this process. Epidermal expression of *WAG2* at the leaf apex and at the hydathodes is consistent with a role of *WAG2* in vein patterning as leaf apex and hydathodes are area of formation of epidermal PIN1 convergent points that are associated with formation of midvein and lateral veins. Because *WAG2* is not expressed in subepidermal tissues, however, it probably does not function in formation of marginal or higher-order veins.

PID and *WAG2* genetically interact with *PIN1* in vein patterning

The appearance of a new phenotype class (Class VI – "trumpet-shaped leaf") in *pid;pin1* leaves, a class that had been previously observed in the double mutant (Schuetz et al., 2008), suggests that *PID* and *PIN1* work in functionally overlapping pathways in vein patterning. Such genetic interaction is consistent with a function of *PID* in regulation of PIN1 polarity in vein development. Although statistical analysis shows that this new phenotypic class is not significant, this is likely an artifact due to small sample size. While *wag1;pin1* leaves are indistinguishable from *pin1* leaves, *wag2;pin1* leaves have vein pattern defects that are more severe than those of *pin1*, suggesting that, as *PID* and *PIN1*, *WAG2* and *PIN1* function in overlapping pathways.

It is important to consider that each of these mutants, though strong or null for the function of one specific gene, is not null for the function of the pathway in which the gene functions. For example, *wag1*, *wag2*, or *pid* single or double mutants do not completely lack serine-threonine phosphorylation activity as there is at least one WT member of the family in those mutant backgrounds that can perform this function. Similarly, *pin1* is not null for polar auxin transport mediated by plasma-membrane-localized PIN proteins, as PIN2, PIN3, PIN4, PIN5, and PIN7—all localized to the plasma membrane (Galweiller et al. 1998; Muller et al., 1998; Friml et al., 2002; Friml et al., 2002b; Vieten et al., 2005)—can still perform this function in the absence of *PIN1* function. Therefore, the quintuple *pin1*;*2*;*3*;*4*;*7*, and triple *pid*;*wag1*;*wag2* will have to be analyzed to completely expose the relation between AGCVIIIa kinases and PIN transporters and correctly interpret the contributions of each gene to the vein patterning pathway.

CHAPTER FOUR: CONCLUSIONS AND FUTURE DIRECTIONS

The first question I aimed to address with my research was whether auxin-dependent leaf and vein formation was controlled by light signalling. To address this, I visualized the expression of the nine genes encoding members of the three main photoreceptor families of Arabidopsis—*CRYPTOCHROMES* (UV-A/blue light; *CRY1, CRY2*), *PHOTOTROPINS* (UV-A/blue light; *PHOT1, PHOT2*), and *PHYTOCHROMES* (red/far red; *PHYA-PHYE*)—in vein development. My results suggest that specific photoreceptors perceiving UV-A/blue or red/far-red light are expressed at early stages of vein development. To investigate whether light perception was required for vein patterning, I examined vein patterns of single, double, triple, and quadruple intra-family photoreceptor mutants. My results suggests that blue/UV-A (*cry1;2, phot1, phot1;2*) and red/far-red (triple and quadruple mutants in *phyA, phyB, phyD,* and *phyE*) show defects in vein network complexity. Further, my data suggest that *PHYC* function antagonizes function of *PHYA, PHYB, PHYD,* and *PHYE* in vein development. To test whether light perception contributed to vein formation through auxin transport or auxin signalling pathways, I analyzed double mutants between *phyB* and *pin1* or *mp*. My results suggest that red/far-red light perception mediated by PHYB antagonizes the role of PIN1-dependent polar auxin transport in vein development. In conclusion, I found that vein formation is controlled by the perception of UV-A/blue, and red/far-red light. Furthermore, red/far-red light perception contributes to vein formation in a way that antagonizes the function of auxin transport in this process.

In the future, it may be interesting to further explore the role of the PHOTOTROPIN family in vein formation as it is the photoreceptor family with the most profound impact on vein network formation. Functional translational fusions of *PHOT1* and *PHOT2* would accurately report where and when these genes are expressed, and markers of vascular (PIN1[Scarpella et al,, 2006; Wenzel et al., 2007], MP [Wenzel et al., 2007] and *ATHB8* [Donner et al., 2009]) and mesophyll (e.g., RbcS1A, RbcS3B, Lhca, Lhcb1.1, and Lhcb4.2; Sawchuk et al., 2008) development in single and double *phot* mutants would clarify the nature of the mutants' primary defects. Mutant combinations between *phot1, phot2, pin,* and *mp* would uncover relations between *PHOT*-dependent and auxin-dependent vein development.

My second research question asked whether the AGCVIIIa subfamily of serine threonine kinases, which regulate PIN polarity in the root epidermis and cortex (Dhonukshe et al., 2010), played a role in leaf vascular development. To address this question, I studied the expression of the four members of this subfamily—*PID, WAG1, WAG2,* and *AGC3.4*—in leaf development. I found that PID, WAG1, and WAG2 are all expressed in the epidermis and localize to the plasma membrane. However, while *WAG1* and *WAG2* are not expressed in subepidermal tissues, *PID* is expressed ubiquitously at early stages of subepidermal tissue development. *PID* expression rapidly declines in subepidermal cells that begin to undergo vascular development. The fourth member of the subfamily, AGC3.4, is expressed in veins and accumulates primarily in the nucleus. Because PID, WAG1, and WAG2 are localized to the plasma membrane of leaf cells, where they could regulate PIN polarity, I asked whether they functioned in vein patterning. My results suggest that non-redundant vein patterning functions are limited to *PID* and that *WAG2* provides redundant vein patterning functions. Expression and genetic data are consistent with a role for PID and WAG2 in regulation of PIN1 polarity during formation of epidermal convergence points, which are associated with formation of midvein and lateral veins. As *PID* is also expressed in subepidermal tissues, it is possible that it is also required to regulate PIN1

polarity during the formation of marginal and higher-order veins. Ubiquitous *PID* expression at early stages of subepidermal tissue development would endow all ground cells with the possibility to redirect PIN1 polarity as required by the formation of vein-associated subepidermal PIN1 expression domains (PEDs) and thus provide flexibility to the process of vein path selection. Termination of *PID* expression in developing veins may suggest that stable PIN1 polarity is required for vein formation. I next asked whether the function of *PID* and *WAG2*—and possibly *WAG1*—overlapped with that of PIN1during vein formation. My results suggest that *PID* and *WAG2* function in a vein patterning pathway that overlaps with that in which *PIN1* functions. This could suggest *PID* and *WAG2* redundantly regulate PIN1 polarity during vein patterning. In the future, it may be interesting to test functionality of the translational fusion constructs of *PID*, *WAG1*, and *WAG2* by assessing their ability to rescue mutant defects. The triple *pid;wag1;wag2* mutant would uncover potential redundant functions of *WAG1* in vein patterning. PIN1 localization in *pid*, *pid;wag2*, and *pid;wag1;wag2* would verify the redundant functions of *PID* and *WAG2* in regulating PIN1 polarity during vein formation. Finally, it could be interesting to examine the effects of ectopic *PID* expression in developing veins, for example by expressing *PID* by the *ATHB8* promoter.

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