Transcriptional Control of Auxin-Transport-Dependent Vein Patterning in *Arabidopsis thaliana*

by

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Abstract

Most multicellular organisms solve the problem of long-distance transport of signals and nutrients by means of tissue networks such as the vascular system of vertebrate embryos and the vein network of plant leaves; therefore, how vascular networks form is a key question in biology. In vertebrates, the formation of the embryonic vascular system relies on direct cell-cell interaction and, at least in part, on cell migration, both of which are precluded in plants by a cell wall that keeps cells apart and in place; therefore, vein networks form differently in plant leaves.

How leaf vein networks form is unclear, but available evidence suggests that the polar transport of the plant signal auxin is required for vein patterning. Functions of polar auxin transport in vein patterning in turn depend on functions of the PIN-FORMED1 (PIN1) auxin transporter. At early stages of leaf tissue development, PIN1 polar localization at the plasma membrane of epidermal cells is directed toward single cells along the marginal epidermis of developing leaves. These "convergence points" of epidermal PIN1 polarity are associated with broad domains of PIN1 expression in the inner tissue of the leaf; these broad domains will over time become restricted to the narrow sites where major veins will form.

Consistent with those observations, for the past 15 years the prevailing hypotheses of leaf vein patterning have proposed that convergence points of epidermal PIN1 polarity lead to the formation of local peaks of auxin level in the epidermis, and that that auxin is transported by PIN1 from the epidermal convergence points into the inner tissue where it will lead to vein formation. As such, these hypotheses predict that epidermal PIN1 expression is strictly required for vein patterning. I tested this prediction in *Arabidopsis*

thaliana by a combination of targeted gene expression, molecular genetic analysis, and cellular imaging, and found it unsupported: epidermal PIN1 expression is neither required nor sufficient for *PIN1*-dependent vein patterning, whereas PIN1 expression in the inner tissues of the leaf turns out to be both required and sufficient for *PIN1*-dependent vein patterning.

To identify regulatory inputs upstream of *PIN1*-dependent vein patterning, I next sought regulatory elements that are necessary for that component of PIN1 expression in the inner tissues of the leaf that is required for *PIN1*-dependent vein patterning. By means of a combination of promoter deletion, molecular genetic analysis, and cellular imaging, I found that vascular expression of PIN1 is required for *PIN1*-dependent vein patterning; that such vascular expression of PIN1 depends on the 151-bp region of the *PIN1* promoter from -645 to -495; and that that region of the *PIN1* promoter contains putative binding sites for members of an uncharacterized plant-specific family of transcription factors.

Finally, for the future characterization of such putative upstream regulators of PIN1 expression, I identified and characterized GAL4/GFP enhancer-trap lines for the targeted misexpression of genes of interest in specific cells and tissues of developing leaves.

In conclusion, my results refute all vein patterning hypotheses based on polar auxin transport from the epidermis and suggest alternatives for future tests. Further, my results have identified regulatory inputs that are required for *PIN1*-dependent vein patterning, and have generated the resources to characterize those regulatory inputs.

Preface

A part of my thesis has been published.

Chapter 4 of this thesis has been published as Amalraj, B., Govindaraju, P., Krishna, A., Lavania, D., Linh, N.M., Ravichandran, S.J., Scarpella, E., 2019. "GAL4/GFP enhancer-trap lines for identification and manipulation of cells and tissues in developing Arabidopsis leaves." *bioRxiv*, 801357. All authors equally shared the responsibility to conceiving, designing, and writing the paper.

All the authors and the publisher have given their permission for the inclusion of these publications in this thesis.

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List of Abbreviations

ABCB	ATP-BINDING CASSETTE B
AFB	AUXIN SIGNALING F-BOX
ARF	AUXIN RESPONSE FACTOR
ATHB8	ARABIDOPSIS THALIANA HOMEOBOX8
ATML1	ARABIDOPSIS THALIANA MERISTEM LAYER1
AUX/IAA	AUXIN/INDOLE-3-ACETIC ACID
cf.	Compare
CFP	CYAN FLUORESCENT PROTEIN
Col-0	Columbia-0
cPIN1	*PIN-FORMED1* coding sequence
DAG	Days after germination
EAR	ETHYLENE-RESPONSIVE-ELEMENT-BINDING
Fig	Figure
GFP	GREEN FLUORESCENT PROTEIN
GN	GNOM
gPIN1	*PIN-FORMED1* genomic sequence
GRAS	GIBBERELLIC ACID INSENSITIVE, REPRESSOR OF gibberellic acid1-3, and SCARECROW
HD-ZIP III	HOMEODOMAIN-LEUCINE ZIPPER class III
IAA	Indole-3-acetic acid
LAX	LIKE-AUX1

LUT	Look-up table
MP	MONOPTEROS
PIN	PIN-FORMED
PM	Plasma membrane
RPS5A	RIBOSOMAL PROTEIN S5A
SCL32	SCARECROW-LIKE32
SHR	SHORT-ROOT
TF	Transcription Factor
TIR1	TRANSPORT INHIBITOR RESPONSE1
WT	Wild type
YFP	YELLOW FLUORESCENT PROTEIN
ZHD	ZINC-FINGER HOMEODOMAIN
ΔPIN1	*PIN-FORMED1* promoter fragments

Notations

WT gene	Uppercase, italics (e.g., *PIN1*)
Mutant gene	Lowercase, italics (e.g., *pin1*)
WT protein	Uppercase (e.g., PIN1)
Fusions between promoter *A* and gene *A*	A::A (e.g., PIN1::gPIN1)
Fusions between gene *A* and gene *B*	A:B (e.g., PIN1:GFP)

Linked genes will be separated by a comma (e.g., *pin1*,*pin3* or *pin1*,*3*) and unlinked genes by a semicolon (e.g., *pin4*;*pin7* or *pin4*;*7*).

Gene Coordinates

All gene coordinates are relative to the adenine (position +1) of the start codon.

Chapter 1: General Introduction

1.1 The Plant Vascular Network

In most multicellular organisms, the long-distance transport of signals and nutrients occurs through tissue networks such as the vascular network. In animals, the formation of vascular networks often relies on direct cell-cell interaction and cell movement (e.g., Noden, 1988; Xue et al., 1999). By contrast, cell-cell interaction and cell movement are precluded in plants by a wall that holds cells apart and in place; therefore, vascular networks form differently in plants.

Plant vascular networks are composed of continuous vascular strands that connect with one another the different parts of an organ and the different organs of the plant (Esau, 1965). In different organs, vascular strands are named differently: for example, veins in flat organs such as cotyledons, leaves, sepals, and petals; vascular bundles in the stem; and vascular cylinder in the root.

Mature vascular strands are cylinders composed of two vascular tissues — xylem and phloem (Esau, 1965). Xylem is found at the center of vascular strands in cylindrical organs and at the upper side of vascular strands in flat organs. Phloem is found at the periphery of vascular strands in cylindrical organs and at the lower side of vascular strands in flat organs. Xylem is composed of tracheary elements, parenchyma cells, and fibers, and mainly transports water and minerals. Phloem is composed of sieve elements, parenchyma cells, fibers, and sclereids, and mainly transports photosynthesis products.

Vascular cells first form during embryogenesis. In *Arabidopsis thaliana* L. Heyn. (Arabidopsis hereafter), the dermatogen-stage embryo is composed of eight outer cells and eight inner cells (Mansfield and Briarty, 1991). Longitudinal division of the four basalmost inner cells results in four innermost cells that elongate to become procambial cells, the precursors of all mature vascular cells (Esau, 1965; Mansfield and Briarty, 1991). Procambial cells also form de novo during the development of flat organs like leaves (Foster,

1952; Pray, 1955). Throughout the life of a plant, vascular strands lengthen and thicken as procambial cells continually divide transversely and longitudinally (Esau, 1965).

1.2 Leaf Vein Patterns

In most rounded leaves of dicots such as Arabidopsis, the vein network is composed of a central midvein; lateral veins, which branch from the midvein and connect to distal veins to form loops; and minor veins, which branch from the midvein and loops, and may connect to other veins. Minor veins and loops curve near the leaf margin to give rise to a scalloped vein-network outline (Telfer and Poethig, 1994; Nelson and Dengler, 1997; Kinsman and Pyke, 1998; Candela et al., 1999; Mattsson et al., 1999; Sieburth, 1999; Steynen and Schultz, 2003; Sawchuk et al., 2013; Verna et al., 2015).

In most elongated leaves of monocots such as maize, vein loops are stretched and laterally compressed, giving rise to a network in which midvein and lateral veins seem to run parallel to one another along the length of the leaf (Troll, 1939; Gifford and Foster, 1988; Nelson and Dengler, 1997).

1.3 Leaf Vein Development

During leaf development, veins form de novo from the inner tissue of the leaf, which also gives rise to the mesophyll tissue (Esau, 1965). In Arabidopsis, the expression and polar localization at the plasma membrane of PIN-FORMED1 (PIN1), which catalyzes cellular efflux of the plant signaling molecule auxin (Petrasek et al., 2006), suggests that veins are formed by two different mechanisms (Scarpella et al., 2006; Wenzel et al., 2007).

The midvein seems to form from sites of convergence of PIN1 polarity in the epidermis of the shoot apical meristem (Benkova et al., 2003; Reinhardt et al., 2003; Carraro et al., 2006; Scarpella et al., 2006; Lee et al., 2008; Bayer et al., 2009; Johnston et al., 2015) (Fig. 1.1). These “convergence points” mark the positions where the new leaf primordia will be formed and are associated with broad PIN1-expression domains in the inner tissue of the leaf primordium; over time, these broad PIN1-expression domains become restricted to the site of midvein formation, and PIN1 becomes localized to the basal side of the plasma

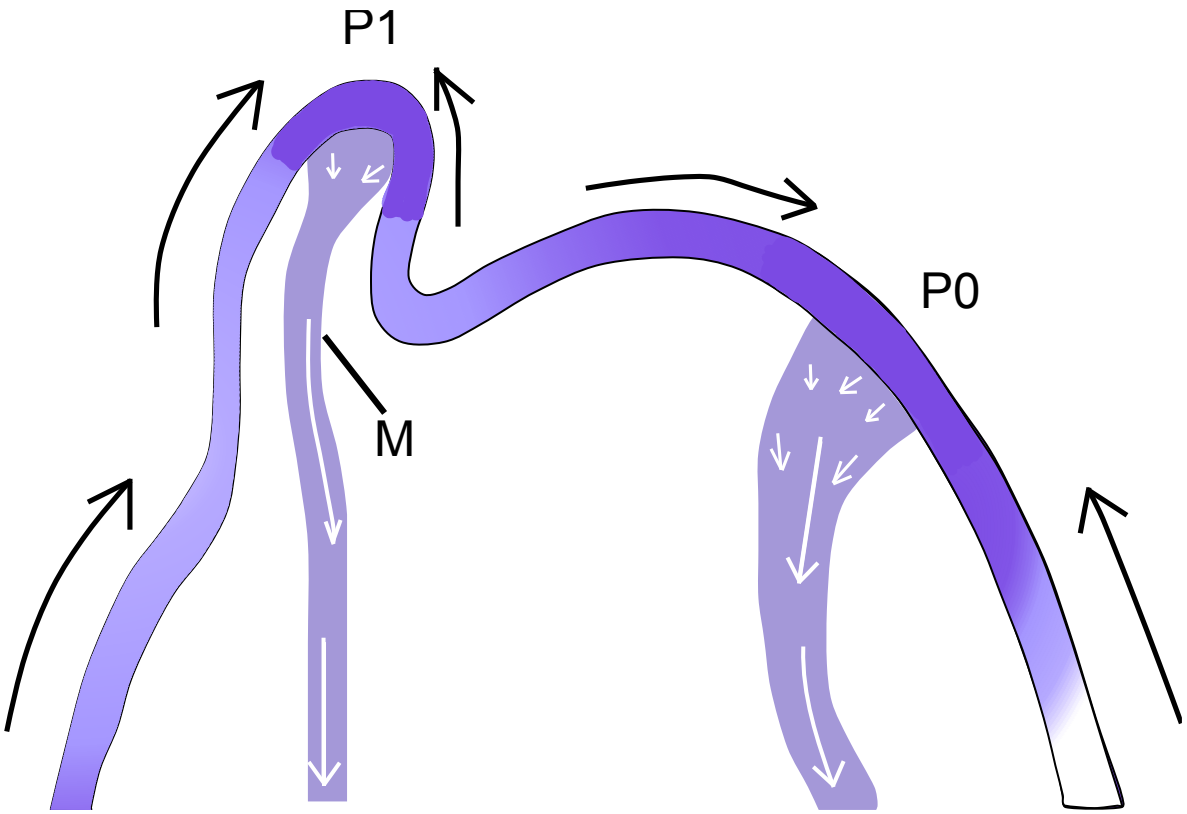

Figure 1.1. Auxin Transport During Leaf Development. P0, P1 indicate successive stages of leaf development. Arrows indicate directions of auxin transport. M, midvein. Redrawn from Scarpella and Helariutta, 2010.

membrane in the vascular cells of the midvein. Likewise, lateral veins seem to form from convergence points of PIN1 polarity in the marginal epidermis of the developing leaf (Hay et al., 2006; Scarpella et al., 2006; Wenzel et al., 2007) (Fig. 1.2). These convergence points are associated with broad PIN1-expression domains in the inner tissue of the developing leaf; over time, these broad PIN1-expression domains become restricted to the sites of lateral vein formation, and in the vascular cells of the lateral veins PIN1 becomes localized to the side of the plasma membrane facing the midvein.

By contrast, minor veins form from PIN1 expression domains that branch from pre-existing veins and have no association with epidermal convergence points of PIN1 polarity (Scarpella et al., 2006; Wenzel et al., 2007; Marcos and Berleth, 2014) (Fig. 1.2). Initially, all minor veins end freely in the inner tissues of the leaf, and PIN1 is localized to the side of the plasma membrane facing the pre-existing veins the minor veins connect to. However, over time minor veins can become connected to pre-existing veins on both sides. At the ends of these "connected" veins, PIN1 is localized to the side of the plasma membrane facing the pre-existing veins the minor veins connect to; the resulting opposite PIN1 polarities are joined by a "bipolar" cell, a cell where PIN1 is localized to two opposite sides of the plasma membrane.

PIN1 expression during loop formation suggests that each loop is formed by a minor vein branching from a lateral vein (Scarpella et al., 2006; Wenzel et al., 2007) (Fig. 1.2). Initially, the minor vein ends freely in the inner tissue of the leaf, but over time it connects to the midvein or to other lateral veins. Like in all connected veins, at the ends of each loop PIN1 is localized to the side of the plasma membrane facing the pre-existing veins the loop connects to, and the two, opposite PIN1 polarities are joined by a bipolar cell.

Domains of PIN1 expression in the leaf inner tissue are initially broad and overlap with broad domains of expression of the auxin-response transcription factor MONOPTEROS (MP) (Donner et al., 2009; Wenzel et al., 2007). Just like for the broad domains of PIN1 expression, over time the broad domains of MP expression become gradually restricted to sites of vein formation. Within the broad expression domains of PIN1 and *MP*, cells that will differentiate into procambial cells express the class III HOMEODOMAIN-LEUCINE ZIPPER (HD-ZIP III) gene *ARABIDOPSIS THALIANA*

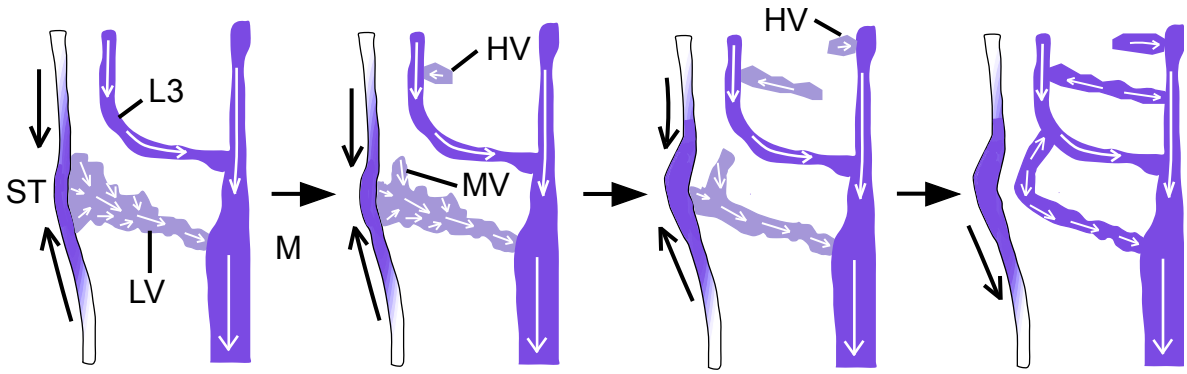

Figure 1.2. Auxin Transport During Lateral Vein, Minor Vein, and Marginal Vein Development. Arrows indicate directions of auxin transport. ST, serration tip; L3, third loop; LV, lateral vein; M, midvein; HV, minor vein; MV, marginal vein. Redrawn from Scarpella and Helariutta, 2010.

HOMEOBOX8 (*ATHB8*) and the GRAS (after GIBBERELLIC ACID INSENSITIVE, REPRESSOR OF gibberellic acid1-3, and SCARECROW) gene *SHORT-ROOT* (*SHR*) (Donner et al., 2009; Gardiner et al., 2011).

1.4 Auxin Transport and Vascular Strand Formation

The plant signaling molecule auxin is the only known molecular that can induce vascular strand formation when applied to plant tissues. The auxin-induced vascular-differentiation response is characterized by five properties (Sachs, 1981; Berleth et al., 2000): (1) the response is local, as the vascular strands are initiated at the site of auxin application; (2) the response is polar, as the newly formed vascular strand are oriented toward the pre-existing vascular strands basal to the site of auxin application; (3) the response is continuous, as it results in the formation of uninterrupted files of vascular cells; (4) the response is constrained laterally, as only narrow strips of cells, rather than all the cells near the site of auxin application, differentiate into vascular cells; (5) the response requires polarly transported auxins, and auxin transport inhibitors obstruct the response (Thompson and Jacobs, 1966; Dalessandro and Roberts, 1971; Gersani, 1987), suggesting that the auxin-induced vascular differentiation response recruits a polar process that already exists in plant tissues and that may correspond to the polar transport of auxin itself.

Auxin is indeed primarily synthesized in young shoot organs, such as leaf and flower primordia, and is mainly transported to the root tip through the vascular strands (Michniewicz et al., 2007; Normanly, 2010; Zhao, 2010). This apical-basal polarity of auxin transport depends on the polar localization of auxin transporters of the PIN-FORMED (PIN) family at the basal plasma membrane of auxin-transporting cells (Wisniewska et al., 2006). Indeed, the weak acid indole-3-acetic acid (IAA), the most abundant auxin in plants, is non-charged in the acidic extracellular space and can therefore freely diffuse into the cells through their plasma membrane (Rubery and Sheldrake, 1974; Raven, 1975). By contrast, in the more alkaline intracellular space, IAA becomes negatively charged and can therefore leave the cell only through specialized efflux carrier proteins.

These observations form the basis of the “auxin canalization hypothesis”, which proposes that auxin transport through a cell positively feeds back on the cell’s auxin

conductivity (Sachs, 1981). This positive feedback would gradually restrict an initially dispersed auxin flow to preferential auxin-transport through narrow strips of cells, which would eventually differentiate into vascular strands.

In Arabidopsis, the localization of PIN proteins at the plasma membrane marks the presumed site of cellular auxin efflux (Petrasek et al., 2006; Wisniewska et al., 2006). Therefore, directions of polar auxin transport can be deduced from the localization of PIN proteins at the plasma membrane. Consistent with predictions of the auxin canalization hypothesis, local application of auxin induces PIN1 expression in broad domains that connect the applied auxin to the pre-existing vasculature and that over time become restricted to sites of vascular strand formation (Sauer et al., 2006; Scarpella et al., 2006). In these domains, PIN1 is initially distributed homogeneously throughout the plasma membrane; over time, however, PIN1 distribution becomes polarized to suggest auxin transport away from the site of auxin application and toward the pre-existing vasculature basal to the site of auxin application. Consistent with a role for auxin transport in vascular strand formation, mutation of *PIN* genes or development in the presence of auxin transport inhibitors delays the restriction of PIN1 expression domains and the polarization of PIN1 localization, and leads to defects in vein network formation (Mattsson et al., 1999; Sieburth, 1999; Scarpella et al., 2006; Sawchuk et al., 2013; Verna et al., 2015).

1.5 Auxin Signaling and Vein Formation

Once auxin is transported into a cell, it triggers a signal transduction cascade that leads to the activation or repression of auxin responsive genes by the transcription factors of the AUXIN RESPONSE FACTOR (ARF) family (Chapman and Estelle, 2009).

When the concentration of cellular auxin is low, the transcriptional repressors of the AUXIN/INDOLE-3-ACETIC ACID (AUX/IAA) family interact with ARFs; this prevents ARFs from inducing the transcription of their target genes (Mockaitis and Estelle, 2008). When the concentration of auxin is high, TRANSPORT INHIBITOR RESPONSE1/AUXIN SIGNALING F-BOX PROTEIN (TIR1/AFB) receptor complexes and AUX/IAAs bind auxin, thereby forming a TIR1/AFB-auxin-AUX/IAA complex. This association leads to the ubiquitination of AUX/IAAs, followed by their degradation by the 26S proteasome. In this

way, ARFs are released from inhibition and can induce the transcription of their target genes. This model, however, fails to explain the mechanism of action of the ARFs that act as transcriptional repressors (Guilfoyle and Hagen, 2007).

Available evidence suggests that auxin signaling is required for vein formation:

(1) Expression domains of targets of activating ARFs and expression domains of synthetic auxin-responsive promoters overlap with sites of vein formation (Mattsson et al., 2003; Donner et al., 2009; Konishi et al., 2015).

(2) Leaves lacking the function of auxin signaling components or their targets have vein defects (Przemeck et al., 1996; Hardtke and Berleth, 1998; Alonso-Peral et al., 2006; Strader et al., 2008; Esteve-Bruna et al., 2013).

1.6 Scope and Outline of the Thesis

The evidence discussed above suggests that vein patterning is controlled by auxin transport and that auxin transport is in turn controlled by *PIN1*. PIN1 is expressed in all the cells of the leaf at early stages of tissue development; over time, however, epidermal expression becomes restricted to the basal-most cells, and inner tissue expression becomes restricted to developing veins (Benkova et al., 2003; Reinhardt et al., 2003; Heisler et al., 2005; Scarpella et al., 2006; Hay et al., 2006; Wenzel et al., 2007; Bayer et al., 2009; Sawchuk et al., 2013; Marcos and Berleth, 2014). The scope of my M.Sc. thesis was to understand the function of PIN1 expression in the different tissues of the leaf in *PIN1*-dependent vein patterning, and what controlled that component of PIN1 expression that is required for *PIN1*-dependent vein patterning.

The current hypotheses of vein patterning by auxin transport propose that in the epidermis of the developing leaf PIN1-mediated auxin transport converges toward peaks of auxin level. From those convergence points of epidermal PIN1 polarity, auxin would be transported in the inner tissue of the leaf where it would give rise to the midvein and lateral veins. In Chapter 2, we tested predictions of this hypothesis and found them unsupported: epidermal PIN1 expression is neither required nor sufficient for *PIN1*-dependent vein patterning, whereas inner-tissue PIN1 expression turns out to be both required and sufficient

for *PIN1*-dependent vein patterning. Our results refute all vein patterning hypotheses based on auxin transport from the epidermis and suggest alternatives for future tests.

In Chapter 3, we sought to identify the cis-regulatory element that are required for that component of PIN1 expression in the inner tissues of the leaf that is relevant to *PIN1*-dependent vein patterning. We found that vascular expression of PIN1 is required for *PIN1*-dependent vein patterning and that such vascular expression of PIN1 depends on the 151-bp region of the *PIN1* promoter from -645 to -495.

Testing the function in *PIN1*-dependent vein patterning expression in the different tissues of the leaf (Chapter 2) required expression of *PIN1* by different promoters. This imposed the burden of generating different constructs for different promoter::PIN1 combinations. This approach could be simplified if GAL4/GFP enhancer-trap lines existed in Columbia-0, the genotype of reference in Arabidopsis (Koornneef and Meinke, 2010), with which to drive expression of genes of interest in desired cells and tissues of developing leaves. Unfortunately, such lines were not available when I started my M.Sc.. In Chapter 4, we addressed this limitation and provided GAL4/GFP enhancer-trap lines in the Col-0 background of Arabidopsis for the identification and manipulation of cells and tissues in developing leaves.

Finally, in Chapter 5 I propose and discuss a hypothesis on the upstream regulators of PIN1 functional expression in *PIN1*-dependent vein patterning.

Chapter 2: Vein Patterning by Tissue-Specific Auxin Transport

2.1 Introduction

Most multicellular organisms solve the problem of long-distant transport of signals and nutrients by means of tissue networks such as the vascular system of vertebrate embryos and the vein networks of plant leaves; therefore, how vascular networks form is a key question in biology. In vertebrates, the formation of the embryonic vascular system relies on direct cell-cell interaction and at least in part on cell migration (e.g., Noden, 1988; Xue et al., 1999), both of which are precluded in plants by a wall that keeps cells apart and in place; therefore, vascular networks form differently in plant leaves.

How leaf vein networks form is unclear, but available evidence suggests that polar transport of the plant signal auxin is non-redundantly required for vein patterning (Mattsson et al., 1999; Sieburth, 1999). Such non-redundant functions of polar auxin transport in vein patterning in turn depend on non-redundant functions of the PIN-FORMED1 (PIN1) auxin transporter (Galweiler et al., 1998; Petrasek et al., 2006; Sawchuk et al., 2013; Verna et al., 2019; Zourelidou et al., 2014). At early stages of leaf development, PIN1 polar localization at the plasma membrane of epidermal cells is directed toward single cells along the marginal epidermis (Bayer et al., 2009; Benkova et al., 2003; Hay et al., 2006; Heisler et al., 2005; Reinhardt et al., 2003; Scarpella et al., 2006; Wenzel et al., 2007). These convergence points of epidermal PIN1 polarity are associated with broad domains of PIN1 expression in the inner tissue of the developing leaf, and these broad domains will over time become restricted to the narrow sites where the midvein and lateral veins will form.

Consistent with those observations, the prevailing hypotheses of vein patterning propose that convergence points of epidermal PIN1 polarity contribute to the formation of local peaks of auxin level in the epidermis, and that that auxin is transported by PIN1 from the epidermal convergence points into the inner tissues of the leaf, where it will lead to vein formation (reviewed in Prusinkiewicz and Runions, 2012; Runions et al., 2014); see also (Alim and Frey, 2010; Hartmann et al., 2019, and references therein). As such, these hypotheses predict that epidermal PIN1 expression is required for vein patterning. Here we

tested this prediction and found it unsupported: epidermal PIN1 expression is neither required nor sufficient for vein patterning; instead, PIN1 expression in the inner tissues turns out to be both required and sufficient for vein patterning. Our results refute all the current hypotheses of vein formation that depend on polar auxin transport from the epidermis and suggest alternatives for future testing.

2.2 Results and Discussion

2.2.1 PIN1 Expression During Arabidopsis Vein Patterning

In Arabidopsis leaf development, the formation of the midvein precedes the formation of the first loops of veins ("first loops"), which in turn precedes the formation of the second loops (Kang and Dengler, 2004; Mattsson et al., 1999; Sawchuk et al., 2007; Scarpella et al., 2004; Sieburth, 1999) (Fig. 2.1A–C). The formation of second loops precedes the formation of third loops and that of minor veins in the area delimited by the midvein and the first loops (Fig. 2.1C,D). Loops and minor veins form first near the top of the leaf and then progressively closer to its bottom, and minor veins form after loops in the same area of the leaf (Fig. 2.1B–D).

Consistent with previous reports (Bayer et al., 2009; Benkova et al., 2003; Heisler et al., 2005; Marcos and Berleth, 2014; Reinhardt et al., 2003; Sawchuk et al., 2007; Sawchuk et al., 2013; Scarpella et al., 2006; Verna et al., 2019; Wenzel et al., 2007), a fusion of the *PIN-FORMED1* (*PIN1*) open reading frame to YFP driven by the *PIN1* promoter (PIN1::gPIN1:YFP) (Xu et al., 2006) was expressed in all the cells of the leaf at early stages of tissue development; over time, however, epidermal expression became restricted to the basalmost cells, and inner-tissue expression became restricted to developing veins (Fig. 2.1E–H).

We asked whether PIN1::gPIN1:YFP expression were recapitulated by the activity of the *PIN1* promoter. To address this question, we imaged expression of a nuclear YFP driven by the *PIN1* promoter (PIN1::nYFP) in first leaves 2, 2.5, 3, and 4 days after germination (DAG).

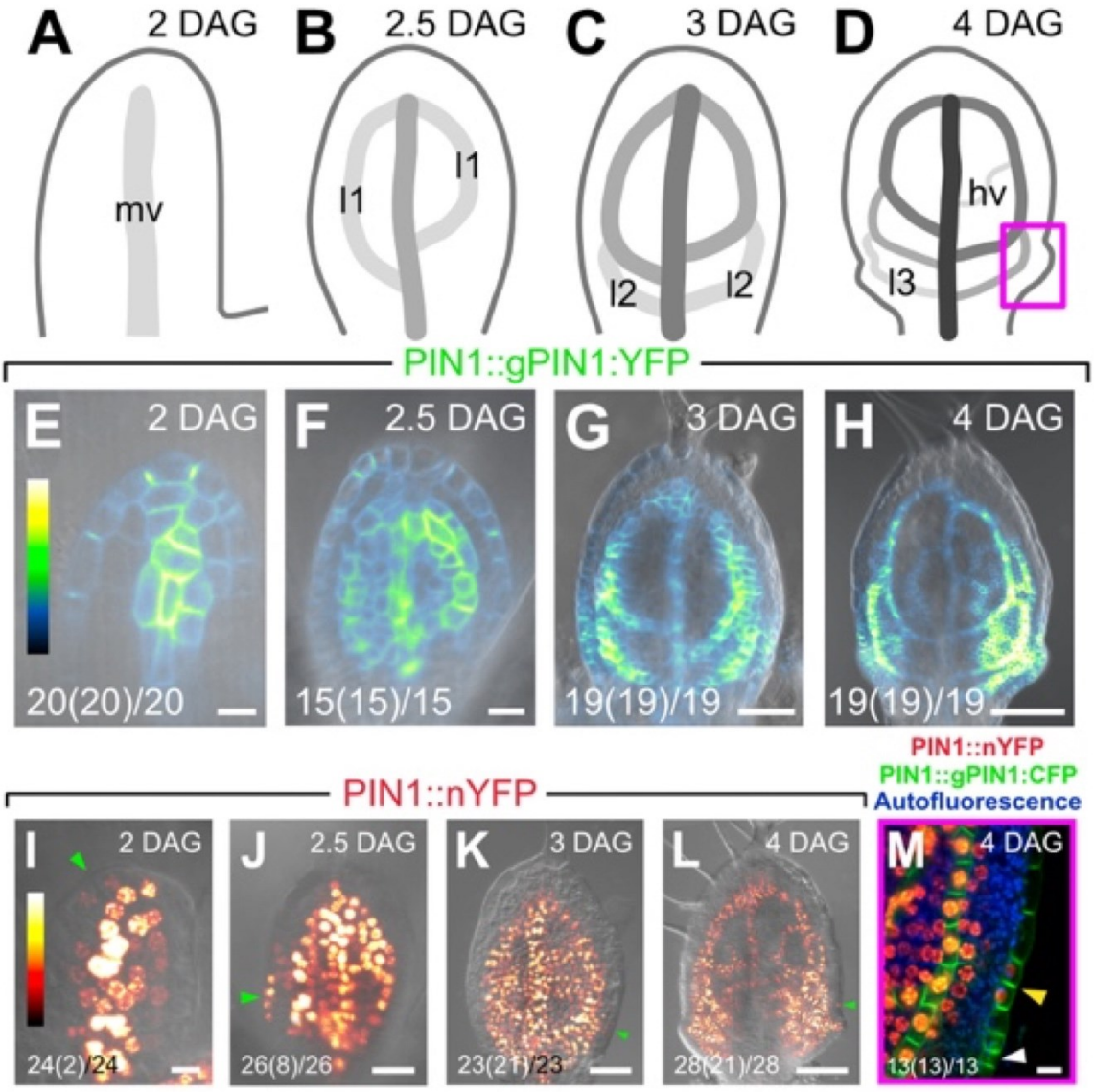

Figure 2.1. PIN1 Expression During Arabidopsis Vein Patterning. (A–M). Top right: leaf age in days after germination (DAG). Abaxial side to the left in (A,E,I). (A–D) Midvein, loops, and minor veins form sequentially during leaf development (Kang and Dengler, 2004; Mattsson et al., 1999; Sawchuk et al., 2007; Scarpella et al., 2004; Sieburth, 1999); increasingly darker grays depict progressively later stages of vein development. Box in (D) illustrates position of closeup in (M) and in Figs. 2.2D,J and 2.4D,J. (E–M) Confocal laser scanning microscopy with (E–L) or without (M) transmitted light. Bottom left: reproducibility index, i.e. no. of leaves with the displayed inner-tissue expression (no. of leaves with the displayed epidermal expression) / no. of leaves analyzed. Look-up tables in

(E–H) — ramp in (E) — and in (I–L) — ramp in (I) — visualize expression levels. Green arrowheads in (I–L) and yellow arrowhead in (M) point to epidermal expression; white arrowhead in (M) points to convergence point of PIN1 polarity. hv, minor vein; l1, first loop; l2, second loop; l3, third loop; mv, midvein. Scale bars: (E,I,M) 10 μm; (F,J) 20 μm; (G,K) 50 μm; (H,L) 100 μm.

Just like PIN1::gPIN1:YFP (Fig. 2.1E–H), PIN1::nYFP was expressed in all the inner cells of the leaf at early stages of tissue development, and over time this inner-tissue expression became restricted to developing veins (Fig. 2.1I–L). However, unlike PIN1::gPIN1:YFP and PIN1::gPIN1:CFP (Gordon et al., 2007) (Fig. 2.1E–H,M), PIN1::nYFP was expressed in very few epidermal cells at the tip of 2-DAG primordia and at the margin of 2.5-DAG primordia, and this epidermal expression was very rare (Fig. 2.1I,J). PIN1::nYFP expression in epidermal cells at the leaf margin was more frequent at 3 and 4 DAG but was still limited to very few cells (Fig. 2.1K–M). Moreover, these PIN1::nYFP-expressing epidermal cells were not those that contributed to convergence points of epidermal PIN1 polarity (Fig. 2.1M).

Because a fusion of the *PIN1* coding sequence to GFP driven by the *PIN1* promoter (PIN1::cPIN1:GFP) was hardly expressed in leaf epidermal cells (Fig. 2.2C,D,I,J), we conclude that the already limited activity of the *PIN1* promoter in the leaf epidermis is suppressed post-transcriptionally by the *PIN1* coding sequence and that the leaf epidermal expression characteristic of PIN1 is encoded in the gene's introns.

2.2.2 Tissue-Specific PIN1 Expression in *PIN1* Non-Redundant Functions in Vein Patterning

During leaf development, PIN1 is expressed in all the tissues — the epidermis, the vascular tissue, and the nonvascular inner tissue (Fig. 2.1). We asked what the function in *PIN1*-dependent vein patterning were of PIN1 expression in these tissues. To address this question, we expressed in the WT and *pin1* mutant backgrounds

(1) PIN1::gPIN1:GFP, which like PIN1::gPIN1:YFP and PIN1::gPIN1:CFP (Fig. 2.1E–H,M) is expressed in all the tissues of the developing leaf (Fig. 2.2A,G);
(2) cPIN1:GFP driven by the epidermis-specific *ARABIDOPSIS THALIANA MERISTEM LAYER1* promoter (Sessions et al., 1999) (ATML1::cPIN1:GFP) (Fig. 2.2B,H);
(3) PIN1::cPIN1:GFP, which is expressed in the leaf inner tissues (Fig. 2.2C,D,I,J)

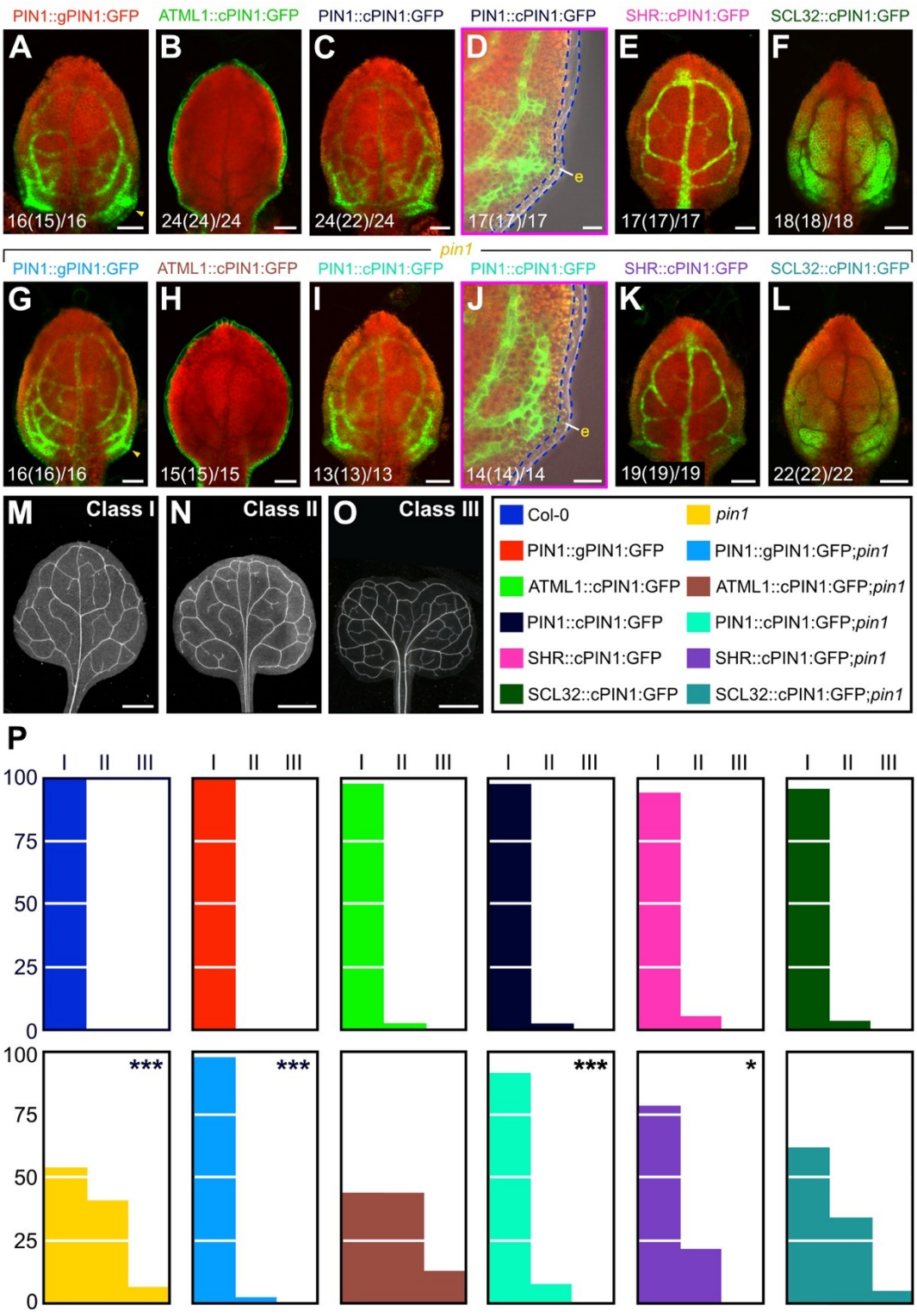

Figure 2.2. Tissue-Specific PIN1 Expression in *PIN1*-dependent Vein Patterning. (A–L). Confocal laser scanning microscopy with (D,J) or without (A–C,E–I,K,L) transmitted light; first leaves 4 DAG. Green, GFP expression; red, autofluorescence. Yellow arrowheads

in (A,G) point to epidermal expression. Bottom left: reproducibility index, i.e. no. of leaves with the displayed inner-tissue expression (no. of leaves with the displayed epidermal expression) / no. of leaves analyzed. (M–O) Dark-field illumination of mature first leaves illustrating phenotype classes (top right): class I, I-shaped midvein (M); class II, Y-shaped midvein (N); class III, fused leaves (O). (P) Percentages of leaves in phenotype classes. Difference between *pin1* and WT, between PIN1::gPIN1:GFP;*pin1* and *pin1*, and between PIN1::cPIN1:GFP;*pin1* and *pin1* was significant at $P<0.001$ (***) by Kruskal-Wallis and Mann-Whitney test with Bonferroni correction. Difference between SHR::cPIN1:GFP;*pin1* and WT, and between SHR::cPIN1:GFP;*pin1* and *pin1* was significant at $P<0.05$ (*) by Kruskal-Wallis and Mann-Whitney test with Bonferroni correction. Sample population sizes: WT, 40; *pin1*, 60; PIN1::gPIN1:GFP, 55; ATML1::cPIN1:GFP, 49; PIN1::cPIN1:GFP, 48; SHR::cPIN1:GFP, 59; SCL32::cPIN1:GFP, 60; PIN1::gPIN1:GFP;*pin1*, 60; ATML1::cPIN1:GFP;*pin1*, 55; PIN1::cPIN1:GFP;*pin1*, 51; SHR::cPIN1:GFP;*pin1*, 60; SCL32::cPIN1:GFP;*pin1*, 58. e, epidermis. Scale bars: (A–C,E–I,K,L) 60 μm; (D,J) 20 μm; (M) 1 mm; (N,O) 2 mm.

(4) cPIN1:GFP driven by the vascular-tissue-specific *SHORT-ROOT* promoter (Gardiner et al., 2011) (SHR::cPIN1:GFP) (Fig. 2.2E,K);

(5) cPIN1:GFP driven by the *SCARECROW-LIKE32* promoter, which is active in the nonvascular inner tissue of the leaf (Gardiner et al., 2011) (SCL32::cPIN1:GFP) (Fig. 2.2F,L).

We then compared vein patterns of mature first leaves of the resulting backgrounds.

Consistent with previous reports (Sawchuk et al., 2013; Verna et al., 2019), the vein patterns of nearly 50% of *pin1* leaves were abnormal (Fig. 2.2M–P). The vein patterns of PIN1::gPIN1:GFP, ATML1::cPIN1:GFP, PIN1::cPIN1:GFP, SHR::cPIN1:GFP, and SCL32::cPIN1:GFP were no different from the WT vein pattern (Fig. 2.2M–P). Both PIN1::gPIN1:GFP and PIN1::cPIN1:GFP normalized the phenotype spectrum of *pin1* vein patterns (Fig. 2.2M–P; Fig. 2.3A,C). SHR::cPIN1:GFP shifted the phenotype spectrum of *pin1* vein patterns toward the WT vein pattern (Fig. 2.2M–P; Fig. 2.3D). The vein pattern defects of ATML1::cPIN1:GFP;*pin1* and SCL32::cPIN1:GFP;*pin1* were no different from those of *pin1* (Fig. 2.2M–P; Fig. 2.3B,E). We observed a similar effect of tissue-specific *PIN1* expression in *PIN1*-dependent cotyledon patterning (Fig. 2.4).

We conclude that PIN1 expression in the epidermis is neither required nor sufficient for *PIN1*-dependent vein patterning. By contrast, PIN1 expression in the inner tissues of the leaf is both required and sufficient for *PIN1*-dependent vein patterning; such function of PIN1 expression mainly depends on PIN1 expression in the vascular tissue.

2.2.3 Expression of PIN3, PIN4, and PIN7 During Vein Patterning

Collectively, *PIN3*, *PIN4*, and *PIN7* act redundantly with *PIN1* in *PIN1*-dependent vein patterning, and like *PIN1* they are expressed in both epidermis and inner tissues of young leaves (Verna et al., 2019). In those leaves, however, the most reproducible features of the Arabidopsis vein pattern can already be recognized (Amalraj et al., 2019; Donner et al., 2009; Donner and Scarpella, 2013; Gardiner et al., 2010; Gardiner et al., 2011; Sawchuk et al., 2013; Verna et al., 2015; Verna et al., 2019). Therefore, to test the possibility that compensatory functions provided by *PIN3*, *PIN4*, and *PIN7* may account for the observation

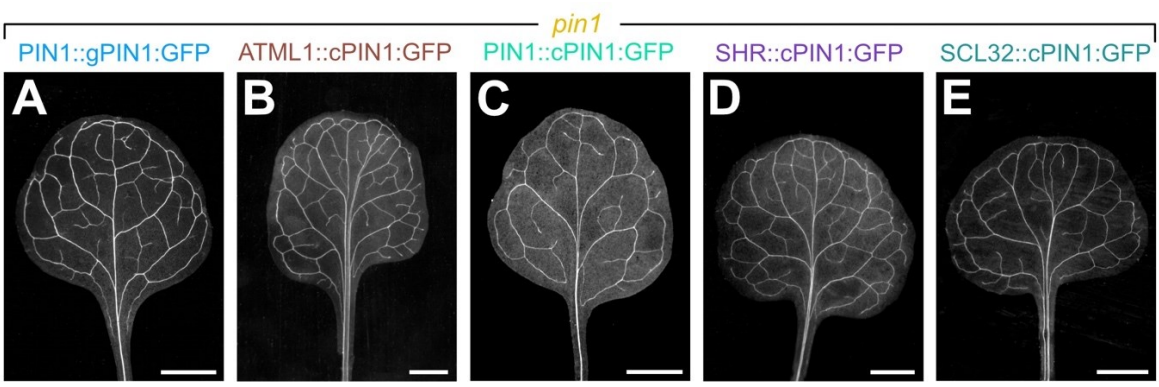

Figure 2.3. Effect of Tissue-Specific PIN1 Expression on *pin1* Vein Patterns. (A-E) Dark-field illumination of mature first leaves. Scale bars: (A-E) 2 mm.

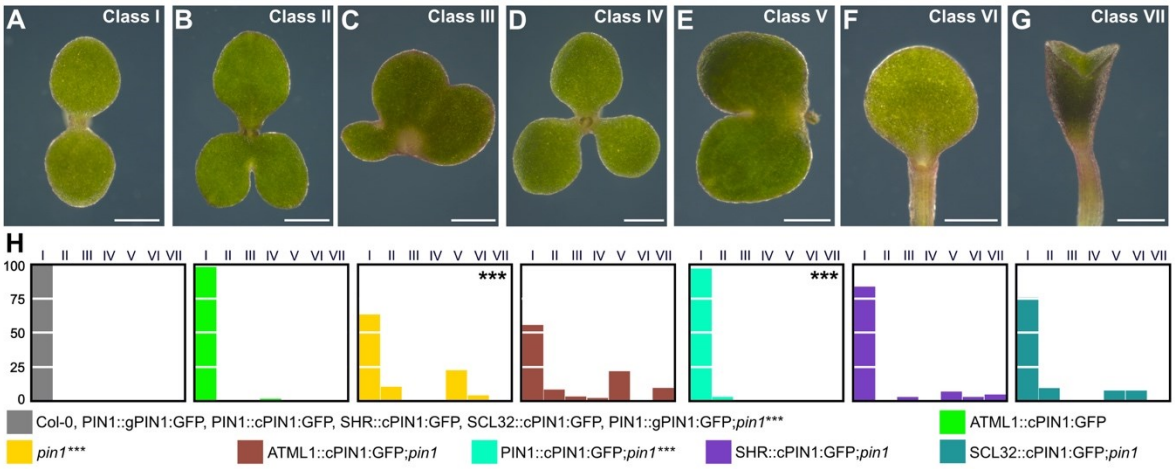

Figure 2.4. Tissue-Specific PIN1 Expression in *PIN1*-dependent Cotyledon Patterning.

(A–G) Dark-field illumination of 3-day-old seedlings illustrating phenotype classes (top right): class I, two separate cotyledons (A); class II, two fused cotyledons and separate single cotyledon (B); class III, three fused cotyledons (C); class IV, three separate cotyledons (D); class V, two fused cotyledons (E); class VI, single cotyledon (F); class VII, cup-shaped cotyledon, side view (G). (H) Percentages of cotyledons in phenotype classes. Difference between *pin1* and WT, between PIN1::gPIN1:GFP;*pin1* and *pin1*, and between PIN1::cPIN1:GFP;*pin1* and *pin1* was significant at $P<0.001$ (***) by Kruskal-Wallis and Mann-Whitney test with Bonferroni correction. Sample population sizes: WT, 99; *pin1*, 50; PIN1::gPIN1:GFP, 110; ATML1::cPIN1:GFP, 113; PIN1::cPIN1:GFP, 115; SHR::cPIN1:GFP, 63; SCL32::cPIN1:GFP, 103; PIN1::gPIN1:GFP;*pin1*, 111; ATML1::cPIN1:GFP;*pin1*, 183; PIN1::cPIN1:GFP;*pin1*, 47; SHR::cPIN1:GFP;*pin1*, 45; SCL32::cPIN1:GFP;*pin1*, 54. Scale bars: (A–G) 0.5 mm.

that *PIN1* expression in the epidermis is dispensable and that *PIN1* expression in the inner tissues of the leaf is sufficient for *PIN1*-dependent vein patterning, we first asked what the expression were of PIN3, PIN4, and PIN7 during vein patterning. To address this question we imaged expression of PIN3::gPIN3:YFP, PIN4::gPIN4:YFP, and PIN7::gPIN7:YFP in first leaves 2, 2.5, 3, and 4 DAG.

2.2.3.1 PIN3 Expression

At 2 DAG, PIN3::gPIN3:YFP was expressed in the abaxial epidermis, though more strongly near its top, and in inner cells on the abaxial side of the primordium, mainly at its bottom (Fig. 2.5A). At 2.5 DAG, PIN3::gPIN3:YFP was expressed in the marginal epidermis, though more strongly near its top (Fig. 2.5B). Inner expression was restricted to the top and bottom of the midvein and to and around the top of the first loops. At 3 DAG, PIN3::gPIN3:YFP expression persisted in the marginal epidermis, but strong expression had spread toward the bottom of the primordium (Fig. 2.5C). Inner expression had spread to the whole midvein but was stronger at its top and bottom; inner expression had also spread toward the bottom of the primordium but was stronger in and around the first loops. At 4 DAG, PIN3::gPIN3:YFP expression continued to persist in the marginal epidermis, but strong expression had spread to the whole lamina (Fig. 2.5D). Inner expression persisted in the midvein and remained stronger at its top and bottom; furthermore, inner expression had spread to the entire lamina but was stronger in and around loops and minor veins.

2.2.3.2 PIN4 Expression

At 2 DAG, PIN4::gPIN4:YFP was expressed in both the adaxial and abaxial epidermis, though more strongly at the top of the primordium (Fig. 2.5E). Inner expression was restricted to the bottom of the midvein and to very few cells scattered across the primordium. At 2.5 DAG, PIN4::gPIN4:YFP was expressed in the marginal epidermis, though more strongly at its top (Fig. 2.5F). Inner expression persisted at the bottom of the midvein and in very few cells scattered across the primordium. At 3 DAG, PIN4::gPIN4:YFP expression persisted in the marginal epidermis, though expression was stronger at its top and bottom (Fig. 2.5G). Inner expression had spread to the whole midvein and to small groups of cells scattered across the primordium. At 4 DAG, PIN4::gPIN4:YFP continued to be expressed

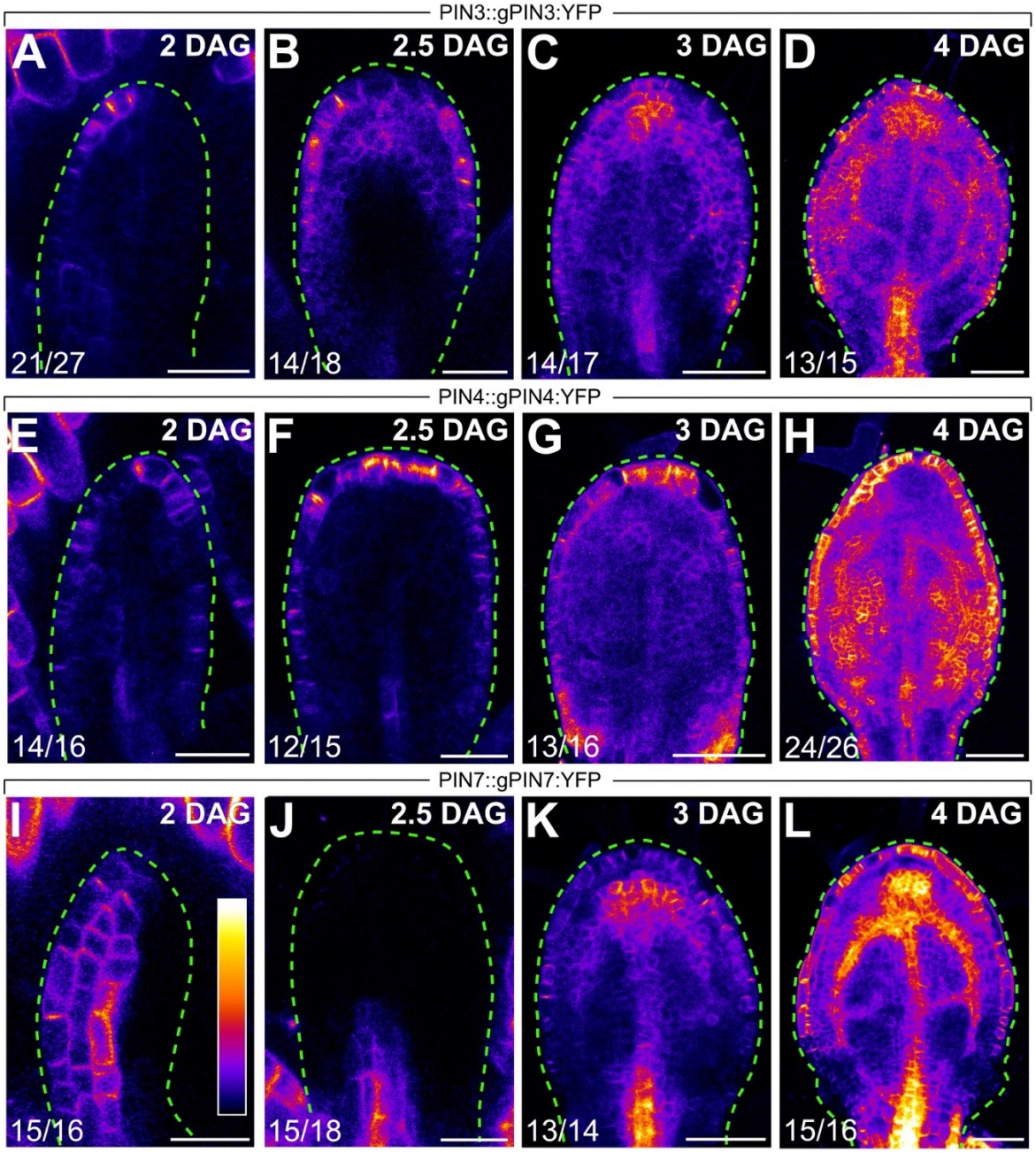

Figure 2.5. Expression of PIN3, PIN4, and PIN7 During Vein Patterning. (A–L) Confocal laser scanning microscopy. Top right: leaf age in DAG; bottom left: reproducibility index, i.e. no. of leaves with the displayed expression / no. of leaves analyzed. Look-up table — ramp in (I) — visualizes expression levels. Abaxial side to the left in (A,E,I). Scale bars: (A,B,E,F,I,J) 30 µm; (C,D,G,H,K,L) 60 µm.

in the marginal epidermis, but expression had become more homogeneous (Fig. 2.5H). Inner expression in the midvein and had spread to and around loops and larger groups of cells scattered across the lamina.

2.2.3.3 PIN7 Expression

At 2 DAG, PIN7::gPIN7:YFP was expressed in the abaxial epidermis and in inner cells on the abaxial side of the primordium (Fig. 2.5I). At 2.5 DAG, PIN7::gPIN7:YFP was expressed at the bottom of the midvein (Fig. 2.5J). At 3 DAG, PIN7::gPIN7:YFP became expressed in the marginal epidermis, though expression was stronger near the top of the primordium (Fig. 2.5K). Inner expression had spread to the whole midvein but was stronger at its top and bottom; inner expression had also spread to and around the first loops, though expression was stronger at their top. At 4 DAG, PIN7::gPIN7:YFP expression had spread to the whole marginal epidermis but was weaker at its bottom (Fig. 2.5L). Inner expression persisted in the midvein and remained stronger at its top and bottom; furthermore, inner expression had spread to the whole lamina, though expression was stronger in and around loops and minor veins.

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In conclusion, during vein patterning PIN3, PIN4, and PIN7 are collectively expressed in the epidermis, in developing veins, and — more weakly — in the nonvascular inner tissue of the leaf.

2.2.4 Tissue-Specific PIN1 Expression in *PIN1* Redundant Functions in Vein Patterning

Collectively, *PIN3*, *PIN4*, and *PIN7* act redundantly with *PIN1* in *PIN1*-dependent vein patterning (Verna et al., 2019), and they are expressed in the leaf epidermis and inner tissues during vein patterning (Fig. 2.5). Therefore, to test the possibility that compensatory functions provided by *PIN3*, *PIN4*, and *PIN7* may account for the observation that PIN1 expression in the epidermis is dispensable and that PIN1 expression in the inner tissues of the leaf is sufficient for *PIN1*-dependent vein patterning, we next expressed in the *pin3*;*pin4*;*pin7* (*pin3*;*4*;*7* hereafter) and *pin1*,*3*;*4*;*7* mutant backgrounds

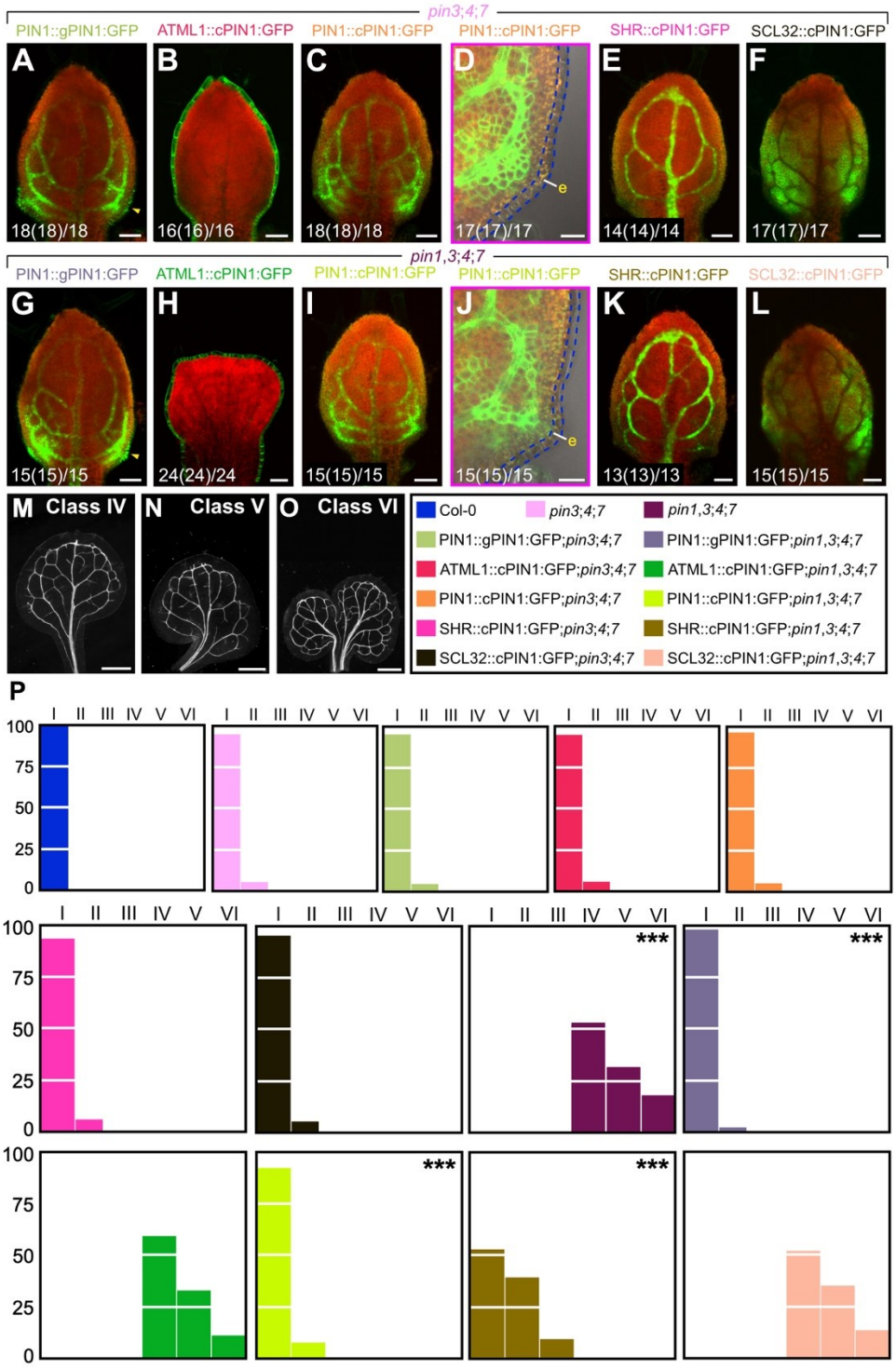

Figure 2.6. Tissue-Specific PIN1 Expression in *PIN1*/*PIN3*/*PIN4*/*PIN7*-dependent Vein Patterning. (A–L). Confocal laser scanning microscopy with (D,J) or without (A–C,E–I,K,L) transmitted light; first leaves 4 DAG. Green, GFP expression; red, autofluorescence.

Yellow arrowheads in (A,G) point to epidermal expression. Bottom left: reproducibility index, i.e. no. of leaves with the displayed inner-tissue expression (no. of leaves with the displayed epidermal expression) / no. of leaves analyzed. (M–O) Dark-field illumination of mature first leaves illustrating phenotype classes (top right): class IV, I-shaped midvein and thick veins (M); class V, Y-shaped midvein and thick veins (N); class VI, fused leaves with thick veins (O). (P) Percentages of leaves in phenotype classes. Difference between *pin1*,*3*;*4*;*7* and WT, between PIN1::gPIN1:GFP;*pin1*,*3*;*4*;*7* and *pin1*,*3*;*4*;*7*, between PIN1::cPIN1:GFP;*pin1*,*3*;*4*;*7* and *pin1*,*3*;*4*;*7*, between SHR::cPIN1:GFP;*pin1*,*3*;*4*;*7* and *pin3*;*4*;*7*, and between SHR::cPIN1:GFP;*pin1*,*3*;*4*;*7* and *pin1*,*3*;*4*;*7* was significant at $P<0.001$ (***) by Kruskal-Wallis and Mann-Whitney test with Bonferroni correction. Sample population sizes: WT, 48; *pin3*;*4*;*7*, 45; *pin1*,*3*;*4*;*7*, 70; PIN1::gPIN1:GFP;*pin3*;*4*;*7*, 60; ATML1::cPIN1:GFP;*pin3*;*4*;*7*, 37; PIN1::cPIN1:GFP;*pin3*;*4*;*7*, 28; SHR::cPIN1:GFP;*pin3*;*4*;*7*, 50; SCL32::cPIN1:GFP;*pin3*;*4*;*7*, 38; PIN1::gPIN1:GFP;*pin1*,*3*;*4*;*7*, 45; ATML1::cPIN1:GFP;*pin1*,*3*;*4*;*7*, 57; PIN1::cPIN1:GFP;*pin1*,*3*;*4*;*7*, 53; SHR::cPIN1:GFP;*pin1*,*3*;*4*;*7*, 62; SCL32::cPIN1:GFP;*pin1*,*3*;*4*;*7*, 69. e, epidermis. Scale bars: (A–C,E–I,K,L) 60 μm; (D,J) 20 μm; (M,N,O) 0.75 mm.

(1) PIN1::gPIN1:GFP, which is expressed in all the tissues of the developing leaf (Fig. 2.6A,G);
(2) ATML1::cPIN1:GFP, which is only expressed in the epidermis (Fig. 2.6B,H);
(3) PIN1::cPIN1:GFP, which is expressed in the leaf inner tissues (Fig. 2.6C,D,I,J);
(4) SHR::cPIN1:GFP, which is only expressed in the vascular tissue (Fig. 2.6E,K);
(5) SCL32::cPIN1:GFP, which is expressed in the nonvascular inner tissue of the leaf (Fig. 2.6F,L).

We then compared vein patterns of mature first leaves of the resulting backgrounds.

As previously shown (Verna et al., 2019), the vein pattern of *pin3*;*4*;*7* was no different from that of WT, and none of the *pin1*,*3*;*4*;*7* leaves had a WT vein pattern (Fig. 2.6M–P). The vein patterns of PIN1::gPIN1:GFP;*pin3*;*4*;*7*, ATML1::cPIN1:GFP;*pin3*;*4*;*7*, PIN1::cPIN1:GFP;*pin3*;*4*;*7*, SHR::cPIN1:GFP;*pin3*;*4*;*7*, and SCL32::cPIN1:GFP;*pin3*;*4*;*7* were no different from the WT vein pattern (Fig. 2.6M–P). Both PIN1::gPIN1:GFP and PIN1::cPIN1:GFP normalized the phenotype spectrum of *pin1*,*3*;*4*;*7* vein patterns (Fig. 2.6M–P; Fig. 2.7A,C). SHR::cPIN1:GFP shifted the phenotype spectrum of *pin1*,*3*;*4*;*7* vein patterns toward the WT vein network pattern, to match the phenotype spectrum of *pin1* vein patterns (Fig. 2.6M–P; Fig. 2.7D; cf. Fig. 2.2M–P). The vein pattern defects of ATML1::cPIN1:GFP;*pin1*,*3*;*4*;*7* and SCL32::cPIN1:GFP;*pin3*;*4*;*7* were no different from those of *pin1*,*3*;*4*;*7* (Fig. 2.6M–P; Fig. 2.7B,E). We observed a similar effect of tissue-specific *PIN1* expression on that component of cotyledon patterning that depends on *PIN1*, *PIN3*, *PIN4*, and *PIN7* (Fig. 2.8).

Therefore, that PIN1 expression in the epidermis is dispensable and that PIN1 expression in the inner tissues of the leaf is sufficient for *PIN1*-dependent vein patterning cannot be accounted for by compensatory functions provided by *PIN3*, *PIN4*, and *PIN7*. Such compensatory functions are also unlikely provided by the remaining PIN proteins, by the ABCB1 and ABCB19 auxin efflux carriers, or by the AUX1/LAX auxin influx carriers because none of these proteins are either expressed in the epidermis or have functions in vein patterning, whether in WT or in auxin-transport-inhibited leaves (Sawchuk et al., 2013; Verna et al., 2015; Verna et al., 2019). As such, we conclude that

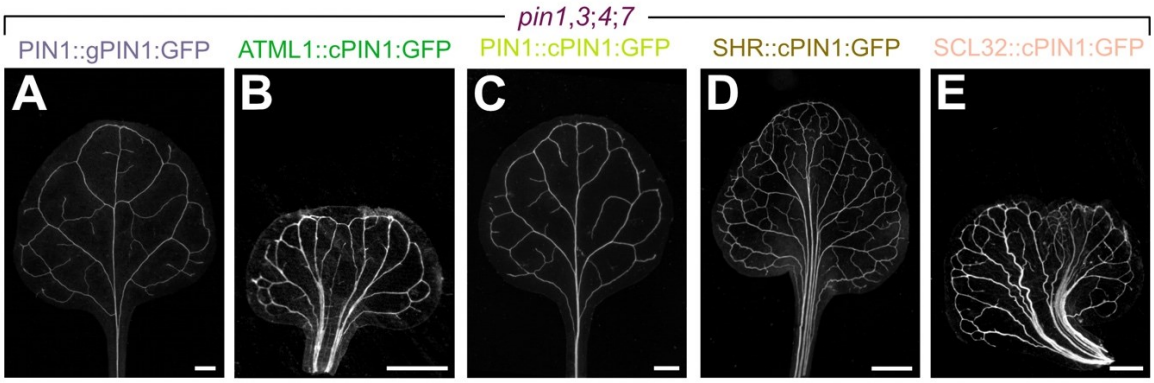

Figure 2.7. Effect of Tissue-Specific PIN1 Expression on *pin1*,*3*;*4*;*7* Vein Patterns. (A-E) Dark-field illumination of mature first leaves. Scale bars: (A,C,D) 2 mm; (B,E) 1 mm.

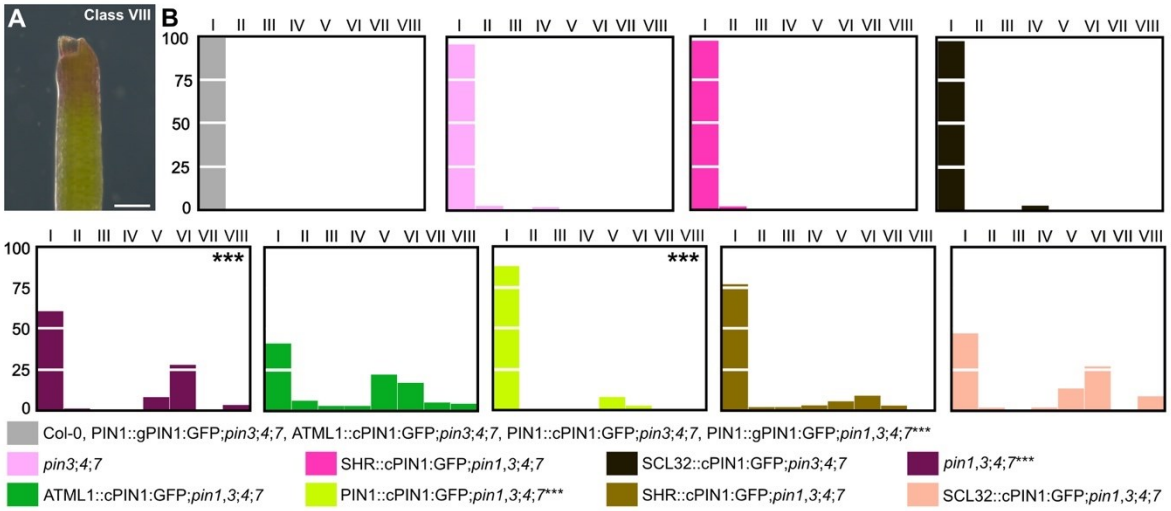

Figure 2.8. Tissue-Specific PIN1 Expression in *PIN1/PIN3/PIN4/PIN7*-dependent Cotyledon Patterning. (A) Dark-field illumination of 3-day-old seedlings illustrating phenotype class VIII (top right) — small, hood-like outgrowth (side view). (H) Percentages of cotyledons in phenotype classes (classes I–VII defined in Figure S1). Difference between *pin1*,*3*;*4*;*7* and WT, between PIN1::gPIN1:PIN1;*pin1*,*3*;*4*;*7* and *pin1*,*3*;*4*;*7*, and between PIN1::cPIN1:PIN1;*pin1*,*3*;*4*;*7* and *pin1*,*3*;*4*;*7* was significant at $P<0.001$ (***) by Kruskal-Wallis and Mann-Whitney test with Bonferroni correction. Sample population sizes: WT, 102; *pin3*;*4*;*7*, 51; *pin1*,*3*;*4*;*7*, 130; PIN1::gPIN1:GFP;*pin3*;*4*;*7*, 65; ATML1::cPIN1:GFP;*pin3*;*4*;*7*, 108; PIN1::cPIN1:GFP;*pin3*;*4*;*7*, 107; SHR::cPIN1:GFP;*pin3*;*4*;*7*, 71; SCL32::cPIN1:GFP;*pin3*;*4*;*7*, 49; PIN1::gPIN1:GFP;*pin1*,*3*;*4*;*7*, 42; ATML1::cPIN1:GFP;*pin1*,*3*;*4*;*7*, 83; PIN1::cPIN1:GFP;*pin1*,*3*;*4*;*7*, 85; SHR::cPIN1:GFP;*pin1*,*3*;*4*;*7*, 60; SCL32::cPIN1:GFP;*pin1*,*3*;*4*;*7*, 49. Scale bar: (A) 0.25 mm.

PIN1 expression in the epidermis is dispensable for auxin-transport-dependent vein patterning. This conclusion is consistent with the observation that *cup-shaped cotyledon2* mutants lack convergent points of epidermal PIN1 polarity and yet have normal vein patterns (Bilsborough et al., 2011). By contrast, PIN1 expression in inner tissues is required and sufficient for auxin-transport-dependent vein patterning; such function of PIN1 expression mainly depends on PIN1 expression in the vascular tissue.

In conclusion, vein patterning hypotheses based on polar auxin transport from the epidermis (reviewed in Prusinkiewicz and Runions, 2012; Runions et al., 2014; see also Alim and Frey, 2010; Hartmann et al., 2019, and references therein) are unsupported by experimental evidence. Our results do not rule out an influence of the epidermis on vein patterning, for example through local auxin production (e.g., Abley et al., 2016), but they do exclude that such influence is brought about by polar auxin transport. Alternatively, patterning of local epidermal features, such as peaks of auxin production or response, and of the processes that depend on those features may be mediated by auxin transport in underlying tissues; there is evidence for such possibility (e.g., Deb et al., 2015), and our results are consistent with that evidence. In the future, it will be interesting to test these and other possibilities, but already now our results refute all the vein patterning hypotheses that depend on polar auxin transport from the epidermis.

2.3 Materials & Methods

2.3.1 Notation

In agreement with (Crittenden et al., 1996), linked genes or mutations (<2,500 kb apart, which in Arabidopsis on an average corresponds to ~10 cM (Lukowitz et al., 2000)) are separated by a comma, and unlinked genes or mutations are separated by a semicolon.

2.3.2 Plants

Origin and nature of lines, genotyping strategies, and oligonucleotide sequences are in Tables 2.1, 2.2, and 2.3, respectively. Seeds were sterilized and sown as in (Sawchuk et al., 2008). Stratified seeds were germinated and seedlings were grown at 22°C under continuous

Table 2.1. Origin and Nature of Lines.

Line	Origin/Nature
PIN1::gPIN1:YFP	(Xu et al., 2006)
PIN1::nYFP	Transcriptional fusion of *PIN1* (AT1G73590; -4,171 to -1; primers: "PIN1 transc 4171 forw" and "PIN1 transc rev") to HTA6:EYFP (Zhang et al., 2005)
PIN1::gPIN1:CFP	(Gordon et al., 2007)
pin1-051	NASC; GK-051A10-012139 (Kleinboelting et al., 2012); contains a T-DNA insertion after +2234 of *PIN1*
PIN1::gPIN1:GFP	Xu et al. 2006
ATML1::cPIN1:GFP	Transcriptional fusion of *ATML1* (AT4G21750; -5,016 to -1,597; primers "XhoI ATML1 p F" and "BamHI ATML1p R") to translational fusion of *PIN1* cDNA (GenBank accession no. AY093960; ABRC clone no. U12338; primers "BamHI PIN1 cDNA F" and "KpnI PIN1 cDNA R") to EGFP (Clontech; insertion after +651 of *PIN1*; primers "XhoI GFP no ATG Fwd" and "XhoI GFP no* Rev")
PIN1::cPIN1:GFP	Transcriptional fusion of *PIN1* (-4,168 to -14; primers "XhoI full length PIN1p F" and "BamHI PIN1p rev") to translational fusion of *PIN1* cDNA (GenBank accession no. AY093960; ABRC clone no. U12338; primers "BamHI PIN1 cDNA F" and "KpnI PIN1 cDNA R") to EGFP (Clontech; insertion after +651 of *PIN1*; primers "XhoI GFP no ATG Fwd" and "XhoI GFP no* Rev")
SHR::cPIN1:GFP	Transcriptional fusion of *SHR* (AT4G37650; -2505 to -16; primers "SHR prom SalI Forw2" and "SHR prom BamHI Rev") to translational fusion of *PIN1* cDNA (GenBank accession no. AY093960; ABRC clone no. U12338; primers "BamHI PIN1 cDNA F" and "KpnI PIN1 cDNA R") to

	EGFP (Clontech; insertion after +651 of *PIN1*; primers "XhoI GFP no ATG Fwd" and "XhoI GFP no* Rev")
SCL32::cPIN1:GFP	Transcriptional fusion of *SCL32* (AT3G49950; -2888 to -2; primers "SCL32 Translational FWD" and "SCL32 prom BamHI Rev") to translational fusion of *PIN1* cDNA (GenBank accession no. AY093960; ABRC clone no. U12338; primers "BamHI PIN1 cDNA F" and "KpnI PIN1 cDNA R") to EGFP (Clontech; insertion after +651 of *PIN1*; primers "XhoI GFP no ATG Fwd" and "XhoI GFP no* Rev")
PIN3::gPIN3:YFP	ABRC; (Zhou et al., 2011)
PIN4::gPIN4:YFP	ABRC; (Zhou et al., 2011)
PIN7::gPIN7:YFP	ABRC; (Zhou et al., 2011)
pin1-1	ABRC; WT at the *TTG1* (AT5G24520) locus (Galweiler et al., 1998; Goto N, 1987; Sawchuk et al., 2013)
pin3-3	(Friml et al., 2002b)
pin4-2	(Friml et al., 2002a)
$pin7^{En}$	(Blilou et al., 2005)

Table 2.2. Genotyping Strategies.

Line	Strategy
pin1-051	*PIN1*: "pin1 GK LP" and "pin1 GK RP"; *pin1:* "pin1 GK RP" and "o8409"
pin1-1	"pin1-1 F" and "pin1-1 R"; *Tat*I
pin3-3	"pin3-3 F" and "pin3-3 R"; *Sty*I
pin4-2	*PIN4*: "PIN4 forw geno II" and "PIN4en rev Ikram"; *pin4*: "PIN4en rev Ikram" and "en primer"
$pin7^{En}$	*PIN7*: "PIN7en forw Ikram" and "PIN7en rev"; *pin7*: "PIN7en rev Ikram II" and "en primer"

Table 2.3. Oligonucleotide Sequences.

Name	Sequence (5' to 3')
PIN1 transc 4171 forw	GGGGACAAGTTTGTACAAAAAAGCAGGCTATGATCCGATTGGATTCG
PIN1 transc rev	GGGGACCACTTTGTACAAGAAAGCTGGGTCTTTTGTTCGCCGGAGAAG
pin GK LP	ACTCTTTGGCAAACACAAACG
pin1 GK RP	CTCTCAGATGCAGGTCTAGGC
o8409	ATATTGACCATCATACTCATTGC
XhoI ATML1 p F	GCCCTCGAGTTTACATTGATTCTGAACTG
BamHI ATML1p R	GATGGATCCTAACCGGTGGATTCAGGGAG
BamHI PIN1 cDNA F new	TTAGGATCCATGATTACGGCGGCGGACTTC
KpnI PIN1 cDNA R	CTCGGTACCTCATAGACCCAAGAGAATGTAG
XhoI GFP no ATG Fwd	TTACTCGAGAGTGAGCAAGGGCGAGGAGCTGTT
XhoI GFP no* Rev	TATCTCGAGTACTTGTACAGCTCGTCCATGCCGAG
XhoI full length PIN1p F	TGTCTCGAGATCCGATTGGATTCGGTCTG
BamHI PIN1p rev	AAGGGATCCGAGAAGAGAGAGGGAAGAGAG
SHR prom SalI Forw2	AAAGTCGACCGAAGAAAGGGACAAAGAAGC
SHR prom BamHI Rev	TGGGGATCCTTAATGAATAAGAAAATGAATAGAAGAAAGGG
SCL32 Translational FWD	AGAGTCGACATCTTAGTAGAAATAAGCGAAC
SCL32 prom BamHI Rev	ACTGGATCCGAGTCTGGTTTTAGAGAGAAATG
pin1-1 F	ATGATTACGGCGGCGGACTTCTA
pin1-1 R	TTCCGACCACCACCAGAAGCC

pin3-3 F	GGAGCTCAAACGGGTCACCCG
pin3-3 R	GCTGGATGAGCTACAGCTATATTC
PIN4 forw geno II	GTCCGACTCCACGGCCTTC
PIN4en rev Ikram	ATCTTCTTCTTCACCTTCCACTCT
en primer	GAGCGTCGGTCCCCACACTTCTATAC
PIN7en forw Ikram	CCTAACGGTTTCCACACTCA
PIN7en rev	TAGCTCTTTAGGGTTTAGCTC
PIN7en rev Ikram II	GGTTTAGCTCTGCTGTGGAGTT

fluorescent light (~80 µmol $m^{-2}s^{-1}$). Plants were grown at 25°C under fluorescent light (~100 µmol $m^{-2}s^{-1}$) in a 16-h-light/8-h-dark cycle. Plants were transformed and representative lines were selected as in (Sawchuk et al., 2008).

2.3.3 Imaging

Developing leaves were mounted and YFP was imaged as in (Sawchuk et al., 2013). CFP, YFP, and autofluorescence were imaged as in (Sawchuk et al., 2013). GFP and autofluorescence were imaged as in (Amalraj et al., 2019). Images were stacked, aligned with the Scale Invariant Feature Transform algorithm (Lowe, 2004), and maximum-intensity projection was applied to aligned image stacks in the Fiji distribution (Schindelin et al., 2012) of ImageJ (Rueden et al., 2017; Schindelin et al., 2015; Schneider et al., 2012). Mature leaves were fixed in ethanol : acetic acid 6 : 1, rehydrated in 70% ethanol and water, and mounted in chloral hydrate : glycerol : water 8 : 2 : 1. Mounted leaves were imaged as in (Odat et al., 2014). Greyscaled RGB color images were turned into 8-bit images, and image brightness and contrast were adjusted by linear stretching of the histogram in the Fiji distribution of ImageJ.

Chapter 3: Transcriptional Control of *PIN1*-Dependent Vein Patterning

3.1 Introduction

Most multicellular organisms solve the problem of long-distant transport of signals and nutrient by tissue networks such as the vascular networks of vertebrate embryos and plant leaves; how vascular networks form is therefore a key question in biology. In vertebrates, vascular networks formation relies on direct cell-cell interaction and often cell migration (e.g., Noden, 1988; Xue et al., 1999), both of which are precluded in plants by a wall that keeps cells apart and in place; therefore, vascular networks form differently in plant leaves.

How leaf vascular networks form is unclear but available evidence suggest that vein patterning depends on the polarity of transport of the plant signal auxin across leaf tissues (Mattsson et al., 1999; Sieburth, 1999). Polarity of auxin transport across leaf tissues in turn depends on the polar localization of PIN-FORMED (PIN) auxin transporters at the plasma membrane of auxin-transporting cells (Petrasek et al., 2006; Wisniewska et al., 2006). Of the eight *PIN* genes in Arabidopsis, *PIN1* is the only one with non-redundant functions in vein patterning (Sawchuk et al., 2013). In epidermal cells of the leaf margin, PIN1 polarity is directed toward local peaks of auxin level (Benkova et al., 2003; Reinhardt et al., 2003; Heisler et al., 2005; Hay et al., 2006; Scarpella et al., 2006; Wenzel et al., 2007; Bayer et al., 2009). These "convergence points" of epidermal PIN1 polarity are associated with broad domains of PIN1 expression in the inner tissue of the leaf. Over time, these broad domains of PIN1 expression become restricted to sites of vein formation.

The correlation between convergence points of epidermal PIN1 polarity and sites of vein formation has suggested that auxin is transported by PIN1 from the epidermal convergence points into the inner tissue of the leaf where it will lead to vein formation (reviewed in (Prusinkiewicz and Runions, 2012; Runions et al., 2014); see also (Alim and Frey, 2010; Hartmann et al., 2019), and references therein). However, all the vein patterning hypotheses that depend on auxin transport from the epidermis are refuted by the observation that epidermal PIN1 expression is neither necessary nor sufficient for *PIN1*-dependent vein

patterning; instead, it turns out that PIN1 expression in the inner tissues of the leaf is both necessary and sufficient for *PIN1*-dependent vein patterning (Chapter 2). Because PIN1 expression in the inner tissues of the leaf is controlled by the activity of the *PIN1* promoter (Chapter 2), to identify regulatory inputs upstream of *PIN1*-dependent vein patterning, we sought regulatory elements that are necessary for that component of PIN1 expression in the inner tissues of the leaf that is required for *PIN1*-dependent vein patterning. We found that vascular expression of PIN1 is necessary for *PIN1*-dependent vein patterning and that that expression of PIN1 depends on a 151-bp region of the *PIN1* promoter.

3.2 Results and Discussion

3.2.1 Transcriptional Control of *PIN1* Function in Vein Patterning

We first asked what cis-regulatory elements were required for *PIN1* function in vein patterning. To address this question, we deleted increasingly longer regions from the 5'-end of the 4,168-bp *PIN1* promoter avoiding the disruption of putative transcription-factor binding-sites identified by bioinformatics tools (see Materials & Methods, Table 3.1) (Fig. 3.1A); used the resulting 20 *PIN1* promoter fragments (Fig. 3.1A) — collectively referred to as *ΔPIN1* hereafter — to drive expression of a fusion of the *PIN1* coding sequence to GFP (cPIN1:GFP) in WT and *pin1* mutant backgrounds; and compared vein patterns of mature first leaves of WT, *pin1*, PIN1::cPIN1:GFP, ΔPIN1::cPIN1:GFP, PIN1::cPIN1:GFP;*pin1*, and ΔPIN1::cPIN1:GFP;*pin1*.

Consistent with previous reports (Sawchuk et al., 2013; Verna et al., 2019) and as previously shown (Chapter 2), the vein patterns of nearly 50% of *pin1* leaves were abnormal (Fig. 3.1B). As also shown previously (Chapter 2), the vein pattern of PIN1::cPIN1:GFP was no different from that of WT (Fig. 3.1B). The vein patterns of ΔPIN1::cPIN1:GFP were also no different from the vein pattern of WT (Fig. 3.1B). As previously shown (Chapter 2), PIN1::cPIN1:GFP shifted the phenotype spectrum of the vein patterns of *pin1* toward the WT vein pattern (Fig. 3.1B). Also [-3,750,-14]::cPIN1:GFP, [-3,377,-14]::cPIN1:GFP, [-2,747,-14]::cPIN1:GFP, [-2,320,-14]::cPIN1:GFP, [-1,893,-14]::cPIN1:GFP, [-1,725,-14]::cPIN1:GFP,

Table 3.1. Bioinformatic Analysis Result of the *PIN1* promoter.

Factor or Site Name	Location	Strand	Signal Sequence
PRECONSCRHSP70A	-4165	+	SCGAYNRNNNNNNNNNNNNNNHD
ARR1AT	-4164	+	NGATT
CAATBOX1	-4162	-	CAAT
CCAATBOX1	-4162	-	CCAAT
RBCSCONSENSUS	-4161	-	AATCCAA
ARR1AT	-4159	+	NGATT
TATABOX5	-4144	+	TTATTT
-300ELEMENT	-4142	-	TGHAAARK
GT1CONSENSUS	-4141	-	GRWAAW
GT1GMSCAM4	-4141	-	GAAAAA
GTGANTG10	-4137	-	GTGA
TATABOX5	-4133	-	TTATTT
POLASIG1	-4132	+	AATAAA
EBOXBNNAPA	-4126	-	CANNTG
MYCCONSENSUSAT	-4126	-	CANNTG
EBOXBNNAPA	-4126	+	CANNTG
MYCCONSENSUSAT	-4126	+	CANNTG
ERELEE4	-4124	-	AWTTCAAA
ROOTMOTIFTAPOX1	-4119	-	ATATT
ROOTMOTIFTAPOX1	-4118	+	ATATT
GATABOX	-4101	+	GATA
ROOTMOTIFTAPOX1	-4100	+	ATATT
CAATBOX1	-4098	-	CAAT
CACTFTPPCA1	-4093	-	YACT
GTGANTG10	-4078	+	GTGA
MYB1AT	-4075	+	WAACCA
CAATBOX1	-4070	-	CAAT

CCAATBOX1	-4070	-	CCAAT
SEF3MOTIFGM	-4068	-	AACCCA
GCCCORE	-4049	-	GCCGCC
RHERPATEXPA7	-4045	+	KCACGW
CACTFTPPCA1	-4034	-	YACT
GTGANTG10	-4033	+	GTGA
ARR1AT	-4032	+	NGATT
CAATBOX1	-4030	-	CAAT
ARR1AT	-4010	-	NGATT
RHERPATEXPA7	-4008	-	KCACGW
GTGANTG10	-4006	+	GTGA
DOFCOREZM	-3998	+	AAAG
NODCON1GM	-3998	+	AAAGAT
OSE1ROOTNODULE	-3998	+	AAAGAT
EVENINGAT	-3996	-	AAAATATCT
GATABOX	-3995	+	GATA
LECPLEACS2	-3994	-	TAAAATAT
ROOTMOTIFTAPOX1	-3994	+	ATATT
CACTFTPPCA1	-3988	+	YACT
DOFCOREZM	-3986	-	AAAG
P1BS	-3981	-	GNATATNC
P1BS	-3981	+	GNATATNC
ROOTMOTIFTAPOX1	-3979	+	ATATT
-10PEHVPSBD	-3978	+	TATTCT
DOFCOREZM	-3974	-	AAAG
POLASIG1	-3972	-	AATAAA
MARTBOX	-3971	+	TTWTWTTWTT
TATABOX5	-3971	+	TTATTT
MARTBOX	-3968	+	TTWTWTTWTT
POLASIG1	-3967	-	AATAAA
TATABOX5	-3966	+	TTATTT

GT1CONSENSUS	-3963	-	GRWAAW
GT1GMSCAM4	-3963	-	GAAAAA
POLLEN1LELAT52	-3961	-	AGAAA
NODCON2GM	-3958	+	CTCTT
OSE1ROOTNODULE	-3958	+	CTCTT
DOFCOREZM	-3956	-	AAAG
REALPHALGLHCB21	-3953	-	AACCAA
MYB1AT	-3952	-	WAACCA
GT1CONSENSUS	-3949	-	GRWAAW
GT1GMSCAM4	-3949	-	GAAAAA
GT1CONSENSUS	-3948	-	GRWAAW
PYRIMIDINEBOXOSRAMY1A	-3944	+	CCTTTT
DOFCOREZM	-3943	-	AAAG
INRNTPSADB	-3935	-	YTCANTYY
CACTFTPPCA1	-3933	-	YACT
GTGANTG10	-3932	+	GTGA
POLLEN1LELAT52	-3928	+	AGAAA
GT1CONSENSUS	-3927	+	GRWAAW
INRNTPSADB	-3926	-	YTCANTYY
-300ELEMENT	-3922	+	TGHAAARK
GT1CONSENSUS	-3921	+	GRWAAW
GT1GMSCAM4	-3921	+	GAAAAA
DOFCOREZM	-3918	+	AAAG
POLLEN1LELAT52	-3916	+	AGAAA
DOFCOREZM	-3914	+	AAAG
NODCON2GM	-3913	-	CTCTT
OSE2ROOTNODULE	-3913	-	CTCTT
-300ELEMENT	-3907	+	TGHAAARK
GT1CONSENSUS	-3906	+	GRWAAW
GT1GMSCAM4	-3906	+	GAAAAA
DOFCOREZM	-3903	+	AAAG

NODCON1GM	-3903	+	AAAGAT
OSE1ROOTNODULE	-3903	+	AAAGAT
GATABOX	-3900	+	GATA
IBOXCORE	-3900	+	GATAA
NTBBF1ARROLB	-3896	+	ACTTTA
DOFCOREZM	-3895	-	AAAG
TAAAGSTKST1	-3895	-	TAAAG
POLASIG1	-3894	-	AATAAA
CAATBOX1	-3891	-	CAAT
CAATBOX1	-3884	+	CAAT
ANAERO2CONSENSUS	-3880	+	AGCAGC
DOFCOREZM	-3875	-	AAAG
CACTFTPPCA1	-3841	-	YACT
POLLEN1LELAT52	-3837	+	AGAAA
GT1CONSENSUS	-3836	+	GRWAAW
GT1GMSCAM4	-3836	+	GAAAAA
DOFCOREZM	-3833	+	AAAG
GT1CONSENSUS	-3827	+	GRWAAW
-300ELEMENT	-3823	-	TGHAAARK
GT1CONSENSUS	-3822	-	GRWAAW
GT1GMSCAM4	-3822	-	GAAAAA
PYRIMIDINEBOXOSRAMY1A	-3812	+	CCTTTT
DOFCOREZM	-3811	-	AAAG
SEF1MOTIF	-3809	-	ATATTTAWW
TATABOXOSPAL	-3808	-	TATTTAA
ROOTMOTIFTAPOX1	-3805	-	ATATT
ROOTMOTIFTAPOX1	-3804	+	ATATT
-10PEHVPSBD	-3803	+	TATTCT
BOXIINTPATPB	-3794	-	ATAGAA
POLLEN1LELAT52	-3789	-	AGAAA
BOXIINTPATPB	-3788	-	ATAGAA

ROOTMOTIFTAPOX1	-3782	+	ATATT
-10PEHVPSBD	-3781	+	TATTCT
DOFCOREZM	-3777	-	AAAG
PYRIMIDINEBOXOSRAMY1A	-3762	+	CCTTTT
DOFCOREZM	-3761	-	AAAG
SEF3MOTIFGM	-3752	+	AACCCA
DOFCOREZM	-3731	+	AAAG
NODCON1GM	-3731	+	AAAGAT
OSE1ROOTNODULE	-3731	+	AAAGAT
ARR1AT	-3729	+	NGATT
SEF4MOTIFGM7S	-3722	+	RTTTTTR
ROOTMOTIFTAPOX1	-3712	-	ATATT
DOFCOREZM	-3697	+	AAAG
DOFCOREZM	-3681	-	AAAG
GT1CONSENSUS	-3679	-	GRWAAW
GT1GMSCAM4	-3679	-	GAAAAA
GTGANTG10	-3675	-	GTGA
CAATBOX1	-3671	-	CAAT
ARR1AT	-3669	+	NGATT
POLLEN1LELAT52	-3633	-	AGAAA
DOFCOREZM	-3630	-	AAAG
LTRECOREATCOR15	-3624	-	CCGAC
E2FCONSENSUS	-3621	-	WTTSSCSS
DOFCOREZM	-3613	-	AAAG
CAATBOX1	-3605	-	CAAT
-300ELEMENT	-3603	+	TGHAAARK
DOFCOREZM	-3590	-	AAAG
TAAAGSTKST1	-3590	-	TAAAG
POLASIG1	-3589	-	AATAAA
POLASIG3	-3586	-	AATAAT
CAATBOX1	-3583	-	CAAT

ARR1AT	-3581	+	NGATT
POLLEN1LELAT52	-3574	-	AGAAA
ARR1AT	-3548	-	NGATT
ASF1MOTIFCAMV	-3543	+	TGACG
WRKY71OS	-3543	+	TGAC
DOFCOREZM	-3538	+	AAAG
CACTFTPPCA1	-3536	-	YACT
GATABOX	-3532	+	GATA
GT1CONSENSUS	-3532	+	GRWAAW
IBOXCORE	-3532	+	GATAA
GT1CONSENSUS	-3526	+	GRWAAW
GT1GMSCAM4	-3526	+	GAAAAA
EBOXBNNAPA	-3520	-	CANNTG
MYCCONSENSUSAT	-3520	-	CANNTG
EBOXBNNAPA	-3520	+	CANNTG
MYCCONSENSUSAT	-3520	+	CANNTG
CAREOSREP1	-3515	-	CAACTC
BIHD1OS	-3495	-	TGTCA
WRKY71OS	-3495	+	TGAC
EBOXBNNAPA	-3492	-	CANNTG
DPBFCOREDCDC3	-3492	-	ACACNNG
MYCCONSENSUSAT	-3492	-	CANNTG
EBOXBNNAPA	-3492	+	CANNTG
MYCCONSENSUSAT	-3492	+	CANNTG
CACTFTPPCA1	-3490	-	YACT
BIHD1OS	-3488	+	TGTCA
WRKY71OS	-3487	-	TGAC
GTGANTG10	-3486	-	GTGA
CACTFTPPCA1	-3477	+	YACT
NTBBF1ARROLB	-3476	+	ACTTTA
DOFCOREZM	-3475	-	AAAG

TAAAGSTKST1	-3475	-	TAAAG
POLASIG2	-3474	-	AATTAAA
GT1CONSENSUS	-3470	-	GRWAAW
GT1CONSENSUS	-3469	-	GRWAAW
GT1CONSENSUS	-3460	-	GRWAAW
DOFCOREZM	-3436	-	AAAG
POLLEN1LELAT52	-3415	-	AGAAA
DOFCOREZM	-3401	-	AAAG
POLLEN1LELAT52	-3400	-	AGAAA
CACTFTPPCA1	-3393	+	YACT
DOFCOREZM	-3388	-	AAAG
TAAAGSTKST1	-3388	-	TAAAG
POLASIG1	-3387	-	AATAAA
CAATBOX1	-3384	-	CAAT
POLLEN1LELAT52	-3379	-	AGAAA
GTGANTG10	-3374	-	GTGA
RHERPATEXPA7	-3374	+	KCACGW
ABRELATERD1	-3373	-	ACGTG
T/GBOXATPIN2	-3373	-	AACGTG
ACGTATERD1	-3372	-	ACGT
ACGTATERD1	-3372	+	ACGT
RAV1AAT	-3357	-	CAACA
ARR1AT	-3354	+	NGATT
POLASIG3	-3352	-	AATAAT
TATABOX5	-3351	+	TTATTT
INRNTPSADB	-3347	+	YTCANTYY
GTGANTG10	-3346	-	GTGA
CACTFTPPCA1	-3345	+	YACT
RAV1AAT	-3340	+	CAACA
SEF4MOTIFGM7S	-3329	-	RTTTTTR
TATABOX5	-3326	-	TTATTT

TAAAGSTKST1	-3318	+	TAAAG
DOFCOREZM	-3317	+	AAAG
POLASIG2	-3310	+	AATTAAA
TATABOXOSPAL	-3308	-	TATTTAA
CACTFTPPCA1	-3300	-	YACT
SEF3MOTIFGM	-3296	+	AACCCA
SEF4MOTIFGM7S	-3286	-	RTTTTTR
CIACADIANLELHC	-3280	+	CAANNNNATC
RAV1AAT	-3280	+	CAACA
HDZIP2ATATHB2	-3275	+	TAATMATTA
ARR1AT	-3274	-	NGATT
LTRECOREATCOR15	-3259	-	CCGAC
GATABOX	-3252	+	GATA
GTGANTG10	-3247	+	GTGA
GT1CONSENSUS	-3235	-	GRWAAW
GT1GMSCAM4	-3235	-	GAAAAA
GT1CONSENSUS	-3234	-	GRWAAW
REBETALGLHCB21	-3229	+	CGGATA
P1BS	-3228	-	GNATATNC
MYBST1	-3228	+	GGATA
P1BS	-3228	+	GNATATNC
GATABOX	-3227	+	GATA
GATABOX	-3225	-	GATA
MYBST1	-3225	-	GGATA
ARR1AT	-3216	-	NGATT
CGACGOSAMY3	-3201	-	CGACG
CBFHV	-3200	-	RYCGAC
ARR1AT	-3198	+	NGATT
EBOXBNNAPA	-3176	-	CANNTG
MYCCONSENSUSAT	-3176	-	CANNTG
EBOXBNNAPA	-3176	+	CANNTG

MYCCONSENSUSAT	-3176	+	CANNTG
MYBCORE	-3173	+	CNGTTR
RAV1AAT	-3172	-	CAACA
PYRIMIDINEBOXOSRAMY1A	-3158	-	CCTTTT
DOFCOREZM	-3157	+	AAAG
CURECORECR	-3138	-	GTAC
CURECORECR	-3138	+	GTAC
ARR1AT	-3126	-	NGATT
WBOXHVISO1	-3116	-	TGACT
WBOXNTERF3	-3116	-	TGACY
WRKY71OS	-3115	-	TGAC
MYBCOREATCYCB1	-3107	-	AACGG
CACTFTPPCA1	-3098	-	YACT
ARR1AT	-3094	+	NGATT
POLASIG3	-3092	-	AATAAT
TATABOX5	-3091	+	TTATTT
ANAERO1CONSENSUS	-3088	-	AAACAAA
ARR1AT	-3081	+	NGATT
CAATBOX1	-3075	+	CAAT
DOFCOREZM	-3060	-	AAAG
TAAAGSTKST1	-3060	-	TAAAG
RAV1AAT	-3021	+	CAACA
RYREPEATLEGUMINBOX	-3018	+	CATGCAY
RYREPEATGMGY2	-3018	+	CATGCAT
RYREPEATBNNAPA	-3018	+	CATGCA
POLASIG3	-3018	-	AATAAT
TATABOX5	-3013	+	TTATTT
GT1CONSENSUS	-3012	-	GRWAAW
LTRE1HVBLT49	-3010	-	CCGAAA
GTGANTG10	-3008	+	GTGA
DOFCOREZM	-2997	+	AAAG

CAATBOX1	-2993	-	CAAT
CURECORECR	-2979	-	GTAC
CURECORECR	-2976	+	GTAC
CACTFTPPCA1	-2976	+	YACT
DOFCOREZM	-2975	-	AAAG
POLASIG2	-2969	-	AATTAAA
POLASIG2	-2967	+	AATTAAA
HDZIP2ATATHB2	-2956	+	TAATMATTA
ARR1AT	-2955	-	NGATT
ARR1AT	-2944	+	NGATT
POLLEN1LELAT52	-2941	-	AGAAA
DOFCOREZM	-2938	-	AAAG
POLASIG2	-2936	-	AATTAAA
RYREPEATBNNAPA	-2928	+	CATGCA
EBOXBNNAPA	-2924	-	CANNTG
MYCCONSENSUSAT	-2924	-	CANNTG
MYB2CONSENSUSAT	-2924	-	YAACKG
EBOXBNNAPA	-2924	+	CANNTG
MYBCORE	-2924	+	CNGTTR
MYCCONSENSUSAT	-2924	+	CANNTG
CURECORECR	-2912	-	GTAC
CURECORECR	-2912	+	GTAC
CACTFTPPCA1	-2911	+	YACT
PREATPRODH	-2906	-	ACTCAT
ARR1AT	-2898	+	NGATT
CAATBOX1	-2896	-	CAAT
GATABOX	-2893	+	GATA
IBOXCORE	-2893	+	GATAA
MYB1AT	-2891	+	WAACCA
MYBPLANT	-2890	+	MACCWAMC
REALPHALGLHCB21	-2890	+	AACCAA

POLLEN1LELAT52	-2874	-	AGAAA
DOFCOREZM	-2871	-	AAAG
POLLEN1LELAT52	-2869	-	AGAAA
ARR1AT	-2865	+	NGATT
GT1CONSENSUS	-2855	-	GRWAAW
IBOXCORE	-2854	-	GATAA
SREATMSD	-2853	+	TTATCC
GATABOX	-2853	-	GATA
MYBST1	-2853	-	GGATA
AMYBOX2	-2853	+	TATCCAT
TATCCAYMOTIFOSRAMY3D	-2853	+	TATCCAY
TATCCAOSAMY	-2853	+	TATCCA
EBOXBNNAPA	-2849	-	CANNTG
MYCCONSENSUSAT	-2849	-	CANNTG
EBOXBNNAPA	-2849	+	CANNTG
MYCCONSENSUSAT	-2849	+	CANNTG
POLASIG1	-2838	-	AATAAA
MYBCORE	-2832	-	CNGTTR
POLLEN1LELAT52	-2822	-	AGAAA
CAATBOX1	-2816	-	CAAT
ARR1AT	-2814	+	NGATT
RAV1AAT	-2806	+	CAACA
CAATBOX1	-2803	+	CAAT
WBOXHVISO1	-2798	-	TGACT
WBOXNTERF3	-2798	-	TGACY
WRKY71OS	-2797	-	TGAC
GTGANTG10	-2796	-	GTGA
CCAATBOX1	-2792	+	CCAAT
CAATBOX1	-2791	+	CAAT
WBOXHVISO1	-2788	+	TGACT
WRKY71OS	-2788	+	TGAC

WBOXNTERF3	-2788	+	TGACY
SURECOREATSULTR11	-2781	+	GAGAC
PREATPRODH	-2778	+	ACTCAT
POLASIG3	-2763	+	AATAAT
POLASIG2	-2760	+	AATTAAA
EBOXBNNAPA	-2748	-	CANNTG
MYCCONSENSUSAT	-2748	-	CANNTG
CAATBOX1	-2748	+	CAAT
EBOXBNNAPA	-2748	+	CANNTG
MYCCONSENSUSAT	-2748	+	CANNTG
CAATBOX1	-2746	-	CAAT
SORLIP1AT	-2743	-	GCCAC
EBOXBNNAPA	-2739	-	CANNTG
MYCCONSENSUSAT	-2739	-	CANNTG
EBOXBNNAPA	-2739	+	CANNTG
MYCCONSENSUSAT	-2739	+	CANNTG
SEF4MOTIFGM7S	-2725	-	RTTTTTR
RAV1AAT	-2719	+	CAACA
NODCON1GM	-2715	-	AAAGAT
OSE1ROOTNODULE	-2715	-	AAAGAT
DOFCOREZM	-2713	-	AAAG
SEF4MOTIFGM7S	-2705	+	RTTTTTR
GTGANTG10	-2694	+	GTGA
NTBBF1ARROLB	-2680	-	ACTTTA
TAAAGSTKST1	-2680	+	TAAAG
INRNTPSADB	-2679	-	YTCANTYY
DOFCOREZM	-2679	+	AAAG
CACTFTPPCA1	-2677	-	YACT
GTGANTG10	-2676	+	GTGA
CACTFTPPCA1	-2666	+	YACT
-300ELEMENT	-2665	-	TGHAAARK

DOFCOREZM	-2664	-	AAAG
MYBCORE	-2653	-	CNGTTR
MYB2CONSENSUSAT	-2653	+	YAACKG
MYBCOREATCYCB1	-2652	+	AACGG
CARGCW8GAT	-2631	-	CWWWWWWWWG
CARGCW8GAT	-2631	+	CWWWWWWWWG
ROOTMOTIFTAPOX1	-2627	+	ATATT
CAATBOX1	-2625	-	CAAT
TATABOX5	-2610	+	TTATTT
-300ELEMENT	-2598	+	TGHAAARK
GT1CONSENSUS	-2597	+	GRWAAW
GT1GMSCAM4	-2597	+	GAAAAA
TATABOX5	-2594	-	TTATTT
POLASIG3	-2593	+	AATAAT
ARR1AT	-2590	-	NGATT
CACTFTPPCA1	-2585	-	YACT
CURECORECR	-2584	-	GTAC
CURECORECR	-2584	+	GTAC
CAATBOX1	-2581	+	CAAT
POLASIG3	-2580	+	AATAAT
GT1CONSENSUS	-2576	-	GRWAAW
POLLEN1LELAT52	-2574	-	AGAAA
DOFCOREZM	-2571	-	AAAG
POLLEN1LELAT52	-2570	-	AGAAA
DOFCOREZM	-2533	+	AAAG
NODCON1GM	-2533	+	AAAGAT
OSE1ROOTNODULE	-2533	+	AAAGAT
GATABOX	-2530	+	GATA
CAATBOX1	-2517	+	CAAT
CCAATBOX1	-2490	+	CCAAT
CAATBOX1	-2489	+	CAAT

WUSATAg	-2468	-	TTAATGG
GT1CORE	-2465	-	GGTTAA
MYB1AT	-2464	+	WAACCA
CACTFTPPCA1	-2459	-	YACT
ARR1AT	-2443	-	NGATT
CAATBOX1	-2440	+	CAAT
ROOTMOTIFTAPOX1	-2439	-	ATATT
GATABOX	-2437	-	GATA
ROOTMOTIFTAPOX1	-2432	-	ATATT
ROOTMOTIFTAPOX1	-2431	+	ATATT
GT1CONSENSUS	-2429	-	GRWAAW
POLLEN1LELAT52	-2427	-	AGAAA
WUSATAg	-2410	-	TTAATGG
ARR1AT	-2405	-	NGATT
MYBCORE	-2399	+	CNGTTR
RAV1AAT	-2398	-	CAACA
GATABOX	-2394	+	GATA
GT1CONSENSUS	-2394	+	GRWAAW
IBOXCORE	-2394	+	GATAA
NTBBF1ARROLB	-2389	+	ACTTTA
DOFCOREZM	-2388	-	AAAG
TAAAGSTKST1	-2388	-	TAAAG
CACTFTPPCA1	-2369	-	YACT
ARR1AT	-2366	-	NGATT
NODCON1GM	-2365	-	AAAGAT
OSE1ROOTNODULE	-2365	-	AAAGAT
DOFCOREZM	-2363	-	AAAG
POLASIG1	-2360	-	AATAAA
TATABOX5	-2359	+	TTATTT
GT1CONSENSUS	-2356	-	GRWAAW
IBOXCORE	-2355	-	GATAA

GATABOX	-2354	-	GATA
NODCON1GM	-2353	-	AAAGAT
OSE1ROOTNODULE	-2353	-	AAAGAT
DOFCOREZM	-2351	-	AAAG
CCAATBOX1	-2344	+	CCAAT
CARGCW8GAT	-2343	-	CWWWWWWWWG
CAATBOX1	-2343	+	CAAT
CARGCW8GAT	-2343	+	CWWWWWWWWG
POLASIG1	-2342	+	AATAAA
DOFCOREZM	-2337	+	AAAG
NODCON1GM	-2337	+	AAAGAT
OSE1ROOTNODULE	-2337	+	AAAGAT
ARR1AT	-2335	+	NGATT
WBBOXPCWRKY1	-2330	-	TTTGACY
WBOXHVISO1	-2330	-	TGACT
WBOXNTERF3	-2330	-	TGACY
WBOXATNPR1	-2329	-	TTGAC
WRKY71OS	-2329	-	TGAC
PRECONSCRHSP70A	-2318	-	SCGAYNRNNNNNNNN NNNNNNNHD
POLASIG3	-2316	-	AATAAT
CAATBOX1	-2308	-	CAAT
CCAATBOX1	-2308	-	CCAAT
TBOXATGAPB	-2294	-	ACTTTG
DOFCOREZM	-2293	+	AAAG
INRNTPSADB	-2288	+	YTCANTYY
ERELEE4	-2285	+	AWTTCAAA
MYB1AT	-2279	+	WAACCA
MYB1LEPR	-2269	+	GTTAGTT
CACTFTPPCA1	-2260	+	YACT
DOFCOREZM	-2246	+	AAAG

PREATPRODH	-2238	-	ACTCAT
ARR1AT	-2227	+	NGATT
AMYBOX1	-2223	-	TAACARA
MYBGAHV	-2223	-	TAACAAA
GAREAT	-2223	-	TAACAAR
CACTFTPPCA1	-2213	-	YACT
GTGANTG10	-2212	+	GTGA
GATABOX	-2210	+	GATA
ROOTMOTIFTAPOX1	-2209	+	ATATT
LTRE1HVBLT49	-2206	-	CCGAAA
TATAPVTRNALEU	-2198	-	TTTATATA
TATABOX4	-2198	+	TATATAA
TATABOX2	-2196	+	TATAAAT
POLLEN1LELAT52	-2184	+	AGAAA
SEF3MOTIFGM	-2180	+	AACCCA
DOFCOREZM	-2174	+	AAAG
POLLEN1LELAT52	-2172	+	AGAAA
DOFCOREZM	-2170	+	AAAG
GATABOX	-2145	-	GATA
MYBST1	-2145	-	GGATA
TATCCAYMOTIFOSRAMY3D	-2145	+	TATCCAY
TATCCAOSAMY	-2145	+	TATCCA
TATCCACHVAL21	-2145	+	TATCCAC
CACTFTPPCA1	-2141	+	YACT
IBOX	-2139	-	GATAAG
IBOXCORE	-2138	-	GATAA
GATABOX	-2137	-	GATA
CAATBOX1	-2127	-	CAAT
ARR1AT	-2125	+	NGATT
GARE2OSREP1	-2121	+	TAACGTA
ACGTATERD1	-2119	-	ACGT

ACGTATERD1	-2119	+	ACGT
POLASIG1	-2114	-	AATAAA
CAATBOX1	-2111	-	CAAT
ARR1AT	-2107	+	NGATT
CIACADIANLELHC	-2106	-	CAANNNNATC
CRTDREHVCBF2	-2097	-	GTCGAC
CBFHV	-2097	-	RYCGAC
CRTDREHVCBF2	-2097	+	GTCGAC
CBFHV	-2097	+	RYCGAC
DOFCOREZM	-2092	-	AAAG
CACTFTPPCA1	-2087	-	YACT
GATABOX	-2085	-	GATA
INRNTPSADB	-2071	+	YTCANTYY
GTGANTG10	-2070	-	GTGA
CACTFTPPCA1	-2069	+	YACT
NTBBF1ARROLB	-2068	+	ACTTTA
DOFCOREZM	-2067	-	AAAG
TAAAGSTKST1	-2067	-	TAAAG
ROOTMOTIFTAPOX1	-2059	+	ATATT
TAAAGSTKST1	-2055	+	TAAAG
DOFCOREZM	-2054	+	AAAG
QELEMENTZMZM13	-2049	+	AGGTCA
ELRECOREPCRP1	-2048	-	TTGACC
WBOXNTERF3	-2048	-	TGACY
WBOXATNPR1	-2047	-	TTGAC
WRKY71OS	-2047	-	TGAC
CAATBOX1	-2045	+	CAAT
ROOTMOTIFTAPOX1	-2044	-	ATATT
NTBBF1ARROLB	-2033	-	ACTTTA
TAAAGSTKST1	-2033	+	TAAAG
DOFCOREZM	-2032	+	AAAG

CACTFTPPCA1	-2030	-	YACT
WBBOXPCWRKY1	-2020	-	TTTGACY
WBOXHVISO1	-2020	-	TGACT
WBOXNTERF3	-2020	-	TGACY
WBOXATNPR1	-2019	-	TTGAC
WRKY71OS	-2019	-	TGAC
DOFCOREZM	-2008	-	AAAG
TAAAGSTKST1	-2008	-	TAAAG
POLASIG1	-2007	-	AATAAA
MYB1AT	-2001	+	WAACCA
MYBPLANT	-2000	+	MACCWAMC
REALPHALGLHCB21	-2000	+	AACCAA
GATABOX	-1992	-	GATA
CACTFTPPCA1	-1987	+	YACT
NODCON1GM	-1983	+	AAAGAT
OSE2ROOTNODULE	-1983	+	CTCTT
DOFCOREZM	-1981	-	AAAG
GT1CONSENSUS	-1980	-	GRWAAW
GT1CONSENSUS	-1973	-	GRWAAW
GT1GMSCAM4	-1973	-	GAAAAA
GT1CONSENSUS	-1972	-	GRWAAW
MYBCOREATCYCB1	-1967	-	AACGG
EBOXBNNAPA	-1961	-	CANNTG
DPBFCOREDCDC3	-1961	-	ACACNNG
MYCCONSENSUSAT	-1961	-	CANNTG
EBOXBNNAPA	-1961	+	CANNTG
MYCCONSENSUSAT	-1961	+	CANNTG
CACTFTPPCA1	-1959	-	YACT
2SSEEDPROTBANAPA	-1958	-	CAAACAC
CANBNNAPA	-1958	-	CNAACAC
DOFCOREZM	-1948	+	AAAG

NODCON1GM	-1948	+	AAAGAT
OSE1ROOTNODULE	-1948	+	AAAGAT
CAATBOX1	-1942	+	CAAT
POLASIG2	-1938	-	AATTAAA
GT1CONSENSUS	-1934	-	GRWAAW
POLLEN1LELAT52	-1932	-	AGAAA
CACTFTPPCA1	-1920	-	YACT
PYRIMIDINEBOXOSRAMY1A	-1910	-	CCTTTT
DOFCOREZM	-1909	+	AAAG
QELEMENTZMZM13	-1907	+	AGGTCA
WBOXNTERF3	-1906	-	TGACY
WRKY71OS	-1905	-	TGAC
GTGANTG10	-1897	-	GTGA
MYB1AT	-1890	+	WAACCA
WBOXHVISO1	-1882	-	TGACT
WBOXNTERF3	-1882	-	TGACY
WBOXATNPR1	-1881	-	TTGAC
WRKY71OS	-1881	-	TGAC
CACTFTPPCA1	-1861	-	YACT
CPBCSPOR	-1859	+	TATTAG
POLLEN1LELAT52	-1855	+	AGAAA
GT1CONSENSUS	-1854	+	GRWAAW
GT1GMSCAM4	-1854	+	GAAAAA
ACGTTBOX	-1849	-	AACGTT
ACGTTBOX	-1849	+	AACGTT
ACGTATERD1	-1848	-	ACGT
ACGTATERD1	-1833	+	ACGT
CAATBOX1	-1832	+	CAAT
ROOTMOTIFTAPOX1	-1831	-	ATATT
ROOTMOTIFTAPOX1	-1823	+	ATATT
TBOXATGAPB	-1822	-	ACTTTG

DOFCOREZM	-1815	+	AAAG
EBOXBNNAPA	-1815	-	CANNTG
MYCCONSENSUSAT	-1815	-	CANNTG
MYB2CONSENSUSAT	-1815	-	YAACKG
EBOXBNNAPA	-1815	+	CANNTG
MYBCORE	-1815	+	CNGTTR
MYCCONSENSUSAT	-1815	+	CANNTG
GAREAT	-1809	+	TAACAAR
ARR1AT	-1803	+	NGATT
EECCRCAH1	-1802	+	GANTTNC
RAV1AAT	-1794	+	CAACA
DOFCOREZM	-1789	+	AAAG
NODCON1GM	-1789	+	AAAGAT
OSE1ROOTNODULE	-1789	+	AAAGAT
INRNTPSADB	-1787	-	YTCANTYY
ARR1AT	-1787	+	NGATT
CAATBOX1	-1785	-	CAAT
DOFCOREZM	-1781	+	AAAG
NODCON1GM	-1781	+	AAAGAT
OSE1ROOTNODULE	-1781	+	AAAGAT
ARR1AT	-1779	+	NGATT
WUSATAg	-1776	+	TTAATGG
ARR1AT	-1771	+	NGATT
CACTFTPPCA1	-1766	-	YACT
SEBFCONSSTPR10A	-1765	-	YTGTCWC
GTGANTG10	-1765	+	GTGA
BIHD1OS	-1764	-	TGTCA
WRKY71OS	-1764	+	TGAC
TATABOX5	-1760	-	TTATTT
POLASIG3	-1759	+	AATAAT
DOFCOREZM	-1750	+	AAAG

NODCON1GM	-1750	+	AAAGAT
OSE1ROOTNODULE	-1750	+	AAAGAT
AGMOTIFNTMYB2	-1748	+	AGATCCAA
ROOTMOTIFTAPOX1	-1740	-	ATATT
GATABOX	-1738	-	GATA
GTGANTG10	-1718	+	GTGA
GT1CONSENSUS	-1716	+	GRWAAW
GT1GMSCAM4	-1716	+	GAAAAA
ARR1AT	-1711	-	NGATT
INTRONLOWER	-1708	-	TGCAGG
CAATBOX1	-1704	+	CAAT
MARTBOX	-1701	+	TTWTWTTWTT
MARTBOX	-1700	+	TTWTWTTWTT
MARTBOX	-1699	+	TTWTWTTWTT
MARTBOX	-1698	+	TTWTWTTWTT
MARTBOX	-1697	+	TTWTWTTWTT
MARTBOX	-1696	+	TTWTWTTWTT
MARTBOX	-1695	+	TTWTWTTWTT
SEF4MOTIFGM7S	-1678	-	RTTTTTR
INRNTPSADB	-1672	+	YTCANTYY
CAATBOX1	-1670	+	CAAT
DOFCOREZM	-1665	-	AAAG
DOFCOREZM	-1660	+	AAAG
REALPHALGLHCB21	-1656	+	AACCAA
CCAATBOX1	-1654	+	CCAAT
CAATBOX1	-1653	+	CAAT
POLASIG3	-1643	-	AATAAT
TATABOX5	-1642	+	TTATTT
BIHD1OS	-1637	+	TGTCA
WRKY71OS	-1636	-	TGAC
CACTFTPPCA1	-1633	-	YACT

BOXIINTPATPB	-1622	+	ATAGAA
POLLEN1LELAT52	-1620	+	AGAAA
CACTFTPPCA1	-1614	+	YACT
ARR1AT	-1610	+	NGATT
TATABOX2	-1608	-	TATAAAT
SEF4MOTIFGM7S	-1601	+	RTTTTTR
SURE1STPAT21	-1595	+	AATAGAAAA
BOXIINTPATPB	-1594	+	ATAGAA
POLLEN1LELAT52	-1592	+	AGAAA
GT1CONSENSUS	-1591	+	GRWAAW
GT1GMSCAM4	-1591	+	GAAAAA
WBOXHVISO1	-1577	-	TGACT
WBOXNTERF3	-1577	-	TGACY
WRKY71OS	-1576	-	TGAC
CAREOSREP1	-1571	+	CAACTC
NODCON2GM	-1568	+	CTCTT
OSE2ROOTNODULE	-1568	+	CTCTT
DOFCOREZM	-1566	-	AAAG
MARTBOX	-1565	+	TTWTWTTWTT
POLASIG1	-1564	-	AATAAA
TATABOX5	-1563	+	TTATTT
GT1CONSENSUS	-1559	-	GRWAAW
GT1GMSCAM4	-1559	-	GAAAAA
RAV1AAT	-1544	+	CAACA
ROOTMOTIFTAPOX1	-1540	+	ATATT
CACTFTPPCA1	-1535	+	YACT
NTBBF1ARROLB	-1534	+	ACTTTA
DOFCOREZM	-1533	-	AAAG
TAAAGSTKST1	-1533	-	TAAAG
POLASIG1	-1532	-	AATAAA
ARR1AT	-1525	+	NGATT

CAATBOX1	-1523	-	CAAT
CACTFTPPCA1	-1512	+	YACT
DOFCOREZM	-1510	-	AAAG
SURECOREATSULTR11	-1493	-	GAGAC
NODCON2GM	-1491	+	CTCTT
OSE2ROOTNODULE	-1491	+	CTCTT
DOFCOREZM	-1489	-	AAAG
TAAAGSTKST1	-1489	-	TAAAG
POLLEN1LELAT52	-1478	+	AGAAA
EECCRCAH1	-1466	-	GANTTNC
DOFCOREZM	-1465	+	AAAG
ROOTMOTIFTAPOX1	-1454	+	ATATT
CPBCSPOR	-1453	+	TATTAG
CACTFTPPCA1	-1449	-	YACT
CURECORECR	-1448	-	GTAC
CURECORECR	-1448	+	GTAC
CACTFTPPCA1	-1447	+	YACT
ROOTMOTIFTAPOX1	-1441	+	ATATT
POLASIG3	-1439	-	AATAAT
POLASIG3	-1436	-	AATAAT
DOFCOREZM	-1419	+	AAAG
LEAFYATAG	-1409	-	CCAATGT
CAATBOX1	-1407	-	CAAT
CCAATBOX1	-1407	-	CCAAT
GT1CONSENSUS	-1404	+	GRWAAW
GT1CONSENSUS	-1403	+	GRWAAW
GT1GMSCAM4	-1403	+	GAAAAA
MARTBOX	-1402	-	TTWTWTTWTT
TATABOX5	-1400	-	TTATTT
POLASIG1	-1399	+	AATAAA
DOFCOREZM	-1394	+	AAAG

DOFCOREZM	-1386	+	AAAG
POLLEN1LELAT52	-1384	+	AGAAA
GT1CONSENSUS	-1383	+	GRWAAW
GT1GMSCAM4	-1383	+	GAAAAA
MARTBOX	-1382	-	TTWTWTTWTT
DOFCOREZM	-1375	+	AAAG
2SSEEDPROTBANAPA	-1364	-	CAAACAC
CANBNNAPA	-1364	-	CNAACAC
POLLEN1LELAT52	-1344	+	AGAAA
GT1CONSENSUS	-1344	+	GRWAAW
GT1GMSCAM4	-1344	+	GAAAAA
INRNTPSADB	-1342	-	YTCANTYY
NODCON2GM	-1336	-	CTCTT
OSE2ROOTNODULE	-1336	-	CTCTT
MYBCORE	-1327	+	CNGTTR
RAV1AAT	-1326	-	CAACA
ROOTMOTIFTAPOX1	-1317	-	ATATT
ROOTMOTIFTAPOX1	-1316	+	ATATT
CAATBOX1	-1314	-	CAAT
ROOTMOTIFTAPOX1	-1300	-	ATATT
GATABOX	-1298	-	GATA
RAV1AAT	-1295	+	CAACA
CACTFTPPCA1	-1292	+	YACT
GATABOX	-1280	-	GATA
SEF3MOTIFGM	-1273	+	AACCCA
CAATBOX1	-1268	-	CAAT
DOFCOREZM	-1264	-	AAAG
CURECORECR	-1258	-	GTAC
CURECORECR	-1258	+	GTAC
NTBBF1ARROLB	-1252	+	ACTTTA
DOFCOREZM	-1251	-	AAAG

TAAAGSTKST1	-1251	-	TAAAG
POLASIG1	-1250	-	AATAAA
CPBCSPOR	-1248	+	TATTAG
CACTFTPPCA1	-1244	-	YACT
CACTFTPPCA1	-1241	-	YACT
TAAAGSTKST1	-1236	+	TAAAG
DOFCOREZM	-1235	+	AAAG
ARR1AT	-1219	+	NGATT
MYB1AT	-1205	+	WAACCA
NODCON2GM	-1191	+	CTCTT
OSE2ROOTNODULE	-1191	+	CTCTT
DOFCOREZM	-1189	-	AAAG
RAV1AAT	-1184	+	CAACA
GT1CONSENSUS	-1179	-	GRWAAW
GT1GMSCAM4	-1179	-	GAAAAA
POLLEN1LELAT52	-1177	-	AGAAA
DOFCOREZM	-1174	-	AAAG
ACGTTBOX	-1159	-	AACGTT
ACGTTBOX	-1159	+	AACGTT
ACGTATERD1	-1158	-	ACGT
ACGTATERD1	-1158	+	ACGT
CAATBOX1	-1145	+	CAAT
POLASIG1	-1144	+	AATAAA
TBOXATGAPB	-1138	+	ACTTTG
DOFCOREZM	-1137	-	AAAG
AMYBOX1	-1136	-	TAACARA
MYBGAHV	-1136	-	TAACAAA
GAREAT	-1136	-	TAACAAR
CACTFTPPCA1	-1131	+	YACT
GT1CONSENSUS	-1126	-	GRWAAW
POLLEN1LELAT52	-1124	-	AGAAA

ROOTMOTIFTAPOX1	-1113	-	ATATT
LECPLEACS2	-1110	-	TAAAATAT
ROOTMOTIFTAPOX1	-1098	+	ATATT
TBOXATGAPB	-1097	+	ACTTTG
DOFCOREZM	-1096	-	AAAG
AMYBOX1	-1096	-	TAACARA
MYBGAHV	-1096	-	TAACAAA
GAREAT	-1096	-	TAACAAR
CACTFTPPCA1	-1090	-	YACT
CACTFTPPCA1	-1077	-	YACT
CACTFTPPCA1	-1070	-	YACT
DOFCOREZM	-1066	+	AAAG
POLLEN1LELAT52	-1064	+	AGAAA
GT1CONSENSUS	-1063	+	GRWAAW
TATABOX5	-1061	-	TTATTT
PRECONSCRHSP70A	-1043	+	SCGAYNRNNNNNNNNN NNNNNNHD
DOFCOREZM	-1037	+	AAAG
INRNTPSADB	-1034	+	YTCANTYY
ARR1AT	-1034	-	NGATT
CAATBOX1	-1032	+	CAAT
TATABOX5	-1018	-	TTATTT
POLASIG3	-1017	+	AATAAT
EBOXBNNAPA	-996	-	CANNTG
MYBCORE	-996	-	CNGTTR
MYCCONSENSUSAT	-996	-	CANNTG
EBOXBNNAPA	-996	+	CANNTG
MYCCONSENSUSAT	-996	+	CANNTG
MYB2CONSENSUSAT	-996	+	YAACKG
GATABOX	-985	+	GATA
IBOXCORE	-985	+	GATAA

CAATBOX1	-974	+	CAAT
GATABOX	-970	+	GATA
IBOXCORE	-970	+	GATAA
GTGANTG10	-964	-	GTGA
GTGANTG10	-949	-	GTGA
EBOXBNNAPA	-948	-	CANNTG
MYCCONSENSUSAT	-948	-	CANNTG
MYCATERD1	-948	-	CATGTG
EBOXBNNAPA	-948	+	CANNTG
MYCATRD22	-948	+	CACATG
MYCCONSENSUSAT	-948	+	CANNTG
-300ELEMENT	-944	+	TGHAAARK
GT1CONSENSUS	-943	+	GRWAAW
GT1GMSCAM4	-943	+	GAAAAA
DOFCOREZM	-940	+	AAAG
CACTFTPPCA1	-938	-	YACT
GTGANTG10	-937	+	GTGA
ARR1AT	-936	+	NGATT
CACTFTPPCA1	-918	-	YACT
GTGANTG10	-917	+	GTGA
GATABOX	-915	+	GATA
GATABOX	-913	-	GATA
GTGANTG10	-911	-	GTGA
NODCON2GM	-907	-	CTCTT
OSE1ROOTNODULE	-907	-	AAAGAT
DOFCOREZM	-905	-	AAAG
POLLEN1LELAT52	-903	-	AGAAA
DOFCOREZM	-900	-	AAAG
GT1CONSENSUS	-907	-	GRWAAW
IBOXCORE	-897	-	GATAA
GATABOX	-896	-	GATA

TBOXATGAPB	-884	-	ACTTTG
DOFCOREZM	-883	+	AAAG
DOFCOREZM	-878	-	AAAG
REALPHALGLHCB21	-876	-	AACCAA
MYBATRD22	-875	-	CTAACCA
MYB1AT	-875	-	WAACCA
AACACOREOSGLUB1	-862	-	AACAAAC
EBOXBNNAPA	-855	-	CANNTG
MYCCONSENSUSAT	-855	-	CANNTG
CAATBOX1	-855	+	CAAT
EBOXBNNAPA	-855	+	CANNTG
MYCCONSENSUSAT	-855	+	CANNTG
CAATBOX1	-853	-	CAAT
GT1CONSENSUS	-843	-	GRWAAW
GT1GMSCAM4	-843	-	GAAAAA
GT1CONSENSUS	-842	-	GRWAAW
DOFCOREZM	-836	+	AAAG
EECCRCAH1	-833	+	GANTTNC
DOFCOREZM	-831	-	AAAG
NODCON2GM	-821	+	CTCTT
OSE2ROOTNODULE	-821	+	CTCTT
BOXIINTPATPB	-806	-	ATAGAA
GATABOX	-803	-	GATA
NODCON1GM	-802	-	AAAGAT
OSE1ROOTNODULE	-802	-	AAAGAT
DOFCOREZM	-800	-	AAAG
TAAAGSTKST1	-800	-	TAAAG
MYB1AT	-795	+	WAACCA
REALPHALGLHCB21	-794	+	AACCAA
GT1CONSENSUS	-787	-	GRWAAW
POLLEN1LELAT52	-785	-	AGAAA

ARR1AT	-762	-	NGATT
GATABOX	-750	-	GATA
ANAERO1CONSENSUS	-748	+	AAACAAA
GTGANTG10	-745	-	GTGA
CACTFTPPCA1	-744	+	YACT
CAATBOX1	-739	+	CAAT
NAPINMOTIFBN	-737	-	TACACAT
GT1CONSENSUS	-730	-	GRWAAW
IBOXCORE	-729	-	GATAA
GATABOX	-728	-	GATA
RGATAOS	-728	-	CAGAAGATA
MYBCORE	-722	+	CNGTTR
RAV1AAT	-721	-	CAACA
GTGANTG10	-717	+	GTGA
ARR1AT	-716	+	NGATT
GT1CONSENSUS	-713	-	GRWAAW
IBOXCORE	-712	-	GATAA
GATABOX	-711	-	GATA
EBOXBNNAPA	-708	-	CANNTG
MYCCONSENSUSAT	-708	-	CANNTG
CAATBOX1	-708	+	CAAT
EBOXBNNAPA	-708	+	CANNTG
MYCCONSENSUSAT	-708	+	CANNTG
CAATBOX1	-706	-	CAAT
EECCRCAH1	-703	-	GANTTNC
DOFCOREZM	-702	+	AAAG
WBOXHVISO1	-700	-	TGACT
WBOXNTERF3	-700	-	TGACY
WBOXATNPR1	-699	-	TTGAC
WRKY71OS	-699	-	TGAC
CAATBOX1	-697	+	CAAT

SEF4MOTIFGM7S	-691	-	RTTTTTR
ARR1AT	-687	-	NGATT
SORLIP1AT	-683	-	GCCAC
CACTFTPPCA1	-677	+	YACT
ARR1AT	-673	-	NGATT
GTGANTG10	-671	-	GTGA
CAATBOX1	-668	+	CAAT
POLASIG3	-667	+	AATAAT
TATABOX2	-663	-	TATAAAT
SEF4MOTIFGM7S	-655	-	RTTTTTR
TATABOX5	-652	-	TTATTT
POLASIG1	-651	+	AATAAA
CAATBOX1	-640	+	CAAT
SEF4MOTIFGM7S	-638	+	RTTTTTR
S1FBOXSORPS1L21	-628	+	ATGGTA
GT1CONSENSUS	-626	+	GRWAAW
SEF4MOTIFGM7S	-624	-	RTTTTTR
MARTBOX	-623	-	TTWTWTTWTT
MARARS	-622	-	WTTTATRTTTW
ROOTMOTIFTAPOX1	-620	-	ATATT
TATABOX2	-618	+	TATAAAT
HDZIP2ATATHB2	-610	+	TAATMATTA
POLASIG3	-609	+	AATAAT
CACTFTPPCA1	-602	-	YACT
SREATMSD	-594	-	TTATCC
MYBST1	-594	+	GGATA
GATABOX	-593	+	GATA
GT1CONSENSUS	-593	+	GRWAAW
IBOXCORE	-593	+	GATAA
CAATBOX1	-588	-	CAAT
MYBCORE	-582	-	CNGTTR

ARR1AT	-573	+	NGATT
-300ELEMENT	-557	+	TGHAAARK
PYRIMIDINEBOXOSRAMY1A	-555	-	CCTTTT
DOFCOREZM	-554	+	AAAG
MARTBOX	-547	-	TTWTWTTWTT
DOFCOREZM	-540	+	AAAG
NODCON2GM	-539	-	CTCTT
OSE2ROOTNODULE	-539	-	CTCTT
SEF4MOTIFGM7S	-529	-	RTTTTTR
DOFCOREZM	-512	-	AAAG
DOFCOREZM	-499	+	AAAG
GCCCORE	-487	+	GCCGCC
NODCON2GM	-482	+	CTCTT
OSE2ROOTNODULE	-482	+	CTCTT
DOFCOREZM	-480	-	AAAG
GTGANTG10	-477	-	GTGA
CACTFTPPCA1	-476	+	YACT
GATABOX	-473	-	GATA
MYBST1	-473	-	GGATA
DOFCOREZM	-466	+	AAAG
SEF4MOTIFGM7S	-437	+	RTTTTTR
POLASIG1	-434	-	AATAAA
TATABOX5	-433	+	TTATTT
SEF4MOTIFGM7S	-426	+	RTTTTTR
POLASIG1	-423	-	AATAAA
DOFCOREZM	-401	-	AAAG
CAATBOX1	-386	+	CAAT
ARR1AT	-385	-	NGATT
GTGANTG10	-372	-	GTGA
CACTFTPPCA1	-365	+	YACT
RAV1AAT	-357	-	CAACA

AMYBOX1	-345	+	TAACARA
MYBGAHV	-345	+	TAACAAA
GAREAT	-345	+	TAACAAR
ANAERO1CONSENSUS	-330	+	AAACAAA
TATABOX5	-336	-	TTATTT
SEF1MOTIF	-335	-	ATATTTAWW
POLASIG1	-335	+	AATAAA
ROOTMOTIFTAPOX1	-331	-	ATATT
RAV1AAT	-327	-	CAACA
CCAATBOX1	-315	+	CCAAT
CAATBOX1	-314	+	CAAT
-300ELEMENT	-312	-	TGHAAARK
LIBOXATPDF1	-308	-	TAAATGYA
CACTFTPPCA1	-291	+	YACT
ABRELATERD1	-283	-	ACGTG
ACGTATERD1	-282	-	ACGT
ACGTATERD1	-282	+	ACGT
CACTFTPPCA1	-274	+	YACT
DOFCOREZM	-272	-	AAAG
ROOTMOTIFTAPOX1	-266	-	ATATT
ROOTMOTIFTAPOX1	-261	-	ATATT
GATABOX	-259	-	GATA
POLLEN1LELAT52	-254	+	AGAAA
MYB1LEPR	-251	-	GTTAGTT
MYBATRD22	-249	+	CTAACCA
MYB1AT	-248	+	WAACCA
REALPHALGLHCB21	-247	+	AACCAA
TATABOX5	-243	-	TTATTT
POLASIG3	-242	+	AATAAT
ARR1AT	-239	-	NGATT
GT1CONSENSUS	-229	+	GRWAAW

TATABOX5	-227	-	TTATTT
MARTBOX	-226	-	TTWTWTTWTT
POLASIG3	-226	+	AATAAT
POLASIG1	-223	+	AATAAA
ARR1AT	-218	-	NGATT
AMYBOX1	-193	+	TAACARA
MYBGAHV	-193	+	TAACAAA
GAREAT	-193	+	TAACAAR
ROOTMOTIFTAPOX1	-187	-	ATATT
POLASIG2	-175	-	AATTAAA
POLASIG2	-172	+	AATTAAA
ROOTMOTIFTAPOX1	-164	-	ATATT
S1FSORPL21	-161	+	ATGGTATT
S1FBOXSORPS1L21	-161	+	ATGGTA
GT1CONSENSUS	-156	-	GRWAAW
POLLEN1LELAT52	-154	-	AGAAA
MYBCORE	-149	-	CNGTTR
MYB2CONSENSUSAT	-149	+	YAACKG
MYBCOREATCYCB1	-148	+	AACGG
MARTBOX	-141	-	TTWTWTTWTT
MARTBOX	-139	-	TTWTWTTWTT
TATABOX5	-137	-	TTATTT
MARTBOX	-136	-	TTWTWTTWTT
POLASIG1	-136	+	AATAAA
TATABOX5	-132	-	TTATTT
POLASIG1	-131	+	AATAAA
DOFCOREZM	-126	+	AAAG
-10PEHVPSBD	-124	-	TATTCT
ROOTMOTIFTAPOX1	-122	-	ATATT
SEF4MOTIFGM7S	-118	-	RTTTTTR
SURECOREATSULTR11	-108	-	GAGAC

NODCON2GM	-106	+	CTCTT
OSE2ROOTNODULE	-106	+	CTCTT
RAV1AAT	-99	+	CAACA
CACTFTPPCA1	-96	+	YACT
INRNTPSADB	-94	+	YTCANTYY
GTGANTG10	-93	-	GTGA
CACTFTPPCA1	-92	+	YACT
NTBBF1ARROLB	-91	+	ACTTTA
DOFCOREZM	-90	-	AAAG
TAAAGSTKST1	-90	-	TAAAG
CACTFTPPCA1	-87	+	YACT
NODCON2GM	-85	+	CTCTT
OSE2ROOTNODULE	-85	+	CTCTT
DOFCOREZM	-83	-	AAAG
PYRIMIDINEBOXOSRAMY1A	-82	+	CCTTTT
GT1CONSENSUS	-81	-	GRWAAW
GT1GMSCAM4	-81	-	GAAAAA
GT1CONSENSUS	-80	-	GRWAAW
NODCON2GM	-74	+	CTCTT
OSE2ROOTNODULE	-74	+	CTCTT
GTGANTG10	-70	-	GTGA
CACTFTPPCA1	-66	+	YACT
TAAAGSTKST1	-50	+	TAAAG
DOFCOREZM	-49	+	AAAG
DOFCOREZM	-42	+	AAAG
NODCON2GM	-38	+	CTCTT
OSE2ROOTNODULE	-38	+	CTCTT
NODCON2GM	-31	+	CTCTT
OSE2ROOTNODULE	-31	+	CTCTT
NODCON2GM	-20	+	CTCTT
OSE2ROOTNODULE	-20	+	CTCTT

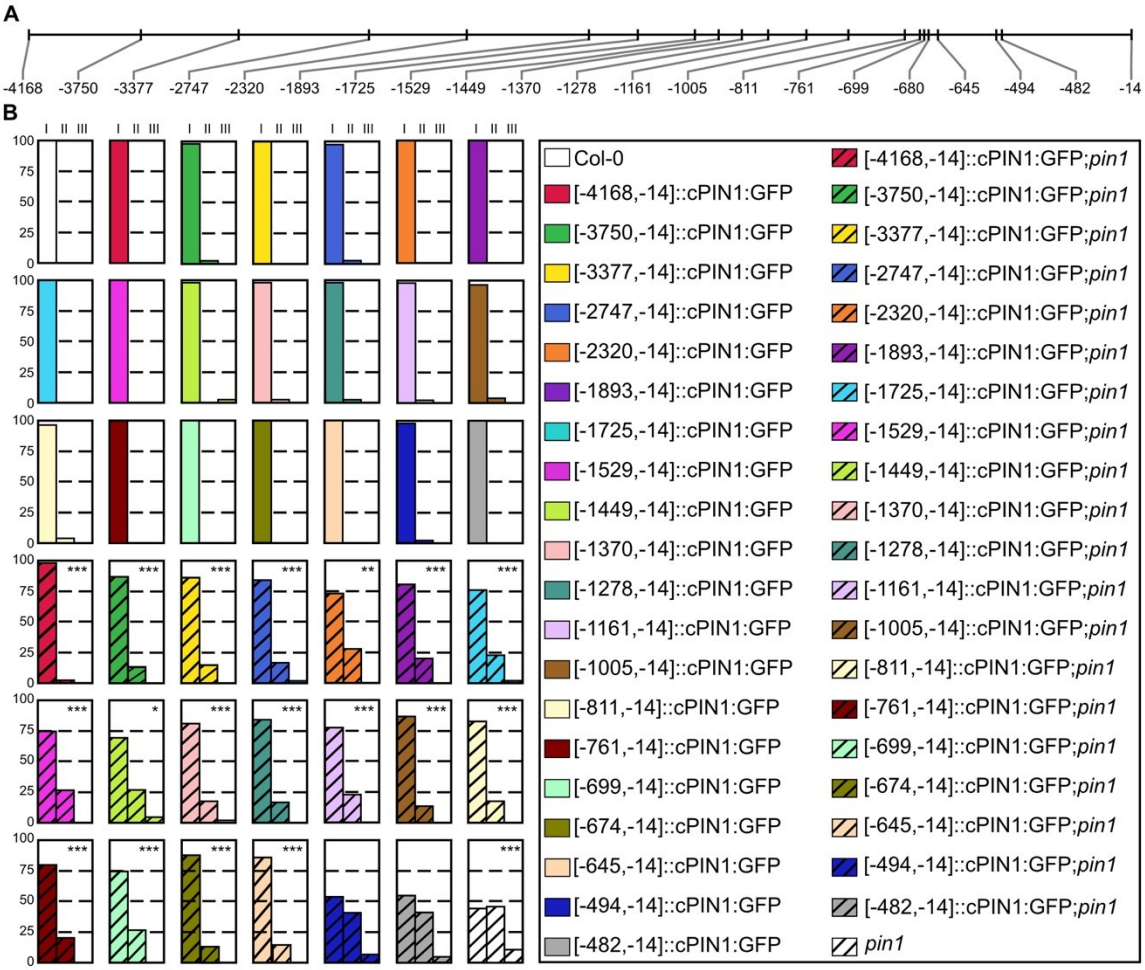

Figure 3.1. Function of *PIN1* Promoter Fragments in *PIN1*-Dependent Vein Patterning. (A) PIN1 promoter. Coordinates relative to start-codon's first nucleotide. (B) Percentages of leaves in phenotype classes: class I, I-shaped midvein; class II, Y-shaped midvein; class III, fused leaves. Difference between pin1 and WT, between [-3,750,-14]::cPIN1:GFP;*pin1* and pin1, between [-3,377,-14]::cPIN1:GFP;*pin1* and *pin1*, between [-2,747,-14]::cPIN1:GFP;*pin1* and *pin1*, between [-2,320,-14]::cPIN1:GFP;*pin1* and *pin1*, between [-1,893,-14]::cPIN1:GFP;*pin1* and *pin1*, between [-1,725,-14]::cPIN1:GFP;*pin1* and *pin1*, between [-1,529,- 14]::cPIN1:GFP;*pin1* and *pin1*, between [-1,449,-14]::cPIN1:GFP;*pin1* and *pin1*, between [-1,370,-14]::cPIN1:GFP;*pin1* and *pin1*, between [-1,278,-14]::cPIN1:GFP;*pin1* and *pin1*, between [-1,161,-14]::cPIN1:GFP;*pin1* and *pin1*, between [-1,005,-14]::cPIN1:GFP;*pin1* and *pin1*, between [-811,-14]::cPIN1:GFP;*pin1* and *pin1*, between [-761,-14]::cPIN1:GFP;*pin1* and *pin1*, between [-699,-14]::cPIN1:GFP;*pin1* and

pin1, between [-674,-14]::cPIN1:GFP;*pin1* and *pin1*, and between [-645,-14]::cPIN1:GFP;*pin1* and *pin1* was significant at P<0.05 (*), P<0.01 (**) or P<0.001 (***) by Kruskal-Wallis and Mann-Whitney test. Sample population sizes: WT, 41; pin1, 57; PIN1::cPIN1:GFP, 47; [-3,750,-14]::cPIN1:GFP, 44; [-3,377,-14]::cPIN1:GFP, 31; [-2,747,-14]::cPIN1:GFP, 32; [-2,320,-14]::cPIN1:GFP, 47; [-1,893,-14]::cPIN1:GFP, 54; [-1,725,-14]::cPIN1:GFP, 51; [-1,529,-14]::cPIN1:GFP, 51; [-1,449,-14]::cPIN1:GFP, 53; [-1,370,-14]::cPIN1:GFP, 50; [-1,278,-14]::cPIN1:GFP, 52; [-1,161,-14]::cPIN1:GFP, 51; [-1,005,-14]::cPIN1:GFP, 53; [-811,-14]::cPIN1:GFP, 27; [-761,-14]::cPIN1:GFP, 51; [-699,-14]::cPIN1:GFP, 49; [-674,-14]::cPIN1:GFP, 24; [-645,-14]::cPIN1:GFP, 25; [-494,-14]::cPIN1:GFP, 47; [-482,-14]::cPIN1:GFP, 52; PIN1::cPIN1:GFP;*pin1*, 54; [-3,750,-14]::cPIN1:GFP;*pin1*, 53; [-3,377,-14]::cPIN1:GFP;*pin1*, 69; [-2,747,-14]::cPIN1:GFP;*pin1*, 62; [-2,320,-14]::cPIN1:GFP;*pin1*, 51; [-1,893,-14]::cPIN1:GFP;*pin1*, 56; [-1,725,-14]::cPIN1:GFP;*pin1*, 58; [-1,529,-14]::cPIN1:GFP;*pin1*, 58; [-1,449,-14]::cPIN1:GFP;*pin1*, 45; [-1,370,-14]::cPIN1:GFP;*pin1*, 58; [-1,278,-14]::cPIN1:GFP;*pin1*, 62; [-1,161,-14]::cPIN1:GFP;*pin1*, 58; [-1,005,-14]::cPIN1:GFP;*pin1*, 52; [-811,- 14]::cPIN1:GFP;*pin1*, 29; [-761,-14]::cPIN1:GFP;*pin1*, 40; [-699,-14]::cPIN1:GFP;*pin1*, 58; [-674,-14]::cPIN1:GFP;*pin1*, 63; [-645,-14]::cPIN1:GFP;*pin1*, 75; [-494,-14]::cPIN1:GFP;*pin1*, 67; [-482,-14]::cPIN1:GFP;*pin1*, 44.

[-1,529,-14]::cPIN1:GFP, [-1,449,-14]::cPIN1:GFP, [-1,370,-14]::cPIN1:GFP, [-1,278,-14]::cPIN1:GFP, [-1,161,-14]::cPIN1:GFP, [-1,005,-14]::cPIN1:GFP, [-811,-14]::cPIN1:GFP, [-761,-14]::cPIN1:GFP, [-699,-14]::cPIN1:GFP, [-674,-14]::cPIN1:GFP, and [-645,-14]::cPIN1:GFP shifted the phenotype spectrum of the vein patterns of *pin1* toward the WT vein pattern (Fig. 3.1B). By contrast, [-494,-14]::cPIN1:GFP and [-482,-14]::cPIN1:GFP failed to do so (Fig. 3.1B).

The [-645,-14] fragment was thus the shortest *PIN1* promoter fragment that drove cPIN1:GFP expression so as to shift the phenotype spectrum of the vein patterns of *pin1* toward the WT vein pattern, and the [-494,-14] fragment was the longest promoter fragment that failed to do so. We therefore conclude that the 151-bp region of the *PIN1* promoter between -645 and -495 is required for *PIN1* function in vein patterning.

3.2.2 Transcriptional Control of PIN1 Functional Expression in Vein Patterning

We then asked what the domains of activity were of the 20 *PIN1* promoter fragments (Fig. 3.1A). To address this question, we imaged ΔPIN1::cPIN1:GFP expression in first leaves 4 days after germination.

Consistent with the activity of the *PIN1* promoter reported by PIN1::nYFP expression and as previously shown (Chapter 2), the 4,168-bp *PIN1* promoter drove cPIN1:GFP expression in all the veins and in nearly all the inner cells in the area delimited by the midvein and by the second and third loops (Fig. 3.2A). The [-3,750,-14], [-3,377,-14], [-2,747,-14], [-2,320,-14], [-1,893,-14], [-1,725,-14], [-1,529,-14], and [-1,449,-14] fragments drove cPIN1:GFP expression in all the veins (Fig. 3.2B–I). The [-1,370,-14], [-1,278,-14], and [-1,161,-14] fragments drove cPIN1:GFP expression in the midvein, first loops, and very few epidermal cells at the leaf margin (Fig. 3.2J–L). The [-1,005,-14], [-811,-14], [-761,-14], [-699,-14], [-674,-14], and [-645,-14] fragments drove cPIN1:GFP expression in the most apical part of the midvein and first loops, and in very few epidermal cells at the leaf margin (Fig. 3.2M–R). Finally, the [-494,-14] and [-482,-14] fragments drove cPIN1:GFP expression only in very few epidermal cells at the leaf margin (Fig. 3.2S,T).

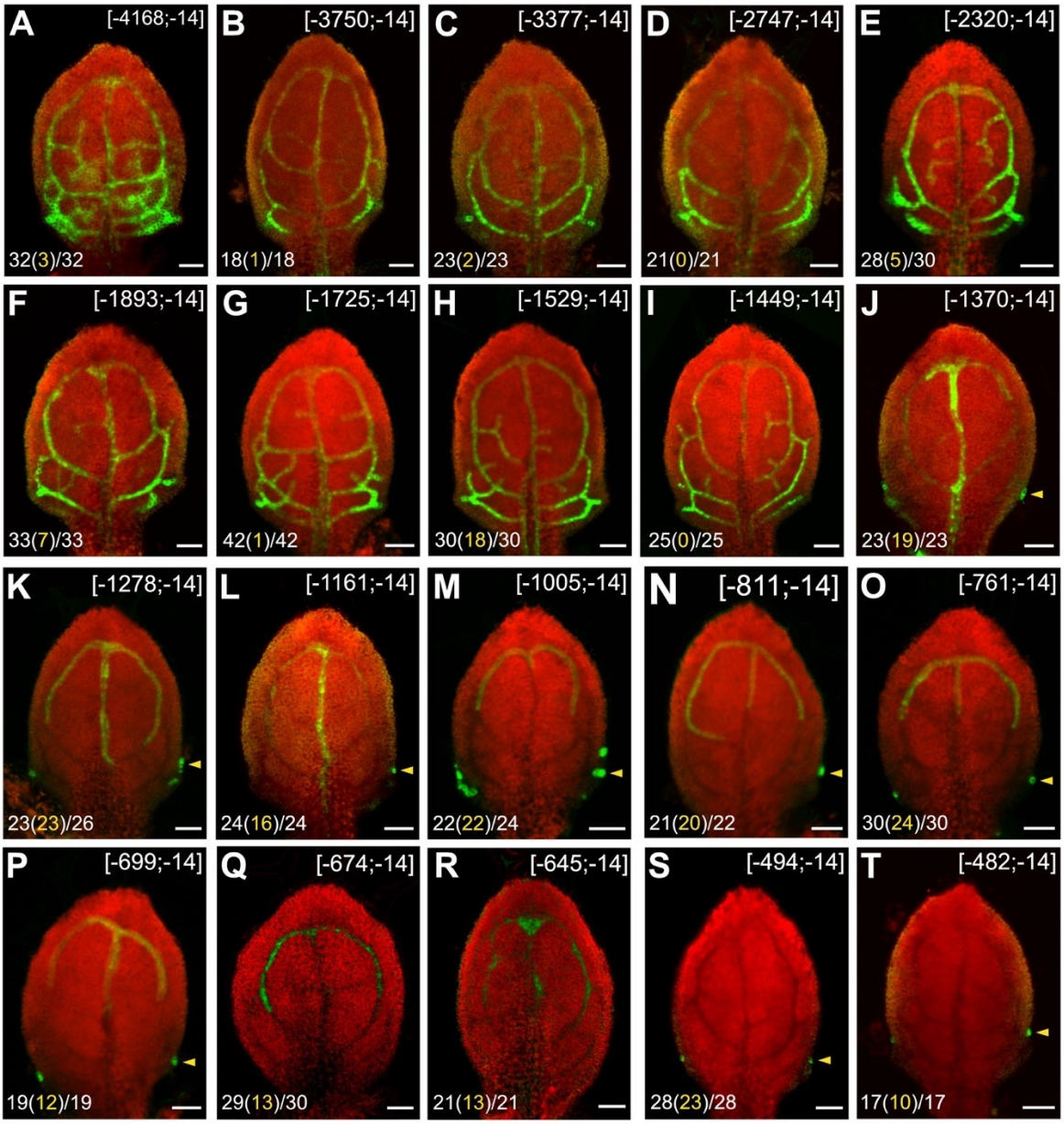

Figure 3.2 Activity of *PIN1* Promoter Fragments in Developing Leaves. (A–T). Confocal laser scanning microscopy; first leaves 4 days after germination. Green, cPIN1:GFP expression; red, autofluorescence. Yellow arrowheads point to epidermal expression. Top right: promoter fragment coordinates; bottom left: reproducibility index (in white for inner-tissue expression; in yellow for epidermal expression). Bars: (A–T) 50 μm.

Because the [-645,-14] fragment was the shortest *PIN1* promoter fragment that drove cPIN1:GFP expression in midvein and first loops, and the [-494,-14] was the longest one that failed to do so, we conclude that the 151-bp region of the *PIN1* promoter between -645 and -495 is required for PIN1 expression in midvein and first loops. Because this same region of the *PIN1* promoter is also required for *PIN1* function in vein patterning (Fig. 3.1B), we further conclude that PIN1 expression in midvein and first loops is required for *PIN1* function in vein patterning.

Though it will be interesting to identify the transcription factors that bind the 151-bp region of the *PIN1* promoter that is required for PIN1 functional expression in vein patterning, our results already define cis-regulation of *PIN1* function in this process.

3.3 Materials & Methods

3.3.1 Plants

Origin and nature of lines, and oligonucleotide sequences are in Tables 3.2 and 3.3. Seeds were sterilized and sown as in (Sawchuk et al., 2008). Stratified seeds were germinated and seedlings were grown at 22°C under continuous fluorescent light (~80 µmol $m^{-2}s^{-1}$). Plants were grown at 25°C under fluorescent light (~100 µmol $m^{-2}s^{-1}$) in a 16-h-light/8-h-dark cycle. *pin1-051* was genotyped with the "pin1 GK LP" and "pin1 GK RP" primers (WT allele) and with the "pin1 GK RP" and "o8409" primers (mutant allele). Plants were transformed and representative lines were selected as in (Sawchuk et al., 2008).

3.3.2 Imaging

Developing leaves were mounted and imaged as in (Sawchuk et al., 2013). GFP was excited with the 488-nm line of a 30-mW Ar laser. For [-674,-14]::cPIN1:GFP and [-645,-14]::cPIN1:GFP, GFP emission and autofluorescence were collected between 508 and 593 nm and separated by linear unmixing (Berg, 2004). For all other lines, GFP emission was collected with a BP 505–530 filter, and autofluorescence was collected between 550 nm and 754 nm. Images were stacked, aligned with the Scale Invariant Feature Transform algorithm (Lowe, 2004), and maximum-intensity projection was applied to aligned image stacks in the

Table 3.2. Origin and Nature of Lines.

Line	Origin/Nature
pin1-051	NASC; GK-051A10-012139 (Kleinboelting et al. 2012); contains a T-DNA insertion after +2234 of *PIN1*
PIN1::cPIN1:GFP	Transcriptional fusion of *PIN1* (-4,168 to -14; primers "XhoI full length PIN1p F" and "BamHI PIN1p rev") to translational fusion of *PIN1* cDNA (GenBank accession no. AY093960; ABRC clone no. U12338; primers "BamHI PIN1 cDNA F" and "KpnI PIN1 cDNA R") to EGFP (Clontech; insertion after +651 of *PIN1*; primers "XhoI GFP no ATG Fwd" and "XhoI GFP no* Rev")
[-3,750,-14]::cPIN1:GFP	Transcriptional fusion of *PIN1* (-3,750 to -14, primers "XhoI_PIN1pF [-3750,-14]" and "BamHI PIN1p rev") to translational fusion of *PIN1* cDNA (GenBank accession no.: AY093960; ABRC clone no.: U12338; primers "BamHI PIN1 cDNA F" and "KpnI PIN1 cDNA R") to a sequence encoding EGFP (Clontech; insertion after +651 of *PIN1*; primers "XhoI GFP no ATG Fwd" and "XhoI GFP no* Rev")
[-3,377,-14]::cPIN1:GFP	Transcriptional fusion of *PIN1* (-3,377 to -14, primers "XhoI_PIN1 p F [-3377,-14]" and "BamHI PIN1p rev") to translational fusion of *PIN1* cDNA (GenBank accession no.: AY093960; ABRC clone no.: U12338; primers "BamHI PIN1 cDNA F" and "KpnI PIN1 cDNA R") to a sequence encoding EGFP (Clontech; insertion after +651 of *PIN1*; primers "XhoI GFP no ATG Fwd" and "XhoI GFP no* Rev")
[-2,747,-14]::cPIN1:GFP	Transcriptional fusion of *PIN1* (-2,747 to -14, primers "XhoI_PIN 1F [-2747,-14]" and "BamHI PIN1p rev") to translational fusion of *PIN1* cDNA (GenBank accession no.: AY093960; ABRC clone no.: U12338; primers "BamHI PIN1 cDNA F" and "KpnI PIN1 cDNA R") to a sequence encoding

	EGFP (Clontech; insertion after +651 of *PIN1*; primers "XhoI GFP no ATG Fwd" and "XhoI GFP no* Rev")
[-2,320,-14]::cPIN1:GFP	Transcriptional fusion of *PIN1* (-2,120 to -14, primers "XhoI 2300 PIN1p F" and "BamHI PIN1p rev") to translational fusion of *PIN1* cDNA (GenBank accession no.: AY093960; ABRC clone no.: U12338; primers "BamHI PIN1 cDNA F" and "KpnI PIN1 cDNA R") to a sequence encoding EGFP (Clontech; insertion after +651 of *PIN1*; primers "XhoI GFP no ATG Fwd" and "XhoI GFP no* Rev")
[-1,893,-14]::cPIN1:GFP	Transcriptional fusion of *PIN1* (-1,893 to -14, primers "SalI 1900 PIN1p F" and "BamHI PIN1p rev") to translational fusion of *PIN1* cDNA (GenBank accession no.: AY093960; ABRC clone no.: U12338; primers "BamHI PIN1 cDNA F" and "KpnI PIN1 cDNA R") to a sequence encoding EGFP (Clontech; insertion after +651 of *PIN1*; primers "XhoI GFP no ATG Fwd" and "XhoI GFP no* Rev")
[-1,725,-14]::cPIN1:GFP	Transcriptional fusion of *PIN1* (-1,725 to -14, primers "SalI 1700 PIN1p F" and "BamHI PIN1p rev") to translational fusion of *PIN1* cDNA (GenBank accession no.: AY093960; ABRC clone no.: U12338; primers "BamHI PIN1 cDNA F" and "KpnI PIN1 cDNA R") to a sequence encoding EGFP (Clontech; insertion after +651 of *PIN1*; primers "XhoI GFP no ATG Fwd" and "XhoI GFP no* Rev")
[-1,529,-14]: cPIN1:GFP	Transcriptional fusion of *PIN1* (-1,529 to -14, primers "PIN1 prom 1.5 SalI Fwd" and "BamHI PIN1p rev") to translational fusion of *PIN1* cDNA (GenBank accession no.: AY093960; ABRC clone no.: U12338; primers "BamHI PIN1 cDNA F" and "KpnI PIN1 cDNA R") to a sequence encoding EGFP (Clontech; insertion after +651 of *PIN1*; primers "XhoI GFP no ATG Fwd" and "XhoI GFP no* Rev")

[-1,449,-14]::cPIN1:GFP	Transcriptional fusion of *PIN1* (-1,449 to -14, primers "SalI 1450 PIN1p F" and "BamHI PIN1p rev") to translational fusion of *PIN1* cDNA (GenBank accession no.: AY093960; ABRC clone no.: U12338; primers "BamHI PIN1 cDNA F" and "KpnI PIN1 cDNA R") to a sequence encoding EGFP (Clontech; insertion after +651 of *PIN1*; primers "XhoI GFP no ATG Fwd" and "XhoI GFP no* Rev")
[-1,370,-14]::cPIN1:GFP	Transcriptional fusion of *PIN1* (-1,370 to -14, primers "SalI 1350 PIN1p F" and "BamHI PIN1p rev") to translational fusion of *PIN1* cDNA (GenBank accession no.: AY093960; ABRC clone no.: U12338; primers "BamHI PIN1 cDNA F" and "KpnI PIN1 cDNA R") to a sequence encoding EGFP (Clontech; insertion after +651 of *PIN1*; primers "XhoI GFP no ATG Fwd" and "XhoI GFP no* Rev")
[-1,278,-14]::cPIN1:GFP	Transcriptional fusion of *PIN1* (-1,278 to -14, primers "SalI 1270 PIN1p F" and "BamHI PIN1p rev") to translational fusion of *PIN1* cDNA (GenBank accession no.: AY093960; ABRC clone no.: U12338; primers "BamHI PIN1 cDNA F" and "KpnI PIN1 cDNA R") to a sequence encoding EGFP (Clontech; insertion after +651 of *PIN1*; primers "XhoI GFP no ATG Fwd" and "XhoI GFP no* Rev")
[-1,161,-14]::cPIN1:GFP	Transcriptional fusion of *PIN1* (-1,161 to -14, primers "SalI 1160 PIN1p F" and "BamHI PIN1p rev") to translational fusion of *PIN1* cDNA (GenBank accession no.: AY093960; ABRC clone no.: U12338; primers "BamHI PIN1 cDNA F" and "KpnI PIN1 cDNA R") to a sequence encoding EGFP (Clontech; insertion after +651 of *PIN1*; primers "XhoI GFP no ATG Fwd" and "XhoI GFP no* Rev")
[-1,005,-14]::cPIN1:GFP	Transcriptional fusion of *PIN1* (-1,005 to -14, primers "SalI 1kb PIN1p F" and "BamHI PIN1p rev") to translational fusion of *PIN1* cDNA (GenBank accession no.: AY093960; ABRC

	clone no.: U12338; primers “BamHI PIN1 cDNA F” and “KpnI PIN1 cDNA R”) to a sequence encoding EGFP (Clontech; insertion after +651 of *PIN1*; primers “XhoI GFP no ATG Fwd” and “XhoI GFP no* Rev”)
[-811,-14]::cPIN1:GFP	Transcriptional fusion of *PIN1* (-811 to -14, primers “SalI 800 PIN1p F” and “BamHI PIN1p rev”) to translational fusion of *PIN1* cDNA (GenBank accession no.: AY093960; ABRC clone no.: U12338; primers “BamHI PIN1 cDNA F” and “KpnI PIN1 cDNA R”) to a sequence encoding EGFP (Clontech; insertion after +651 of *PIN1*; primers “XhoI GFP no ATG Fwd” and “XhoI GFP no* Rev”)
[-761,-14]::cPIN1:GFP	Transcriptional fusion of *PIN1* (-761 to -14, primers “PIN1 prom 0.75 Sal1 Fwd” and “BamHI PIN1p rev”) to translational fusion of *PIN1* cDNA (GenBank accession no.: AY093960; ABRC clone no.: U12338; primers “BamHI PIN1 cDNA F” and “KpnI PIN1 cDNA R”) to a sequence encoding EGFP (Clontech; insertion after +651 of *PIN1*; primers “XhoI GFP no ATG Fwd” and “XhoI GFP no* Rev”)
[-699,-14]::cPIN1:GFP	Transcriptional fusion of *PIN1* (-699 to -14, primers “SalI 700 PIN1p F” and “BamHI PIN1p rev”) to translational fusion of *PIN1* cDNA (GenBank accession no.: AY093960; ABRC clone no.: U12338; primers “BamHI PIN1 cDNA F” and “KpnI PIN1 cDNA R”) to a sequence encoding EGFP (Clontech; insertion after +651 of *PIN1*; primers “XhoI GFP no ATG Fwd” and “XhoI GFP no* Rev”)
[-674,-14]::cPIN1:GFP	Transcriptional fusion of *PIN1* (-674 to -14, primers “SalI 680 PIN1p F” and “BamHI PIN1p rev”) to translational fusion of *PIN1* cDNA (GenBank accession no.: AY093960; ABRC clone no.: U12338; primers “BamHI PIN1 cDNA F” and “KpnI PIN1 cDNA R”) to a sequence encoding EGFP (Clontech;

	insertion after +651 of *PIN1*; primers "XhoI GFP no ATG Fwd" and "XhoI GFP no* Rev")
[-645,-14]::cPIN1:GFP	Transcriptional fusion of *PIN1* (-645 to -14, primers "0.62 PIN1p SalI" and "BamHI PIN1p rev") to translational fusion of *PIN1* cDNA (GenBank accession no.: AY093960; ABRC clone no.: U12338; primers "BamHI PIN1 cDNA F" and "KpnI PIN1 cDNA R") to a sequence encoding EGFP (Clontech; insertion after +651 of *PIN1*; primers "XhoI GFP no ATG Fwd" and "XhoI GFP no* Rev")
[-494,-14]::cPIN1:GFP	Transcriptional fusion of *PIN1* (-494 to -14, primers "PIN1p no DOFs SalI FWD" and "BamHI PIN1p rev") to translational fusion of *PIN1* cDNA (GenBank accession no.: AY093960; ABRC clone no.: U12338; primers "BamHI PIN1 cDNA F" and "KpnI PIN1 cDNA R") to a sequence encoding EGFP (Clontech; insertion after +651 of *PIN1*; primers "XhoI GFP no ATG Fwd" and "XhoI GFP no* Rev")
[-482,-14]::cPIN1:GFP	Transcriptional fusion of *PIN1* (-482 to -14, primers "0.47 PIN1p SalI" and "BamHI PIN1p rev") to translational fusion of *PIN1* cDNA (GenBank accession no.: AY093960; ABRC clone no.: U12338; primers "BamHI PIN1 cDNA F" and "KpnI PIN1 cDNA R") to a sequence encoding EGFP (Clontech; insertion after +651 of *PIN1*; primers "XhoI GFP no ATG Fwd" and "XhoI GFP no* Rev")

Table 3.3. Oligonucleotide Sequences.

Name	Sequence (5' to 3')
pin GK LP	ACTCTTTGGCAAACACAAACG
pin1 GK RP	CTCTCAGATGCAGGTCTAGGC
o8409	ATATTGACCATCATACTCATTGC
XhoI full length PIN1p F	TGTCTCGAGATCCGATTGGATTCGGTCTG
BamHI PIN1p rev	AAGGGATCCGAGAAGAGAGAGGGAAGAGAG
BamHI PIN1 cDNA F new	TTAGGATCCATGATTACGGCGGCGGACTTC
KpnI PIN1 cDNA R	CTCGGTACCTCATAGACCCAAGAGAATGTAG
XhoI GFP no ATG Fwd	TTACTCGAGAGTGAGCAAGGGCGAGGAGCTGTT
XhoI GFP no* Rev	TATCTCGAGTACTTGTACAGCTCGTCCATGCCGAG
XhoI_PIN1pF [-3750,-14]	AACCTCGAGCCAAAACCGTGCAAAAAAAAAG
XhoI_PIN1 p F [-3377,-14]	CCGCTCGAGCTTCACGTTTATAACTATTTGTTG
XhoI_PIN 1F [-2747,-14]	TAACTCGAGATTGTGGCAAATGGCTATGC
XhoI 2300 PIN1p F	CCGCTCGAGTAAATTATTCCATTGGCGTTG
SalI 1900 PIN1p F	ACCGTCGACCATAACCATAAGTCAAGCCG
SalI 1700 PIN1p F	CCTGTCGACTGGAATGTGAAAAAATCCTGC
PIN1 prom 1.5 SalI Fwd	GGCGTCGACTTCGGATTGCATAACCTA
SalI 1450 PIN1p F	CGGGTCGACGTACTATATATTATTATTATGC
SalI 1350 PIN1p F	GGTGTCGACGAACTGTGTTTGTATGGGATG
SalI 1270 PIN1p F	CCTGTCGACCATCAACCCATTGCTTTTTG
SalI 1160 PIN1p F	GGCGTCGACCTACGTATTTATGTTCAATAAAAC
SalI 1kb PIN1p F	ACCGTCGACCGCAACTACAACTGTAAATG
SalI 800 PIN1p F	GCCGTCGACAGACTTCTATCTTTAAAACC
PIN1 prom 0.75 Sal1 Fwd	GCCGTCGACTCGAGCCTTATATCATCA
SalI 700 PIN1p F	GCCGTCGACTCAATACCAAAAATCCCATC
SalI 680 PIN1p F	GCCGTCGACAATCACAATAATTTATAGC
0.62 PIN1p SalI	GCGTCGACTTAACAATTTTTAAACATGGTAA
PIN1p no DOFs SalI FWD	AATGTCGACCACAAGGCCGCCTCTTTCAC
0.47 PIN1p SalI	TAAGTCGAC TCTTTCACTATCCCCAAAGC

Fiji distribution (Schindelin et al., 2012) of ImageJ (Schneider et al., 2012; Schindelin et al., 2015; Rueden et al., 2017). Mature leaves were fixed in ethanol : acetic acid 6 : 1, rehydrated in 70% ethanol and water, and mounted in chloral hydrate : glycerol : water 8 : 2 : 1. Mounted leaves were imaged as in (Odat et al., 2014). Greyscaled RGB color images were turned into 8-bit images, and image brightness and contrast were adjusted by linear stretching of the histogram in the Fiji distribution of ImageJ.

3.3.3 Bioinformatics

Putative transcription-factor binding sites were identified with AthaMap (Steffens et al., 2004) (http://www.athamap.de/), PLACE (Higo et al., 1999) (http://www.dna.affrc.go.jp/PLACE/), Plant CARE (Lescot et al., 2002) (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) and rVISTA 2.0 (Loots and Ovcharenko, 2004) (http://rvista.dcode.org) using the TRANSFAC professional V10.2 library for plants and 0.75-similarity matrix.

Chapter 4: GAL4/GFP Enhancer-Trap Lines for Identification and Manipulation of Cells and Tissues in Developing Arabidopsis Leaves[1]

4.1 Introduction

Understanding developmental processes requires the unambiguous identification of cells and tissues, and the selective manipulation of the properties of those cells and tissues; both requirements can most efficiently be satisfied by the GAL4 system (Brand and Perrimon, 1993). In this system, a minimal promoter in a construct randomly inserted in a genome responds to neighboring regulatory elements and activates the expression of a gene, included in the same construct, encoding a variant of the GAL4 transcription factor of yeast; the same construct also includes a GAL4-responsive, UAS-driven lacZ, GUS, or GFP, which reports GAL4 expression. Independent, WT-looking lines, in which the construct is inserted in different genomic locations, are selected because they reproducibly express the GAL4-responsive reporter in cell- or tissue-specific patterns. These lines are used to identify cells or tissues, and to drive GAL4-responsive cell- or tissue-specific expression in WT or, through crosses, in mutants and transgenics (e.g., Halder and Gehring, 1995; Ito et al., 1997).

The first implementation of the GAL4 system in Arabidopsis was the Haseloff collection of GAL4/GFP enhancer-trap lines, in which an endoplasmic-reticulum-localized GFP (erGFP) responds to the activity of a fusion between the GAL4 DNA-binding domain and the activating domain of the Viral Protein 16 of *Herpex simplex* (Haseloff, 1999). The Haseloff collection is the most extensively used GAL4 system in Arabidopsis (e.g., Sabatini et al., 1999; Sawchuk et al., 2007; Gardner et al., 2009; Wenzel et al., 2012; Weijers et al., 2003; Laplaze et al., 2005), even though it is in the C24 background. This is problematic because the phenotype of hybrids between C24 and Columbia-0 (Col-0), generally considered the reference genotype in Arabidopsis (Koorneef and Meinke, 2010), is different from that of either parent (e.g., Groszmann et al., 2014; Kawanabe et al., 2016; Zhang et al., 2016). The use of GAL4/GFP enhancer-trap lines in the C24 background to investigate

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processes in the Col-0 background thus imposes the burden of laborious generation of ad-hoc control backgrounds. Therefore, most desirable is the generation and characterization of GAL4/GFP enhancer-trap collections in the Col-0 background. Two such collections have been reported: the Berleth collection, which has been used to identify lines that express GAL4/GFP in vascular tissues (Ckurshumova et al., 2009); and the Poethig collection, which has been used to identify lines that express GAL4/GFP in stomata (Gardner et al., 2009).

Here we screened the Poethig collection and provide a set of lines for the specific labeling of cells and tissues during leaf development, and we show that these lines can be used to address key questions in plant developmental biology.

4.2 Results and Discussion

To identify enhancer-trap lines in the Col-0 background of Arabidopsis with reproducible GAL4-driven GFP expression in developing leaves, we screened the collection generated and donated by Scott Poethig to the Arabidopsis Biological Resource Center. We screened 312 lines for GFP expression in developing leaves; 29 lines satisfied this criterion (Table 4.1). In 10 of these 29 lines, GFP was expressed in specific cells or tissues; nine of these 10 lines grew normally (Table 4.1). We imaged GFP expression in first leaves of these nine lines from 2 to 5 days after germination (DAG).

The development of Arabidopsis leaves has been described previously (Pyke and Leech, 1991; Telfer and Poethig, 1994; Kinsman and Pyke, 1998; Candela et al., 1999; Donnelly et al., 1999; Mattsson et al., 1999; Kang and Dengler, 2002; Kang and Dengler, 2004; Mattsson et al., 2003; Scarpella et al., 2004; Larkin et al., 1996). Briefly, at 2 DAG the first leaf is recognizable as a cylindrical primordium with a midvein at its center (Fig. 4.1A). By 2.5 DAG, the primordium has elongated and expanded (Fig. 4.1B). By 3 DAG, the primordium has continued to expand, and the first loops of veins ("first loops") have formed (Fig. 4.1C). By 4 DAG, a lamina and a petiole have become recognizable, second loops have formed, and minor veins have started to form in the top half of the lamina (Fig. 4.1D). By 5 DAG, lateral outgrowths (hydathodes) have become recognizable in the bottom quarter of the lamina, third loops have formed, and minor vein formation has spread toward

Table 4.1. Origin and Nature of Lines.

ABRC stock no.	Donor stock no.	Expression in developing leaves	Tissue- and/or stage-specific expression	Wild-type looking
CS24240	E53	N	…	…
CS24241	E306	N	…	…
CS24242	E337	N	…	…
CS24243	E362	N	…	…
CS24244	E456	N	…	…
CS24245	E513	N	…	…
CS24246	E652	N	…	…
CS24247	E751	N	…	…
CS24248	E788	N	…	…
CS24249	E829	N	…	…
CS24250	E1012	N	…	…
CS24251	E1075	N	…	…
CS24252	E1195	N	…	…
CS24253	E1247	N	…	…
CS24254	E1287	N	…	…
CS24255	E1324	N	…	…
CS24256	E1332	Y	N	…
CS24257	E2042	N	…	…
CS24258	E2065	N	…	…
CS24259	E2072	N	…	…
CS24260	E2119	N	…	…
CS24262	E2168	N	…	…
CS24264	E2242	N	…	…
CS24265	E2263	N	…	…
CS24266	E2271	N	…	…
CS24267	E2306	N	…	…
CS24269	E3191	N	…	…
CS24270	E3597	N	…	…

CS24271	E3604	N	…	…
CS24272	E4259	Y	Y	Y
CS65892	E2331	Y	Y	Y
CS65893	E2023	N	…	…
CS67882	suo-1	N	…	…
CS70001	E1	N	…	…
CS70002	E3	N	…	…
CS70003	E63	N	…	…
CS70004	E66	N	…	…
CS70005	E74	Y	N	…
CS70006	E829	N	…	…
CS70007	E100	Y	Y	Y
CS70008	E103	N	…	…
CS70009	E105	N	…	…
CS70010	E107	N	…	…
CS70011	E135	N	…	…
CS70012	E144	N	…	…
CS70013	E183	N	…	…
CS70014	E191	N	…	…
CS70015	E226	N	…	…
CS70016	E227	Y	N	…
CS70017	E230	N	…	…
CS70018	E232	N	…	…
CS70019	E242	N	…	…
CS70020	E244	N	…	…
CS70021	E254	N	…	…
CS70022	E259	Y	N	…
CS70023	E268	N	…	…
CS70024	E280	N	…	…
CS70025	E292	N	…	…
CS70026	E314	N	…	…

CS70027	E325	N	…	…
CS70028	E336	N	…	…
CS70029	E340	Y	N	…
CS70030	E361	N	…	…
CS70031	E387	N	…	…
CS70032	E434	N	…	…
CS70033	E457	N	…	…
CS70034	E461	N	…	…
CS70035	E462	N	…	…
CS70036	E464	N	…	…
CS70037	E470	N	…	…
CS70038	E491	N	…	…
CS70039	E555-1	N	…	…
CS70040	E555-2	N	…	…
CS70041	E556	N	…	…
CS70042	E583	N	…	…
CS70043	E655	N	…	…
CS70044	E657	Y	N	…
CS70045	E658	N	…	…
CS70046	E668	N	…	…
CS70047	E698	N	…	…
CS70048	E700	N	…	…
CS70049	E719	N	…	…
CS70050	E744	N	…	…
CS70051	E771	N	…	…
CS70052	E790	N	…	…
CS70053	E835	N	…	…
CS70054	E838	N	…	…
CS70055	E861	Y	Y	Y
CS70056	E864	N	…	…
CS70057	E876	N	…	…

CS70058	E884	N	…	…
CS70059	E892	N	…	…
CS70060	E894	N	…	…
CS70061	E903	N	…	…
CS70062	E910	N	…	…
CS70063	E912	N	…	…
CS70065	E939	N	…	…
CS70066	E940	N	…	…
CS70067	E945	N	…	…
CS70068	E951	N	…	…
CS70069	E992	N	…	…
CS70070	E994	N	…	…
CS70071	E1049	N	…	…
CS70072	E1092	N	…	…
CS70073	E1100	N	…	…
CS70074	E1127	N	…	…
CS70075	E1128	N	…	…
CS70076	E1130	N	…	…
CS70077	E1155	N	…	…
CS70078	E1161	N	…	…
CS70079	E1176	N	…	…
CS70080	E1222	N	…	…
CS70081	E1223	N	…	…
CS70082	E1237	N	…	…
CS70083	E1238	N	…	…
CS70084	E1250	N	…	…
CS70085	E1252	N	…	…
CS70086	E1271	N	…	…
CS70087	E1289	Y	N	…
CS70088	E1304	N	…	…
CS70089	E1322	N	…	…

CS70090	E1325	N	…	…
CS70091	E1331	N	…	…
CS70092	E1341	N	…	…
CS70093	E1344	N	…	…
CS70094	E1356	N	…	…
CS70095	E1361	N	…	…
CS70096	E1362	N	…	…
CS70097	E1370	N	…	…
CS70098	E1387	N	…	…
CS70099	E1388	N	…	…
CS70100	E1395	N	…	…
CS70101	E1396	N	…	…
CS70102	E1405	N	…	…
CS70103	E1416	N	…	…
CS70104	E1439	N	…	…
CS70105	E1439m	N	…	…
CS70106	E1457	N	…	…
CS70107	E1567	N	…	…
CS70108	E1570	N	…	…
CS70109	E1607	N	…	…
CS70110	E1626	N	…	…
CS70111	E1627	N	…	…
CS70112	E1628	N	…	…
CS70113	E1638	N	…	…
CS70114	E1644	N	…	…
CS70115	E1662	N	…	…
CS70116	E1663	Y	N	…
CS70117	E1665	N	…	…
CS70118	E1678	N	…	…
CS70119	E1684	N	…	…
CS70120	E1689	N	…	…

CS70121	E1691	N	…	…
CS70122	E1701	N	…	…
CS70123	E1728	N	…	…
CS70125	E1751	N	…	…
CS70126	E1765	N	…	…
CS70127	E1767	N	…	…
CS70128	E1785	N	…	…
CS70129	E1786	N	…	…
CS70130	E1797	N	…	…
CS70131	E1801	N	…	…
CS70132	E1809	N	…	…
CS70133	E1815	N	…	…
CS70134	E1817	N	…	…
CS70135	E1818	N	…	…
CS70136	E1819	N	…	…
CS70137	E1825	N	…	…
CS70138	E1828	N	…	…
CS70139	E1832	N	…	…
CS70140	E1833	N	…	…
CS70141	E1853	N	…	…
CS70142	E1868	N	…	…
CS70143	E1950	N	…	…
CS70144	E1998	N	…	…
CS70145	E2034	N	…	…
CS70146	E217	N	…	…
CS70147	E562	N	…	…
CS70148	E1001	N	…	…
CS70149	E1368	N	…	…
CS70150	E1690	N	…	…
CS70151	E1704-1	N	…	…
CS70152	E1704-3	N	…	…

CS70153	E1715	N	…	…
CS70154	E1723	N	…	…
CS70155	E1735	N	…	…
CS70156	E1935	N	…	…
CS70157	E1967	N	…	…
CS70158	E2014	N	…	…
CS70159	E2057	N	…	…
CS70160	E2207	N	…	…
CS70161	E2406	N	…	…
CS70162	E2408	Y	Y	Y
CS70163	E2410	N	…	…
CS70164	E2415	N	…	…
CS70165	E2425	N	…	…
CS70166	E2425	N	…	…
CS70167	E2441	N	…	…
CS70168	E2443	N	…	…
CS70169	E2448	N	…	…
CS70170	E2491	N	…	…
CS70171	E2502	N	…	…
CS70172	E2513	N	…	…
CS70173	E2563	N	…	…
CS70174	E2609	N	…	…
CS70175	E2633	N	…	…
CS70176	E2676	N	…	…
CS70177	E2692	Y	N	…
CS70178	E2724	N	…	…
CS70179	E2763	N	…	…
CS70180	E2764	N	…	…
CS70181	E2779	N	…	…
CS70182	E2861	N	…	…
CS70183	E2862	N	…	…

CS70184	E2897	N	…	…
CS70185	E2904	N	…	…
CS70186	E2905	N	…	…
CS70187	E2947	N	…	…
CS70188	E2993	N	…	…
CS70189	E3004	N	…	…
CS70190	E3006	N	…	…
CS70191	E3017	N	…	…
CS70192	E3065	N	…	…
CS70193	E3134	N	…	…
CS70194	E3190	N	…	…
CS70195	E3198	N	…	…
CS70196	E3258	N	…	…
CS70197	E3267	N	…	…
CS70198	E3298	N	…	…
CS70199	E3313	N	…	…
CS70200	E3317	Y	Y	N
CS70201	E3430	N	…	…
CS70202	E3459	N	…	…
CS70203	E3462	N	…	…
CS70204	E3474	N	…	…
CS70205	E3478	N	…	…
CS70206	E3501	N	…	…
CS70207	E3505	N	…	…
CS70208	E3530	N	…	…
CS70209	E3531	N	…	…
CS70210	E3598-1	N	…	…
CS70211	E3598-2	N	…	…
CS70212	E3637	N	…	…
CS70213	E3642	N	…	…
CS70214	E3655	Y	N	…

CS70215	E3683	N	…	…
CS70216	E3700	N	…	…
CS70217	E3754	N	…	…
CS70218	E3756	N	…	…
CS70219	E3783	Y	N	…
CS70220	E3806	N	…	…
CS70221	E3816	N	…	…
CS70222	E3826	N	…	…
CS70223	E3876	N	…	…
CS70224	E3879	N	…	…
CS70225	E3880	N	…	…
CS70226	E3885	Y	N	…
CS70227	E3912	Y	Y	Y
CS70228	E3927	N	…	…
CS70229	E3930	Y	N	…
CS70230	E3963	N	…	…
CS70231	E3980	N	…	…
CS70232	E4009	N	…	…
CS70233	E4028	Y	N	…
CS70234	E4058	N	…	…
CS70235	E4096	N	…	…
CS70236	E4104	N	…	…
CS70237	E4105	N	…	…
CS70238	E4110	N	…	…
CS70239	E4118	Y	N	…
CS70240	E4129	N	…	…
CS70241	E4148	N	…	…
CS70242	E4150	N	…	…
CS70243	E4151	N	…	…
CS70244	E4162	N	…	…
CS70245	E4223	N	…	…

CS70246	E4247	N	…	…
CS70247	E4256	N	…	…
CS70248	E4272	N	…	…
CS70249	E4285	N	…	…
CS70250	E4295	Y	Y	Y
CS70251	E4350	N	…	…
CS70252	E4396	N	…	…
CS70253	E4411	N	…	…
CS70254	E4423	N	…	…
CS70255	E4491	N	…	…
CS70256	E4506	Y	N	…
CS70257	E4522	Y	N	…
CS70258	E4583	N	…	…
CS70259	E4589	N	…	…
CS70260	E4633	N	…	…
CS70261	E4680	N	…	…
CS70262	E4695	N	…	…
CS70263	E4715	N	…	…
CS70264	E4716	Y	Y	Y
CS70265	E4722	Y	Y	Y
CS70266	E4751	N	…	…
CS70267	E4791	N	…	…
CS70268	E4801	N	…	…
CS70269	E4811	N	…	…
CS70270	E4812	N	…	…
CS70271	E4820	N	…	…
CS70272	E4856	Y	N	…
CS70273	E4907	N	…	…
CS70274	E4930	N	…	…
CS70275	E4940	N	…	…
CS70276	E4970	N	…	…

CS70277	E5008	N	…	…
CS70278	E5025	N	…	…
CS70279	E5026	N	…	…
CS70280	E5085	N	…	…
CS70281	E5096	Y	N	…

N, no. Y, yes.

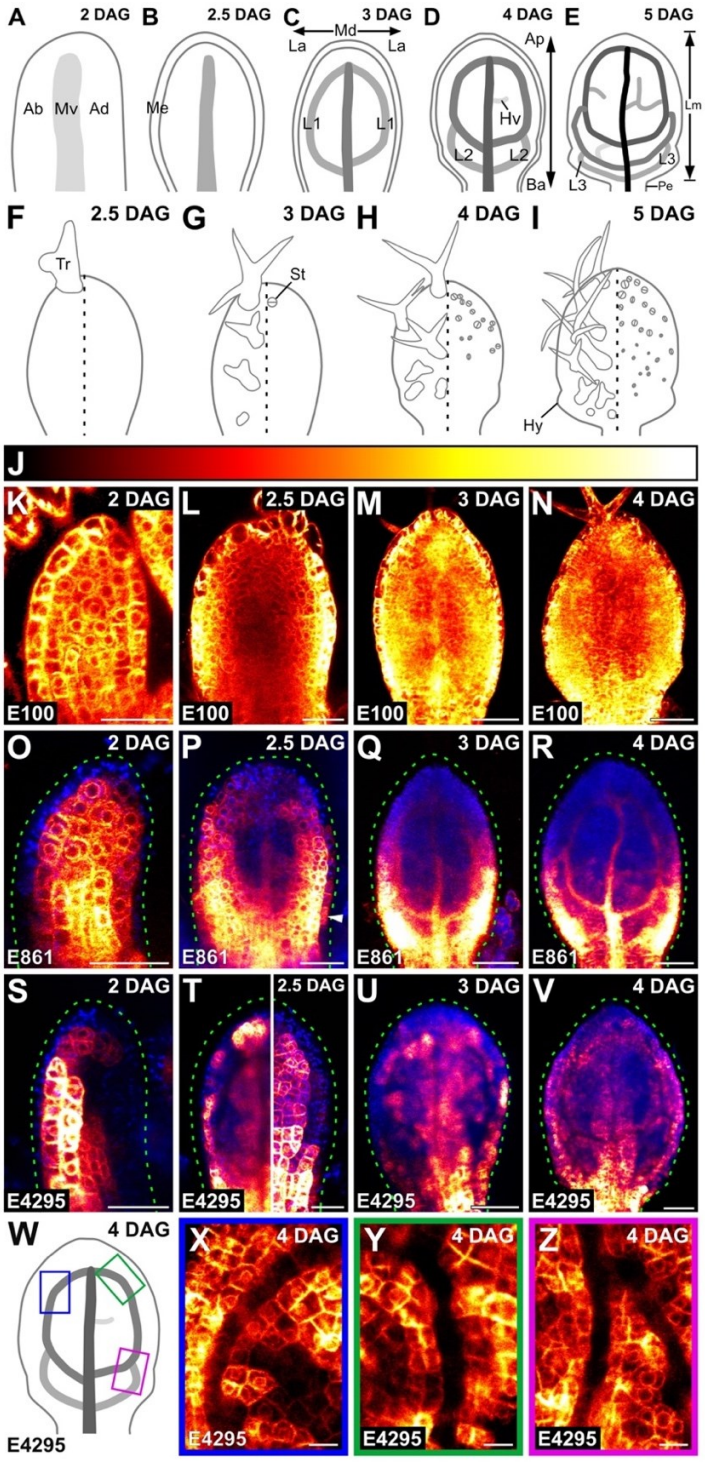

Figure 4.1. Expression of E100>>, E861>> and E4295>>erGFP in Arabidopsis Leaf Development. (A–Z) First leaves. Top right: leaf age in days after germination (DAG). (A–E) Development of leaf and veins; increasingly darker grays depict progressively later stages of vein development. See text for details. (F–I) Development of trichomes and stomata in

adaxial (left) or abaxial (right) epidermis. See text for details. Ab: abaxial; Ad: adaxial; Ap: apical; Ba: basal; Hv: minor vein; Hy: hydathode; L1, L2 and L3: first, second and third loop; La: lateral; Lm: lamina; Md: median; Me: marginal epidermis; Mv: midvein; Pe: petiole; St: stoma; Tr: trichome. (K–V,X–Z) Confocal laser scanning microscopy. Bottom left: genotype. Look-up table (ramp in J) visualizes erGFP expression levels. Blue: autofluorescence. Dashed green line delineates leaf outline. White arrowhead points to epidermal expression. (K–S,U,V,X–Z) Median view (abaxial side to the left in K). (T) Median (left) and abaxial subepidermal (right) views. (W) Increasingly darker grays depict progressively later stages of vein development. Boxes illustrate positions of closeups in X, Y and Z. See Table 4.2 for reproducibility of expression features. Bars: (K,L,O,P,S,T) 30 μm; (M,N,Q,R,U,V) 60 μm; (X–Z) 10 μm

Table 4.2. Reproducibility of Expression and Pattern Features.

Figure	Panel	No. leaves with displayed features / no. analyzed leaves	Assessed expression or pattern features
1	K	15/18	Ubiquitous
1	L	15/17	Ubiquitous
1	M	19/19	Ubiquitous
1	N	33/33	Ubiquitous
1	O	26/29	Inner cells
1	P	29/29	Vascular cells in top half of primordium, inner cells in basal half of primordium
1	Q	31/31	Vascular cells in top half of primordium, inner cells in basal half of primordium
1	R	19/19	Vascular cells in top half of leaf, inner cells in basal half of leaf
1	S	16/19	Abaxial inner cells
1	T	34/36	Abaxial inner cells & middle tissue layer
1	U	24/25	Abaxial inner cells & middle tissue layer
1	V	34/34	Abaxial inner cells & middle tissue layer
1	X	14/14	Inner, nonvascular cells
1	Y	14/14	Inner, nonvascular cells
1	Z	14/14	Inner, nonvascular cells

2	A	15 (adaxial) or 26 (abaxial) / 28	Top third of adaxial epidermis & whole abaxial epidermis
2	B, left	22/23	Top three-quarters of epidermis & trichomes
2	B, right	30/30	Whole epidermis
2	C, left	14/14	Top three-quarters of epidermis & trichomes
2	C, right	15/15	Whole epidermis
2	D, left	16/16	Epidermis of whole lamina and petiole midline & trichomes
2	D, right	18/18	Whole epidermis
2	E	16/16	Trichomes
2	F	17/18	Top three-quarters of marginal epidermis
2	G	14/14	Whole marginal epidermis
2	H	16/16	Whole marginal epidermis
2	I	59/59	Whole epidermis
2	J, left	42/42	All cells of marginal epidermis, except few cells in top half of primordium
2	J, right	45/45	Whole epidermis
2	K, left	33/38	Bottom quarter and few cells in top three-quarters of marginal epidermis

2	K, right	21/21	Whole epidermis, including stomata
2	L, left	31/31	Bottom quarter and few cells in top three-quarters of marginal epidermis
2	L, right	21/21	Whole epidermis, including stomata
2	M	29/30	Absent
2	N	26/26	Top quarter of primordium
2	O	18/18	Top three-quarters of primordium
2	P	18/18	Whole leaf
2	Q	31/33	Absent
2	R	19/21	Top quarter of primordium
2	S	23/28	Top half of lamina
2	T	16/18	Top three-quarters of lamina
3	A	22/22	Midvein
3	B	30/30	Midvein
3	C	16/17	Midvein & first loop
3	D	34/48	Midvein & first and second loop
3	E	25/25	Absent
3	F	20/20	Midvein
3	G	27/37	Midvein & first loop
3	H	24/28	Midvein & first and second loop

4	A	ND	Narrow midvein & scalloped vein-network outline
4	B	19/20	Shapeless vascular cluster
4	C	32/46	Midvein & first and second loop
4	D	21/21	Shapeless vascular domain
4	E	16/23	Midvein & first and second loop
4	F	18/18	Broad vascular domain
4	G	21/21	Narrow midvein & scalloped vein-network outline
4	H	19/19	Broad vascular zone

ND: not determined

the base of the lamina (Fig. 4.1E). Leaf hairs (trichomes) and pores (stomata) can be first recognized at the tip of 2.5- and 3-DAG primordia, respectively, and their formation spreads toward the base of the lamina during leaf development (Fig. 4.1F–I).

Consistent with previous observations (Huang et al., 2014), E100>>erGFP was expressed in all the cells of 2-, 2.5-, 3-, and 4-DAG leaf primordia (Fig. 4.1K–N).

Consistent with previous observations(Krogan and Berleth, 2012), E861>>erGFP was expressed in all the inner cells of the 2-DAG primordium, though more strongly in its innermost cells (Fig. 4.1O). At 2.5 DAG, expression had been activated in the lowermost epidermal cells of the primordium margin and persisted in all the inner cells of the bottom half of the primordium; in the top half of the primordium, weaker expression persisted in inner cells, except near the midvein, where by then it had been terminated (Fig. 4.1P). At 3 DAG, expression continued to persist in all the inner cells of the bottom half of the primordium, though expression was stronger in the areas where second loops were forming; in the top half of the primordium, weaker expression had become restricted to the midvein, first loops and minor veins (Fig. 4.1Q). At 4 DAG, expression in the top half of the leaf remained restricted to the midvein, first loops and minor veins, and in the bottom half of the leaf it had declined in inner cells between the first loops and the developing second loops (Fig. 4.1R). In summary, E861>>erGFP was expressed ubiquitously at early stages of inner-cell development; over time, however, expression became restricted to developing veins. As such, expression of E861>>erGFP closely resembles that of *MONOPTEROS* and *PIN-FORMED1*, which marks the gradual selection of vascular cells from within the leaf inner tissue (Scarpella et al., 2006; Wenzel et al., 2007).

E4295>>erGFP expression was restricted to inner cells in 2-, 2.5-, 3-, and 4-DAG leaf primordia (Fig. 4.1S–V,X–Z). At 2 DAG, E4295>>erGFP was expressed almost exclusively in the inner cells of the abaxial side of the primordium (Fig. 4.1S), but by 2.5 DAG it had spread to the middle tissue layer (Fig. 4.1T), from which veins form (Stewart, 1978; Tilney-Bassett, 1986). Expression persisted in the inner cells of the abaxial side and of the middle tissue layer in 3- and 4- DAG primordia (Fig. 4.1U,V). High-resolution images of the middle tissue layer showed that expression was excluded from developing veins (Fig.

4.1X–Z), suggesting that it marks inner, non-vascular cells. Therefore, expression of E4295>>erGFP closely resembles that of *LIGHT HARVESTING COMPLEX A6* and *SCARECROW-LIKE32* (Sawchuk et al., 2008; Gardiner et al., 2011), and that of J0571>>erGFP in the C24 background (Wenzel et al., 2012).

At 2 DAG, E4259>>erGFP was expressed in the top third of the median adaxial epidermis and in the whole median abaxial epidermis, though expression was stronger in the top half of the primordium (Fig. 4.2A). By 2.5 DAG, strong expression had spread to the whole abaxial and to the top three-quarters of the marginal epidermis; expression had spread to the top three-quarters of the adaxial epidermis too, but it was stronger in the top half of the primordium (Fig. 4.2B,F). At 3 DAG, strong expression had spread to the top three-quarters of the adaxial epidermis and to the whole marginal epidermis, and persisted in the whole abaxial epidermis (Fig. 4.2C,G). At 4 DAG, expression persisted in the whole marginal epidermis, continued to persist in the whole abaxial epidermis, and had spread to the whole lamina and the petiole midline in the adaxial epidermis (Fig. 4.2D,H). At all analyzed stages, E4259>>erGFP was expressed in trichomes but was not expressed in mature stomata (Fig. 4.2B–H). In conclusion, expression of E4259>>erGFP closely resembles that of *ARABIDOPSIS THALIANA MERISTEM LAYER1* (Lu et al., 1996; Sessions et al., 1999), which marks epidermal cells and whose promoter is used to drive epidermis-specific expression (e.g., Takada and Jürgens, 2007; Bilsborough et al., 2011; Kierzkowski et al., 2013).

E4722>>erGFP was expressed in all the epidermal cells of the 2-DAG primordium, though more weakly at its tip (Fig. 4.2I). E4722>>erGFP was expressed in all the epidermal cells of the 2.5-DAG primordium too, except at its margin, where expression had been terminated in a few cells of its top half (Fig. 4.2J). At 3 DAG, expression persisted in all the epidermal cells, except at the primordium margin, where expression had been terminated in most of the cells of its top three-quarters (Fig. 4.2K). At 4 DAG, expression continued to persist in all the epidermal cells, except at the leaf margin, where expression had almost completely been terminated in the cells of its top three-quarters (Fig. 4.2L). Unlike E4259>>erGFP, E4722>>erGFP was expressed in stomata but was not expressed in

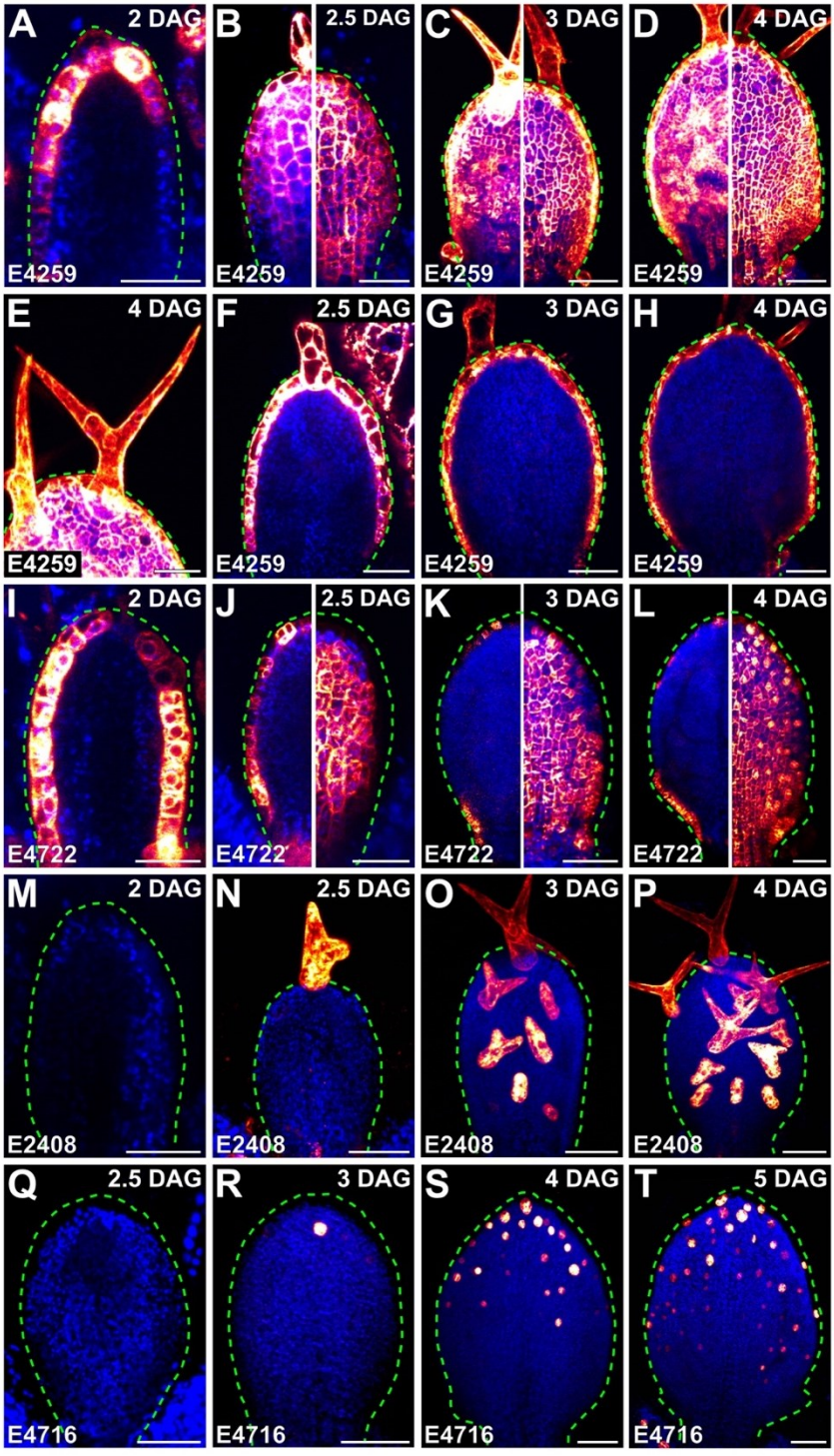

Figure 4.2. Expression of E4259>>, E4722>>, E2408>> and E4716>>erGFP in Leaf Development. (A–T) Confocal laser scanning microscopy. First leaves. Top right: leaf age in days after germination (DAG). Bottom left: genotype. Look-up table (ramp in Fig. 4.1J) visualizes erGFP expression levels. Blue: autofluorescence. Dashed green line delineates

leaf outline. (A,F–I,M) Median view (abaxial side to the left in A,I,M). (B–D) Adaxial (left) and abaxial (right) epidermal views. (E) Closeup of trichome in D, left. (J–L) Median (left) and abaxial epidermal (right) views. (N–P) Adaxial epidermal view. (Q–T) Abaxial epidermal view. See Table 4.2 for reproducibility of expression features. Bars: (A,B,F,I,J,M,N,Q) 30 μm; (C,D,E,G,H,K,L,O,P,R,S,T) 60 μm.

trichomes (Fig. 4.2J–L). At all analyzed stages, expression of E2408>>erGFP and E4716>>erGFP was restricted to trichomes and stomata, respectively. E2408>>erGFP was first expressed in developing trichomes at the tip of the 2.5-DAG primordium (Fig. 4.2M,N). By 3 DAG, expression had spread to developing and mature trichomes in the top three-quarters of the primordium (Fig. 4.2O), and by 4 DAG to those in the whole lamina (Fig. 4.2P). E4716>>erGFP was first expressed in stomata at the tip of the 3-DAG primordium (Fig. 4.2Q,R). By 4 DAG, expression had spread to the stomata in the top half of the lamina (Fig. 4.2S), and by 5 DAG to those in its top three-quarters (Fig. 4.2T).

At all analyzed stages, expression of E2331>>erGFP and E3912>>erGFP was restricted to developing veins. E2331>>erGFP was expressed in both isodiametric and elongated cells of the midvein in 2- and 2.5-DAG primordia (Fig. 4.3A,B). By 3 DAG, it was expressed in first loops, and by 4 DAG in second loops and minor veins (Fig. 4.3C,D). E3912>>erGFP was first expressed in the midvein of the 3-DAG primordium (Fig. 4.3E,F). By 4 DAG, expression had spread to first loops, and by 5 DAG to second loops and minor veins (Fig. 4.3G,H). These observations suggest that expression of E3912>>erGFP is initiated later than that of E2331>>erGFP in vein development. Furthermore, because the expression of E2331>>erGFP appears to be no different from that of the preprocambial markers ATHB8::nYFP, J1721>>erGFP and SHR::nYFP (Sawchuk et al., 2007; Donner et al., 2009; Gardiner et al., 2011), we suggest that E2331>>erGFP expression marks preprocambial stages of vein development, a conclusion that is consistent with E2331>>erGFP expression during embryogenesis (Gillmor et al., 2010). Finally, because E3912>>erGFP expression appears to be no different from that of the procambial marker Q0990>>erGFP in the C24 background (Sawchuk et al., 2007), we suggest that E3912>>erGFP expression marks procambial stages of vein development. To show the informative power of the lines reported here for plant developmental biology, we selected the E2331 line, which marks early stages of vein development (Fig. 4.3A–D).

In WT leaves, the elongated vascular cells are connected to one another into continuous veins (Esau, 1965) (Fig. 4.4A). By contrast, in mature leaves of the *gnom* (*gn*) mutant, putative vascular cells fail to elongate and to connect to one another into continuous

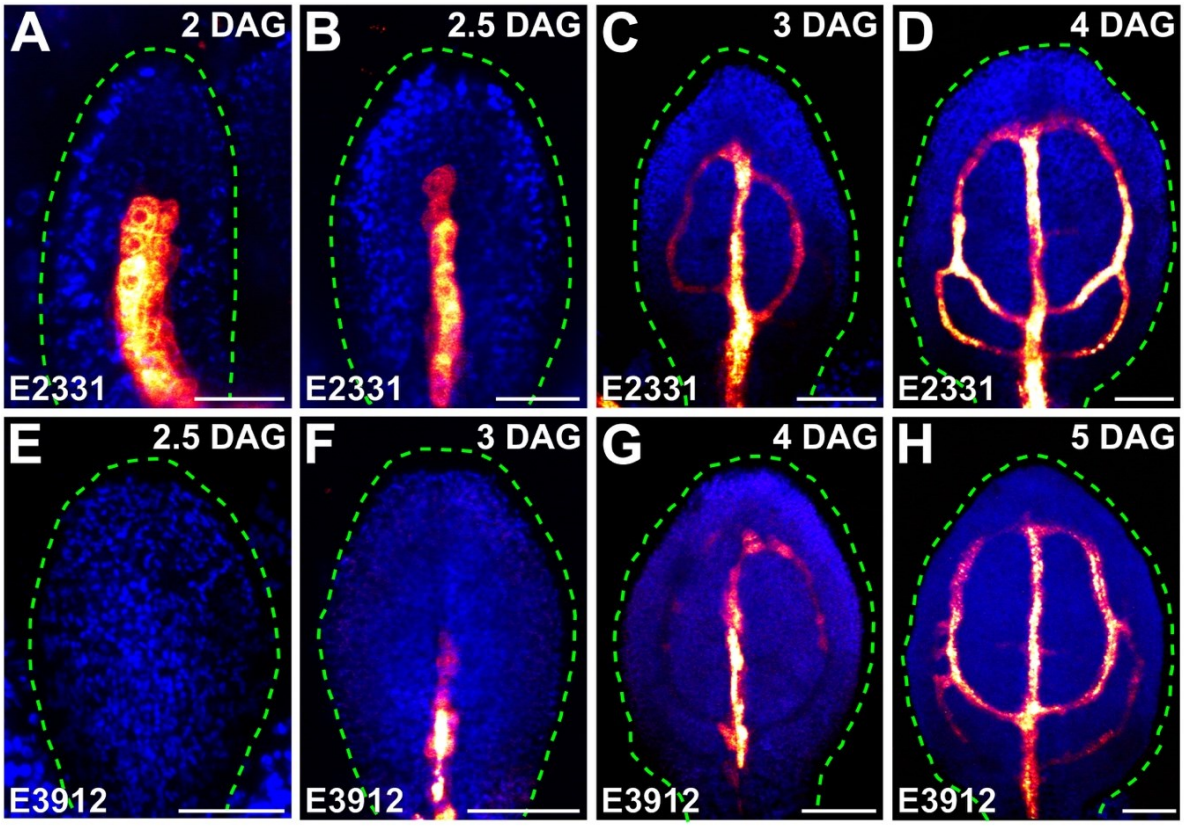

Figure 4.3. Expression of E2331>> and E3912>>erGFP in Leaf Development. (A–H) Confocal laser scanning microscopy. First leaves. Top right: leaf age in days after germination (DAG). Bottom left: genotype. Look-up table (ramp in Fig. 4.1J) visualizes erGFP expression levels. Blue: autofluorescence. Dashed green line delineates leaf outline. Median view (abaxial side to the left in A). See Table 4.2 for reproducibility of expression features. Bars: (A,B,E) 30 µm; (C,D,F–H) 60 µm.

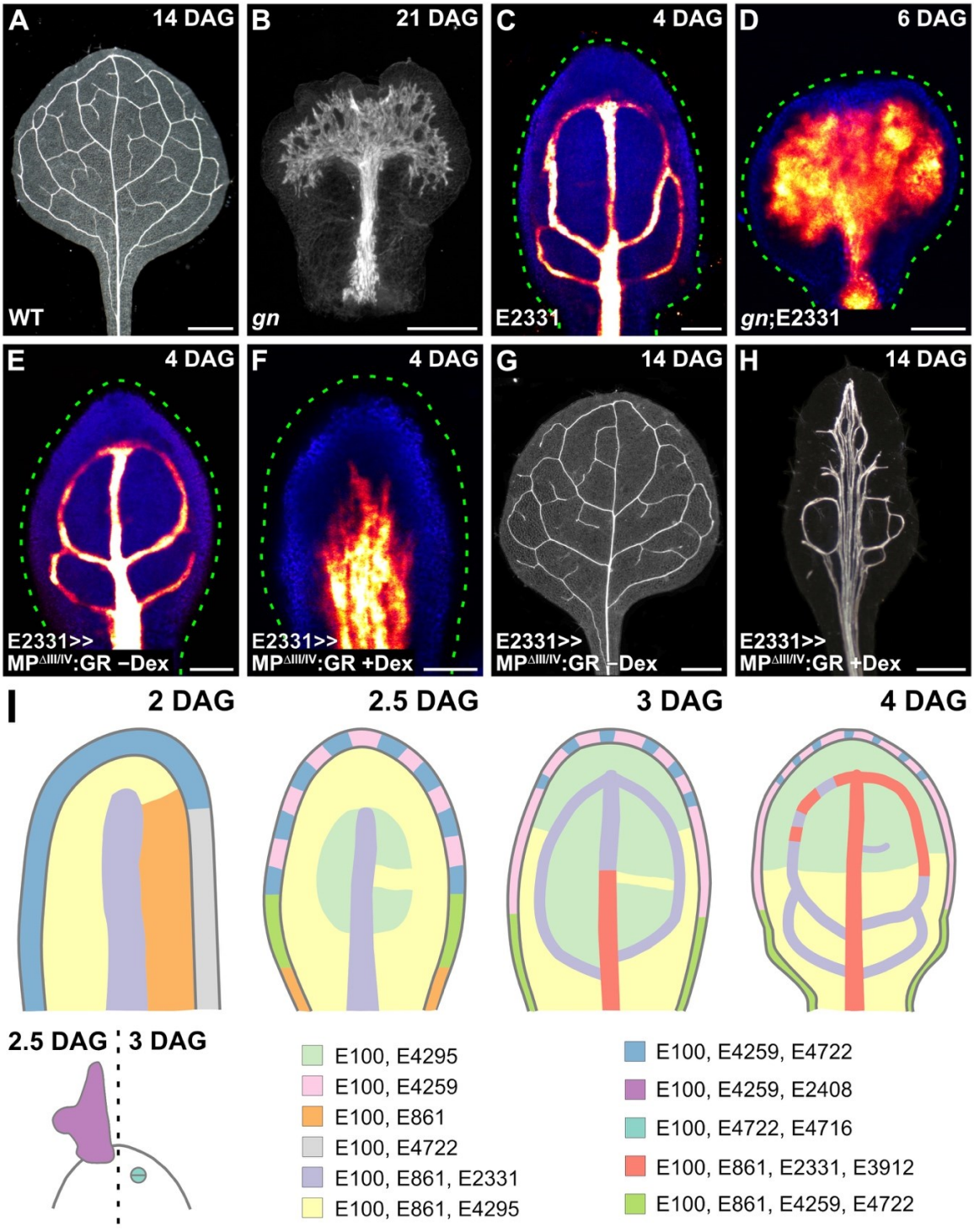

Figure 4.4. E2331-Mediated Visualization and Manipulation of Developing Veins. (A–H) First leaves. Top right: leaf age in days after germination (DAG). Bottom left: genotype and treatment. (A,B,G,H) Dark-field microscopy of cleared leaves. (C–F) Confocal laser scanning microscopy. Look-up table (ramp in Fig. 4.1J) visualizes erGFP expression levels.

Blue: autofluorescence. Dashed green line delineates leaf outline. Median view. See Table 4.2 for reproducibility of expression and pattern features. (I) Expression map of E100>>, E861>>, E4295>>, E4259>>, E4722>>, E2408>>, E4716>>, E2331>> and E3912>>erGFP in leaf development. See text for details. Bars: (A,B,G,H) 500 µm; (C–F) 60 µm.

veins; instead, they accumulate into shapeless clusters of seemingly disconnected and randomly oriented cells (Shevell et al., 2000; Verna et al., 2019) (Fig. 4.4B). Though the cells in these clusters have some features of vascular cells (e.g., distinctive patterns of secondary cell-wall thickenings), they lack others (e.g., elongated shape and end-to-end connection to form continuous veins). Therefore, it is unclear whether the clustered cells in *gn* mature leaves are abnormal vascular cells or nonvascular cells that have recruited a cellular differentiation pathway that is normally, but not always (e.g., Solereder, 1908; Kubo et al., 2005; Yamaguchi et al., 2010), associated with vascular development. To address this question, we imaged E2331>>erGFP expression in developing leaves of WT and *gn*.

As shown above (Fig. 4.3D), E2331>>erGFP was expressed in midvein, first and second loops, and minor veins in WT (Fig. 4.4C). In *gn*, the pattern of E2331>>erGFP expression in developing leaves recapitulated that of vascular differentiation in mature leaves (Fig. 4.4B,D), suggesting that the putative vascular cells in the shapeless clusters are indeed vascular cells, albeit abnormal ones.

Auxin signaling is thought to be required for vein formation because mutations in genes involved in auxin signaling or treatment with inhibitors of auxin signaling leads to the formation of fewer, incompletely differentiated veins (Przemeck et al., 1996; Hardtke and Berleth, 1998; Mattsson et al., 2003; Verna et al., 2019). Increasing auxin signaling by means of broadly expressed mutations or transgenes turns nearly every cell file in the developing leaf into a vein, suggesting that auxin signaling is also sufficient for vein formation (Garrett et al., 2012; Krogan et al., 2012). This interpretation assumes that it is the increased auxin signaling in the cell files that normally would not differentiate into veins that leads those cell files to differentiate in fact into veins.

However, it is also possible that it is the increased auxin signaling in the cell files that normally differentiate into veins that leads the flanking cell files, which normally would not differentiate into veins, to do in fact so. To discriminate between these possibilities, we increased auxin signaling in developing veins by expressing by the E2331 driver a dexamethasone (dex)-inducible MPΔIII/IV (Krogan et al., 2012; Ckurshumova et al., 2014; Smetana et al., 2019) (MPΔIII/IV:GR), and we imaged E2331>>erGFP expression in

developing leaves and vein patterns in mature leaves of E2331>>MPΔIII/IV:GR grown with or without dex.

Consistent with previous observations (Fig. 4.3D; Fig. 4.4C), in developing leaves of E2331>>MPΔIII/IV:GR grown without dex, E2331>>erGFP was expressed in narrow domains (Fig. 4.4E). By contrast, E2331>>erGFP was expressed in broad domains in developing leaves of dex-grown E2331>>MPΔIII/IV:GR (Fig. 4.4F). Whether with or without dex, the patterns of E2331>>erGFP expression in developing leaves of E2331>>MPΔIII/IV:GR presaged those of vein formation in mature leaves: narrow zones of vein formation in the absence of dex; broad areas of vascular differentiation in the presence of dex, often with multiple veins running parallel next to one another (Fig. 4.4G,H). Though the areas of vascular differentiation in dex-grown E2331>>MPΔIII/IV:GR are not as broad as those of leaves in which MPΔIII/IV is expressed in all the inner cells (Krogan et al., 2012), they are broader than those of E2331>>MPΔIII/IV:GR grown without dex. These observations suggest that, at least in part, it is the increased auxin signaling in the cell files that normally differentiate into veins that leads the flanking cell files, which normally would not differentiate into veins, to do in fact so.

In conclusion, we provide a set of GAL4/GFP enhancer-trap lines in the Col-0 background of Arabidopsis for the specific labeling of cells and tissues during leaf development (Fig. 4.4I), and we show that these lines can be used to address key questions in plant developmental biology.

4.3 Materials & Methods

4.3.1 Plants

Origin and nature of GAL4 enhancer-trap lines are in Table 4.1. *gn-13* (SALK_045424; ABRC) (Alonso et al., 2003; Verna et al., 2019) contains a T-DNA insertion after nucleotide +2835 of *GN* and was genotyped with the "SALK_045424 gn LP" (5'-TGATCCAAATCACTGGGTTTC-3') and "SALK_045424 gn RP" (5'-AGCTGAAGATAGGGAATTCGC-3') oligonucleotides (*GN*) and with the "SALK_045424 gn RP" and "LBb1.3" (5'-ATTTTGCCGATTTCGGAAC-3')

oligonucleotides (*gn*). To generate the UAS::MPΔIII/IV:GR construct, the UAS promoter was amplified with the "UAS Promoter SalI Forward" (5'-ATAGTCGACCCAAGCGCGCAATTAACCCTCAC-3') and the "UAS Promoter XhoI Reverse" (5'-AGCCTCGAGCCTCTCCAAATGAAATGAACTTCC-3') oligonucleotides; MPΔIII/IV was amplified with the "MP Delta XhoI Forward" (5'-AAACTCGAGATGATGGCTTCATTGTCTTGTGTT-3') and the "MP EcoRI Reverse" (5'-ATTGAATTCGGTTCGGACGCGGGGTGTCGCAATT-3') oligonucleotides; and a fragment of the rat glucocorticoid (GR) receptor gene was amplified with the "SpeI GR Forward" (5-'GGGACTAGTGGAGAAGCTCGAAAAACAAAG-3') and the "GR ApaI Reverse" (5'-GCGGGGCCCTCATTTTTGATGAAACAG-3') oligonucleotides. Seeds were sterilized and sown as in (Sawchuk et al., 2008). Stratified seeds were germinated and seedlings were grown at 22°C under continuous fluorescent light (~80 µmol m^{-2} s^{-1}). Plants were grown at 24°C under fluorescent light (~85 µmol m^{-2} s^{-1}) in a 16-h-light/8-h-dark cycle. Plants were transformed and representative lines were selected as in (Sawchuk et al., 2008).

4.3.2 Chemicals

Dexamethasone (Sigma-Aldrich, catalogue no. D4902) was dissolved in dimethyl sulfoxide and was added to growth medium just before sowing.

4.3.3 Imaging

Developing leaves were mounted and imaged as in (Sawchuk et al., 2013), except that emission was collected from ~1.5–5-µm-thick optical slices. Fluorophores were excited with the 488-nm line of a 30-mW Ar laser; GFP emission was collected with a BP 505–530 filter, and autofluorescence was collected between 550 and 754 nm. Mature leaves were fixed in 3 : 1 or 6 : 1 ethanol : acetic acid, rehydrated in 70% ethanol and in water, cleared briefly (few seconds to few minutes) — when necessary — in 0.4 M sodium hydroxide, washed in water, mounted in 80% glycerol or in 1 : 2 : 8 or 1 : 3 : 8 water : glycerol : chloral hydrate, and imaged as in (Odat et al., 2014). In the Fiji distribution (Schindelin et al., 2012) of ImageJ, (Schneider et al., 2012; Schindelin et al., 2015; Rueden et al., 2017) grayscaled RGB color images were turned into 8-bit images; when necessary, 8-bit images were combined into stacks, and maximum-intensity projection was applied to stacks; look-up-tables were applied

to images or stacks, and brightness and contrast were adjusted by linear stretching of the histogram.

Chapter 5: General Discussion

5.1 Conclusion Summary

The evidence discussed in Chapter 1 suggests that vein patterning is controlled by auxin transport and that auxin transport is in turn controlled by *PIN1*. PIN1 is expressed in all the cells of the leaf at early stages of tissue development; over time, however, epidermal expression becomes restricted to the basal-most cells, and inner-tissue expression becomes restricted to developing veins (Benkova et al., 2003; Reinhardt et al., 2003; Heisler et al., 2005; Scarpella et al., 2006; Hay et al., 2006; Wenzel et al., 2007; Bayer et al., 2009; Sawchuk et al., 2013; Marcos and Berleth, 2014). The scope of my M.Sc. thesis was to understand what the function in *PIN1*-dependent vein patterning were of PIN1 expression in the different tissues of the leaf, and what controlled that component of PIN1 expression that is required for *PIN1*-dependent vein patterning.

For the past 15 years, the prevailing hypotheses of vein patterning by auxin transport have proposed that in the epidermis of the developing leaf PIN1-mediated auxin transport converges toward peaks of auxin level. From those convergence points of epidermal PIN1 polarity, auxin would be transported in the inner tissues where it would give rise to the midvein and lateral veins. In Chapter 2, we tested predictions of this hypothesis and found them unsupported: epidermal PIN1 expression is neither required nor sufficient for *PIN1*-dependent vein patterning, whereas inner-tissue PIN1 expression turns out to be both required and sufficient for *PIN1*-dependent vein patterning. Our results refute all the vein patterning hypotheses that are based on auxin transport from the epidermis and suggest alternatives for future tests; for example, auxin could diffuse from the epidermis to the inner tissues through plasmodesmata.

In Chapter 3, we sought to identify cis-regulatory elements that are required for that component of PIN1 expression in the inner tissues of the leaf that is relevant to *PIN1*-dependent vein patterning. We found that vascular expression of PIN1 is required for *PIN1*-dependent vein patterning and that such vascular expression of PIN1 depends on the 151-bp region of the *PIN1* promoter from -645 to -495.

Testing the function in *PIN1*-dependent vein patterning of PIN1 expression in the different tissues of the leaf (Chapter 2) required expressing *PIN1* by different promoters. This imposed the burden of generating different constructs for different promoter::PIN1 combinations. This approach could be simplified if GAL4/GFP enhancer-trap lines existed in Columbia-0, the genotype of reference in Arabidopsis (Koornneef and Meinke, 2010), with which to drive expression of genes of interest in desired cells and tissues of developing leaves. Unfortunately, such lines were not available when I started my M.Sc.. In Chapter 4, we addressed this limitation and provided GAL4/GFP enhancer-trap lines in the Col-0 background of Arabidopsis for the identification and manipulation of cells and tissues in developing leaves.

In the discussions of the respective chapters, we provided an account of how we reached those conclusions from the experimental evidence, and how those conclusions could be integrated with one another and with those in studies by others to advance our understanding of vein patterning. Here I instead wish to propose and discuss a hypotheses on the upstream regulators of PIN1 functional expression in *PIN1*-dependent vein patterning. This hypothesis should be understood as an attempt to develop a conceptual framework to guide future experimentation and not as an exhaustive mechanistic account.

5.2 Hypothesis: Zinc Finger - Homeodomain Transcription Factors Regulate PIN1 Functional Expression in Vein Patterning

The results in Chapter 3 suggest that the region of the *PIN1* promoter between -645 and -495 is required for PIN1 functional expression in vein patterning. By manual inspection, I found that this promoter region contains two putative binding sites for transcription factors of the zinc finger - homeodomain (ZHD) family (Figure 5.1) (Tan and Irish, 2006), suggesting that a ZHD transcription factor is an upstream regulator of PIN1 functional expression in vein patterning. In Arabidopsis, the ZHD family is composed of 14 members (Tan and Irish, 2006). ZHD proteins have a conserved N-terminal zinc-finger domain that is

Figure 5.1. Putative ZHD Binding Sites in the *PIN1* Promoter. Sequence of the 151-bp region of the *PIN1* promoter between -645 and -495 that is required for *PIN1* function in vein network patterning. Highlight, putative ZHD transcription-factor binding sites identified by manual inspection.

required for zinc binding and a C-terminal domain that is distantly related to the homeodomain (Windhovel et al., 2001; Tan and Irish, 2006). Though the zinc-finger domain is not involved in DNA binding, it can enhance the interaction between DNA and the homeodomain (Windhovel et al. 2001).

Below I present evidence that is consistent with the hypothesis that the ZHD family of transcription factors regulate PIN1 functional expression in vein patterning.

5.2.1 Evidence from Expression Analysis

The most parsimonious expectation is that ZHD transcription factors and *PIN1* are expressed in overlapping domains. Though the precise site of expression of all the ZHD transcription factors is unknown, RNA in situ hybridization shows that, like *PIN1* (Benkova et al., 2003; Reinhardt et al., 2003; Heisler et al., 2005; Scarpella et al., 2006; Wenzel et al., 2007; Bayer et al., 2009), *ZHD5* is expressed in developing veins (Hu et al., 2008). Further, RT-PCR shows that 13 of the 14 ZHDs are expressed in young seedlings (Hu et al., 2008), which contain developing veins such as those in which PIN1 is expressed.

5.2.2 Evidence from DNA-Binding Studies

Electromobility shift assays show that ZHD5 binds to a core consensus sequence identical to those found in the region of the *PIN1* promoter between -645 and -495 (Fig. 5.1) (Tan & Irish, 2006). Furthermore, the soybean ZHD transcription factors *Glycine max* ZF-HD1 (GmZF-HD1) and GmZF-HD2 bind to that same core consensus sequence in the promoter of the *Glycine max Calmodulin4* (GmCaM4) (Park et al., 2007).

5.2.3 Evidence from Transcriptional Activation Studies

That the region of the *PIN1* promoter between -645 and -495 is required for PIN1 functional expression in leaf vein patterning suggests that that region is bound by a positive regulator of PIN1 expression. In Arabidopsis, ZHD1 binds to the promoter of its target gene *EARLY RESPONSE TO DEHYDRATION 1* (*ERD1*) and activates its expression (Tran et al., 2007). In Soybean, transient expression assays confirmed that GmZF-HD1 functions as an in vivo

transcriptional regulator of the *GmCaM4* gene (Park et al., 2007). These observations suggest that ZHD transcription factors positively regulate the expression of their targets.

5.2.4 Evidence from Genetic Analysis

The most parsimonious expectation is that mutation in a *ZHD* gene results in defects overlapping to those resulting from mutation in *PIN1*. Single mutants in nine *ZHD* genes showed no developmental defects, so currently there is no genetic evidence in support of the hypothesis; however, the evidence is not inconsistent with the hypothesis because the phenotype of mutants in the remaining five *ZHD* genes has not been analyzed yet, and the lack of defects in individual mutants may be the result of functional redundancy among *ZHD* genes (Tan & Irish, 2006).

5.3 Future Approach

The evidence presented above is consistent with the possibility that ZHD transcription factors regulate PIN1 functional expression in vein patterning. Here I wish to suggest how this possibility could be tested experimentally and what the next steps could be should those test instead suggest that ZHD transcription factors fail to regulate PIN1 functional expression in vein patterning.

5.3.1 Step 1

To test whether the putative ZHD binding-sites in the region of the *PIN1* promoter between -645 and -495 are required for PIN1 functional expression in vein patterning, I propose to mutate those putative binding sites to abolish ZHD binding and use the resulting mZHD[-645,-14] promoter fragment to drive PIN1 expression in the *pin1* mutant background. Should the putative ZHD binding sites be required for functional expression of PIN1 in vein patterning, the vein pattern defects of mZHD[-645,-14]::PIN1;*pin1* would be no different from those of *pin1*. By contrast, should those binding sites not be required for functional expression of PIN1 in vein patterning, the vein pattern of mZHD[-645,-14]::PIN1;*pin1* would be no different from that of WT.

5.3.2 Step 2

I propose to identify all the transcription factors that bind to the region of the *PIN1* promoter between -645 and -495 by using this sequence as a bait in a yeast one-hybrid screen.

5.3.3 Step 3

I propose to generate translational fusions to, for example, YFP of ZHD transcription factors or of the transcription factors identified in the yeast one-hybrid screen, and to test whether in leaves their domains of expression overlaps with the domain of activity of the [-645,-14] fragment of the *PIN1* promoter.

5.3.4 Step 4

I propose to use the translational fusions of the transcription factors whose expression domains overlap with the domain of activity of the [-645,-14] fragment of the *PIN1* promoter to test by chromatin immunoprecipitation using anti-YFP antibodies whether those transcription factors bind in vivo to that promoter fragment.

5.3.5 Step 5

I propose to identify mutants of the transcription factors whose expression domains overlap with the domain of activity of the [-645,-14] fragment of the *PIN1* promoter and that bind in vivo that promoter fragment. Should these transcription factors be non-redundantly required for functional expression of PIN1 in vein patterning, the vein pattern defects of those mutants would be similar to those of *pin1*. However, it is possible that the vein patterns of those mutants would be normal. Should that be so, I would test whether that is the result of functional redundancy among transcription factors belonging to the same family by generating and transforming into plants translational fusions between those transcription factors and the portable EAR (after ETHYLENE-RESPONSIVE-ELEMENT-BINDING-FACTOR-associated amphiphilic repression) repressor domain (Hiratsu et al., 2003) (TF:EAR). Should the normal vein pattern of those mutants be the result of functional redundancy among transcription factors belonging to the same family, TF:EAR transgenics would have vein pattern defects. Moreover, should these transcription factors be redundantly

required for functional expression of *PIN1* in vein patterning, the vein pattern defects of those TF:EAR transgenics would be similar to those of *pin1*.

I also propose to analyze the domain of activity of the [-645,-14] fragment of the *PIN1* promoter in the transcription factor mutants or TF:EAR transgenics. Should the transcription factors be non-redundantly required for the domain of activity of the [-645,-14] fragment of the *PIN1* promoter, the domain of activity of that promoter fragment in the transcription factor mutants would be similar to that of the [-494,-14] promoter fragment in WT. By contrast, should the transcription factors be redundantly required for the domain of activity of the [-645,-14] fragment of the *PIN1* promoter, the domain of activity of that promoter fragment in the transcription factor mutants would be similar to that of that promoter fragment in WT; should that be so, however, the domain of activity of that promoter fragment in the TF:EAR transgenics would be similar to that of the [-494,-14] promoter fragment in WT.

Further, I propose to study the genetic interaction between the transcription factor mutants or TF:EAR transgenics and *pin1*. Should the transcription factors be non-redundantly required for *PIN1* functional expression in vein patterning, I expect the vein pattern defects of the double mutants between *pin1* and those transcription factor mutants to be similar to those of the transcription factor mutants. By contrast, should the transcription factors be redundantly required for *PIN1* functional expression in vein patterning, I expect the vein pattern defects of the double mutant between *pin1* and the TF:EAR transgenics to be similar to or worse than those of the TF:EAR transgenics.

Finally, I propose to overexpress *PIN1* in the transcription factor mutants or TF:EAR transgenics by the promoter of the *MONOPTEROS* (Sawchuk et al., 2013) or *RIBOSOMAL PROTEIN S5A* (Weijers et al., 2001) genes, which are active in inner tissues of developing leaves, or by the ubiquitous E100 driver, the *PIN1*-like E861 driver, or the vascular E2331 and E3912 drivers (Chapter 4). I expect the leaf vein pattern defects of those mutants or transgenics to be, at least in part, rescued.

Literature Cited

Abley, K., Sauret-Güeto, S., Maree, A. F. & Coen, E. 2016. Formation of polarity convergences underlying shoot outgrowths. *Elife,* 5**,** e18165.

Alim, K. & Frey, E. 2010. Quantitative predictions on auxin-induced polar distribution of PIN proteins during vein formation in leaves. *The European Physical Journal E,* 33**,** 165-173.

Alonso-Peral, M. M., Candela, H., Del Pozo, J. C., Martínez-Laborda, A., Ponce, M. R. & Micol, J. L. 2006. The *HVE/CAND1* gene is required for the early patterning of leaf venation in Arabidopsis. *Development,* 133**,** 3755-3766.

Alonso, J. M., Stepanova, A. N., Leisse, T. J., Kim, C. J., Chen, H., Shinn, P., Stevenson, D. K., Zimmerman, J., Barajas, P. & Cheuk, R. 2003. Genome-wide insertional mutagenesis of *Arabidopsis thaliana*. *Science,* 301**,** 653-657.

Amalraj, B., Govindaraju, P., Krishna, A., Lavania, D., Linh, N. M., Ravichandran, S. J. & Scarpella, E. 2019. GAL4/GFP enhancer-trap lines for identification and manipulation of cells and tissues in developing Arabidopsis leaves. *bioRxiv,* 801357.

Bayer, E. M., Smith, R. S., Mandel, T., Nakayama, N., Sauer, M., Prusinkiewicz, P. & Kuhlemeier, C. 2009. Integration of transport-based models for phyllotaxis and midvein formation. *Genes & Development,* 23**,** 373-384.

Benková, E., Michniewicz, M., Sauer, M., Teichmann, T., Seifertová, D., Jürgens, G. & Friml, J. 2003. Local, efflux-dependent auxin gradients as a common module for plant organ formation. *Cell,* 115**,** 591-602.

Berg, R. 2004. Evaluation of spectral imaging for plant cell analysis. *Journal of Microscopy,* 214**,** 174-181.

Berleth, T., Mattsson, J. & Hardtke, C. S. 2000. Vascular continuity and auxin signals. *Trends in Plant Science,* 5**,** 387-393.

Bilsborough, G. D., Runions, A., Barkoulas, M., Jenkins, H. W., Hasson, A., Galinha, C., Laufs, P., Hay, A., Prusinkiewicz, P. & Tsiantis, M. 2011. Model for the regulation of *Arabidopsis thaliana* leaf margin development. *Proceedings of the National Academy of Sciences,* 108**,** 3424-3429.

Blilou, I., Xu, J., Wildwater, M., Willemsen, V., Paponov, I., Friml, J., Heidstra, R., Aida, M., Palme, K. & Scheres, B. 2005. The PIN auxin efflux facilitator network controls growth and patterning in Arabidopsis roots. *Nature,* 433**,** 39.

Brand, A. H. & Perrimon, N. 1993. Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development,* 118**,** 401-415.

Candela, H., Martınez-Laborda, A. & Micol, J. L. 1999. Venation pattern formation in *Arabidopsis thaliana* vegetative leaves. *Developmental Biology,* 205**,** 205-216.

Carraro, N., Forestan, C., Canova, S., Traas, J. and Varotto, S., 2006. *ZmPIN1a* and *ZmPIN1b* encode two novel putative candidates for polar auxin transport and plant architecture determination of maize. *Plant Physiology,* 142**,** 254-264.

Chapman, E. J. & Estelle, M. 2009. Mechanism of auxin-regulated gene expression in plants. *Annual Review of Genetics,* 43**,** 265-285.

Ckurshumova, W., Koizumi, K., Chatfield, S. P., Sanchez-Buelna, S. U., Gangaeva, A. E., Mckenzie, R. & Berleth, T. 2009. Tissue-specific GAL4 expression patterns as a resource enabling targeted gene expression, cell type-specific transcript profiling and gene function characterization in the Arabidopsis vascular system. *Plant and Cell Physiology,* 50**,** 141-150.

Ckurshumova, W., Smirnova, T., Marcos, D., Zayed, Y. & Berleth, T. 2014. Irrepressible MONOPTEROS/ARF 5 promotes de novo shoot formation. *New Phytologist,* 204**,** 556-566.

Crittenden, L., Bitgood, J., Burt, D., De Leon, F. P. & Tixier-Boichard, M. 1996. Nomenclature for naming loci, alleles, linkage groups and chromosomes to be used in poultry genome publications and databases. *Genetics Selection Evolution,* 28**,** 289.

Dalessandro, G. & Roberts, L. W. 1971. Induction of xylogenesis in pith parenchyma explants of Lactuca. *American Journal of Botany,* 58**,** 378-385.

Deb, Y., Marti, D., Frenz, M., Kuhlemeier, C. & Reinhardt, D. 2015. Phyllotaxis involves auxin drainage through leaf primordia. *Development,* 142**,** 1992-2001.

Donnelly, P. M., Bonetta, D., Tsukaya, H., Dengler, R. E. & Dengler, N. G. 1999. Cell cycling and cell enlargement in developing leaves of Arabidopsis. *Developmental Biology,* 215**,** 407-419.

Donner, T. J. & Scarpella, E. 2013. Transcriptional control of early vein expression of CYCA2;1 and CYCA2;4 in Arabidopsis leaves. *Mechanisms of Development,* 130**,** 14-24.

Donner, T. J., Sherr, I. & Scarpella, E. 2009. Regulation of preprocambial cell state acquisition by auxin signaling in Arabidopsis leaves. *Development,* 136**,** 3235-3246.

Esau, K. 1965. Plant anatomy. *Plant Anatomy.* 2nd Edition, John Wiley, New York.

Esteve-Bruna, D., Pérez-Pérez, J. M., Ponce, M. R. & Micol, J. L. 2013. *incurvata13*, a novel allele of AUXIN RESISTANT6, reveals a specific role for auxin and the SCF complex in Arabidopsis embryogenesis, vascular specification, and leaf flatness. *Plant Physiology,* 161**,** 1303-1320.

Foster, A. S. 1952. Foliar venation in angiosperms from an ontogenetic standpoint. *American Journal of Botany,* 752-766.

Friml, J., Benková, E., Blilou, I., Wisniewska, J., Hamann, T., Ljung, K., Woody, S., Sandberg, G., Scheres, B. & Jürgens, G. 2002a. AtPIN4 mediates sink-driven auxin gradients and root patterning in Arabidopsis. *Cell,* 108**,** 661-673.

Friml, J., Wiśniewska, J., Benková, E., Mendgen, K. & Palme, K. 2002b. Lateral relocation of auxin efflux regulator PIN3 mediates tropism in Arabidopsis. *Nature,* 415**,** 806.

Gälweiler, L., Guan, C., Müller, A., Wisman, E., Mendgen, K., Yephremov, A. & Palme, K. 1998. Regulation of polar auxin transport by *AtPIN1* in Arabidopsis vascular tissue. *Science,* 282**,** 2226-2230.

Gardiner, J., Donner, T. J. & Scarpella, E. 2011. Simultaneous activation of SHR and ATHB8 expression defines switch to preprocambial cell state in Arabidopsis leaf development. *Developmental Dynamics,* 240**,** 261-270.

Gardiner, J., Sherr, I. & Scarpella, E. 2010. Expression of *DOF* genes identifies early stages of vascular development in Arabidopsis leaves. *International Journal of Developmental Biology,* 54**,** 1389-1396.

Gardner, M. J., Baker, A. J., Assie, J.-M., Poethig, R. S., Haseloff, J. P. & Webb, A. A. 2009. GAL4 GFP enhancer trap lines for analysis of stomatal guard cell development and gene expression. *Journal of Experimental Botany,* 60**,** 213-226.

Garrett, J. J., Meents, M. J., Blackshaw, M. T., Blackshaw, L. C., Hou, H., Styranko, D. M., Kohalmi, S. E. & Schultz, E. A. 2012. A novel, semi-dominant allele of

MONOPTEROS provides insight into leaf initiation and vein pattern formation. *Planta,* 236**,** 297-312.

Gersani, M. 1987. Vessel differentiation along different tissue polarities. *Physiologia Plantarum,* 70**,** 516-522.

Gifford, E. & Foster, A. 1988. Coniferophyta. *Morphology and evolution of vascular plants, eds. D. Kennedy & RB Park***,** 401-453.

Gillmor, C. S., Park, M. Y., Smith, M. R., Pepitone, R., Kerstetter, R. A. & Poethig, R. S. 2010. The MED12-MED13 module of mediator regulates the timing of embryo patterning in Arabidopsis. *Development,* 137**,** 113-122.

Gordon, S. P., Heisler, M. G., Reddy, G. V., Ohno, C., Das, P. & Meyerowitz, E. M. 2007. Pattern formation during de novo assembly of the Arabidopsis shoot meristem. *Development,* 134**,** 3539-3548.

Goto, N. 1987. Effect of gibberellins on flower development of the pin-formed mutant of *Arabidopsis thaliana*. *Arabidopsis Information Service*, 23**,** 66-71.

Groszmann, M., Gonzalez-Bayon, R., Greaves, I. K., Wang, L., Huen, A. K., Peacock, W. J. & Dennis, E. S. 2014. Intraspecific Arabidopsis hybrids show different patterns of heterosis despite the close relatedness of the parental genomes. *Plant Physiology,* 166**,** 265-280.

Guilfoyle, T. J. & Hagen, G. 2007. Auxin response factors. *Current Opinion in Plant Biology,* 10**,** 453-460.

Halder, G., Callaerts, P. & Gehring, W. J. 1995. Induction of ectopic eyes by targeted expression of the eyeless gene in Drosophila. *Science,* 267**,** 1788-1792.

Hardtke, C. S. & Berleth, T. 1998. The Arabidopsis gene *MONOPTEROS* encodes a transcription factor mediating embryo axis formation and vascular development. *The EMBO Journal,* 17**,** 1405-1411.

Hartmann, F. P., De Reuille, P. B. & Kuhlemeier, C. 2019. Toward a 3D model of phyllotaxis based on a biochemically plausible auxin-transport mechanism. *PLoS Computational Biology,* 15**,** e1006896.

Haseloff, J. 1999. GFP variants for multispectral imaging of living cells. *Methods in Cell Biology.* Elsevier.

Hay, A., Barkoulas, M. & Tsiantis, M. 2006. ASYMMETRIC LEAVES1 and auxin activities converge to repress BREVIPEDICELLUS expression and promote leaf development in Arabidopsis. *Development,* 133**,** 3955-3961.

Heisler, M. G., Ohno, C., Das, P., Sieber, P., Reddy, G. V., Long, J. A. & Meyerowitz, E. M. 2005. Patterns of auxin transport and gene expression during primordium development revealed by live imaging of the Arabidopsis inflorescence meristem. *Current Biology,* 15**,** 1899-1911.

Higo, K., Ugawa, Y., Iwamoto, M. & Korenaga, T. 1999. Plant cis-acting regulatory DNA elements (PLACE) database: 1999. *Nucleic Acids Research,* 27**,** 297-300.

Hiratsu, K., Matsui, K., Koyama, T. & Ohme-Takagi, M. 2003. Dominant repression of target genes by chimeric repressors that include the EAR motif, a repression domain, in Arabidopsis. *The Plant Journal,* 34**,** 733-739.

Hu, W., Depamphilis, C. W. & Ma, H. 2008. Phylogenetic analysis of the plant-specific zinc finger-homeobox and mini zinc finger gene families. *Journal of Integrative Plant Biology,* 50**,** 1031-1045.

Huang, T., Harrar, Y., Lin, C., Reinhart, B., Newell, N. R., Talavera-Rauh, F., Hokin, S. A., Barton, M. K. & Kerstetter, R. A. 2014. Arabidopsis *KANADI1* acts as a transcriptional repressor by interacting with a specific cis-element and regulates auxin biosynthesis, transport, and signaling in opposition to HD-ZIPIII factors. *The Plant Cell,* 26**,** 246-262.

Ito, K., Awano, W., Suzuki, K., Hiromi, Y. & Yamamoto, D. 1997. The Drosophila mushroom body is a quadruple structure of clonal units each of which contains a virtually identical set of neurones and glial cells. *Development,* 124**,** 761-771.

Johnston, R., Leiboff, S. & Scanlon, M. J. 2015. Ontogeny of the sheathing leaf base in maize (*Zea mays*). *New Phytologist,* 205**,** 306-315.

Kang, J. & Dengler, N. 2002. Cell cycling frequency and expression of the homeobox gene *ATHB-8* during leaf vein development in Arabidopsis. *Planta,* 216**,** 212-219.

Kang, J. & Dengler, N. 2004. Vein pattern development in adult leaves of *Arabidopsis thaliana*. *International Journal of Plant Sciences,* 165**,** 231-242.

Kawanabe, T., Ishikura, S., Miyaji, N., Sasaki, T., Wu, L. M., Itabashi, E., Takada, S., Shimizu, M., Takasaki-Yasuda, T. & Osabe, K. 2016. Role of DNA methylation in

hybrid vigor in *Arabidopsis thaliana*. *Proceedings of the National Academy of Sciences,* 113**,** E6704-E6711.

Kierzkowski, D., Lenhard, M., Smith, R. & Kuhlemeier, C. 2013. Interaction between meristem tissue layers controls phyllotaxis. *Developmental Cell,* 26**,** 616-628.

Kinsman, E. & Pyke, K. 1998. Bundle sheath cells and cell-specific plastid development in Arabidopsis leaves. *Development,* 125**,** 1815-1822.

Kleinboelting, N., Huep, G., Kloetgen, A., Viehoever, P. & Weisshaar, B. 2012. GABI-Kat SimpleSearch: new features of the *Arabidopsis thaliana* T-DNA mutant database. *Nucleic Acids Research,* 40 (Database issue): D1211–1215.

Konishi, M., Donner, T. J., Scarpella, E. & Yanagisawa, S. 2015. MONOPTEROS directly activates the auxin-inducible promoter of the *Dof5.8* transcription factor gene in *Arabidopsis thaliana* leaf provascular cells. *Journal of Experimental Botany,* 66**,** 283-291.

Koornneef, M. & Meinke, D. 2010. The development of Arabidopsis as a model plant. *The Plant Journal,* 61**,** 909-921.

Krogan, N. T. & Berleth, T. 2012. A dominant mutation reveals asymmetry in MP/ARF5 function along the adaxial-abaxial axis of shoot lateral organs. *Plant Signaling & Behavior,* 7**,** 940-943.

Krogan, N. T., Ckurshumova, W., Marcos, D., Caragea, A. E. & Berleth, T. 2012. Deletion of MP/ARF5 domains III and IV reveals a requirement for Aux/IAA regulation in Arabidopsis leaf vascular patterning. *New Phytologist,* 194**,** 391-401.

Kubo, M., Udagawa, M., Nishikubo, N., Horiguchi, G., Yamaguchi, M., Ito, J., Mimura, T., Fukuda, H. & Demura, T. 2005. Transcription switches for protoxylem and metaxylem vessel formation. *Genes & Development,* 19**,** 1855-1860.

Laplaze, L., Parizot, B., Baker, A., Ricaud, L., Martiniere, A., Auguy, F., Franche, C., Nussaume, L., Bogusz, D. & Haseloff, J. 2005. GAL4-GFP enhancer trap lines for genetic manipulation of lateral root development in *Arabidopsis thaliana*. *Journal of Experimental Botany,* 56**,** 2433-2442.

Larkin, J. C., Young, N., Prigge, M. & Marks, M. D. 1996. The control of trichome spacing and number in Arabidopsis. *Development,* 122**,** 997-1005.

Lee, M.-H., Kim, B., Song, S.K., Heo, J.O., Yu, N.I., Lee, S. A., Kim, M., Kim, D. G., Sohn, S. O. & Lim, C. E. 2008. Large-scale analysis of the *GRAS* gene family in *Arabidopsis thaliana*. *Plant Molecular Biology,* 67**,** 659-670.

Lescot, M., Déhais, P., Thijs, G., Marchal, K., Moreau, Y., Van De Peer, Y., Rouzé, P. & Rombauts, S. 2002. PlantCARE, a database of plant cis-acting regulatory elements and a portal to tools for in silico analysis of promoter sequences. *Nucleic Acids Research,* 30**,** 325-327.

Loots, G. G. & Ovcharenko, I. 2004. rVISTA 2.0: evolutionary analysis of transcription factor binding sites. *Nucleic Acids Research,* 32**,** W217-W221.

Lowe, D. G. 2004. Distinctive image features from scale-invariant keypoints. *International Journal of Computer Vision,* 60**,** 91-110.

Lu, P., Porat, R., Nadeau, J. A. & O'neill, S. D. 1996. Identification of a meristem L1 layer-specific gene in Arabidopsis that is expressed during embryonic pattern formation and defines a new class of homeobox genes. *The Plant Cell,* 8**,** 2155-2168.

Lukowitz, W., Gillmor, C. S. & Scheible, W.R. 2000. Positional cloning in Arabidopsis. Why it feels good to have a genome initiative working for you. *Plant Physiology,* 123**,** 795-806.

Mansfield, S. & Briarty, L. 1991. Early embryogenesis in *Arabidopsis thaliana*. The developing embryo. *Canadian Journal of Botany,* 69**,** 461-476.

Marcos, D. & Berleth, T. 2014. Dynamic auxin transport patterns preceding vein formation revealed by live-imaging of Arabidopsis leaf primordia. *Frontiers in Plant Science,* 5**,** 235.

Mattsson, J., Ckurshumova, W. & Berleth, T. 2003. Auxin signaling in Arabidopsis leaf vascular development. *Plant Physiology,* 131**,** 1327-1339.

Mattsson, J., Sung, Z. R. & Berleth, T. 1999. Responses of plant vascular systems to auxin transport inhibition. *Development,* 126**,** 2979-2991.

Michniewicz, M., Zago, M. K., Abas, L., Weijers, D., Schweighofer, A., Meskiene, I., Heisler, M. G., Ohno, C., Zhang, J. & Huang, F. 2007. Antagonistic regulation of PIN phosphorylation by PP2A and PINOID directs auxin flux. *Cell,* 130**,** 1044-1056.

Mockaitis, K. & Estelle, M. 2008. Auxin receptors and plant development: a new signaling paradigm. *Annual Review of Cell and Developmental Biology,* 24.

Nelson, T. & Dengler, N. 1997. Leaf vascular pattern formation. *The Plant Cell,* 9**,** 1121.

Noden, D. M. 1988. Interactions and fates of avian craniofacial mesenchyme. *Development,* 103**,** 121-140.

Normanly, J. 2010. Approaching cellular and molecular resolution of auxin biosynthesis and metabolism. *Cold Spring Harbor Perspectives in Biology,* 2**,** a001594.

Odat, O., Gardiner, J., Sawchuk, M. G., Verna, C., Donner, T. J. & Scarpella, E. 2014. Characterization of an allelic series in the *MONOPTEROS* gene of Arabidopsis. *Genesis,* 52**,** 127-133.

Park, H. C., Kim, M. L., Lee, S. M., Bahk, J. D., Yun, D.-J., Lim, C. O., Hong, J. C., Lee, S. Y., Cho, M. J. & Chung, W. S. 2007. Pathogen-induced binding of the soybean zinc finger homeodomain proteins GmZF-HD1 and GmZF-HD2 to two repeats of ATTA homeodomain binding site in the calmodulin isoform 4 (*GmCaM4*) promoter. *Nucleic Acids Research,* 35**,** 3612-3623.

Petrášek, J., Mravec, J., Bouchard, R., Blakeslee, J. J., Abas, M., Seifertová, D., Wiśniewska, J., Tadele, Z., Kubeš, M. & Čovanová, M. 2006. PIN proteins perform a rate-limiting function in cellular auxin efflux. *Science,* 312**,** 914-918.

Pray, T. R. 1955. Foliar venation of angiosperms. Histogenesis of the venation of Liriodendron. *American Journal of Botany,* 18-27.

Prusinkiewicz, P. & Runions, A. 2012. Computational models of plant development and form. *New Phytologist,* 193**,** 549-569.

Przemeck, G. K., Mattsson, J., Hardtke, C. S., Sung, Z. R. & Berleth, T. 1996. Studies on the role of the Arabidopsis gene *MONOPTEROS* in vascular development and plant cell axialization. *Planta,* 200**,** 229-237.

Pyke, K., Marrison, J. & Leech, A. 1991. Temporal and spatial development of the cells of the expanding first leaf of *Arabidopsis thaliana* (L.) Heynh. *Journal of Experimental Botany,* 42**,** 1407-1416.

Raven, J. 1975. Transport of indoleacetic acid in plant cells in relation to pH and electrical potential gradients, and its significance for polar IAA transport. *New Phytologist,* 74**,** 163-172.

Reinhardt, D., Pesce, E.R., Stieger, P., Mandel, T., Baltensperger, K., Bennett, M., Traas, J., Friml, J. & Kuhlemeier, C. 2003. Regulation of phyllotaxis by polar auxin transport. *Nature,* 426**,** 255.

Rubery, P. H. & Sheldrake, A. R. 1974. Carrier-mediated auxin transport. *Planta,* 118**,** 101-121.

Rueden, C. T., Schindelin, J., Hiner, M. C., Dezonia, B. E., Walter, A. E., Arena, E. T. & Eliceiri, K. W. 2017. ImageJ2: ImageJ for the next generation of scientific image data. *BMC Bioinformatics,* 18**,** 529.

Runions, A., Smith, R. S. & Prusinkiewicz, P. 2014. Computational models of auxin-driven development. *Auxin and its role in plant development.* Springer.

Sabatini, S., Beis, D., Wolkenfelt, H., Murfett, J., Guilfoyle, T., Malamy, J., Benfey, P., Leyser, O., Bechtold, N. & Weisbeek, P. 1999. An auxin-dependent distal organizer of pattern and polarity in the Arabidopsis root. *Cell,* 99**,** 463-472.

Sachs, T. 1981. The control of the patterned differentiation of vascular tissues. *Advances in Botanical Research.* Elsevier.

Sauer, M., Balla, J., Luschnig, C., Wiśniewska, J., Reinöhl, V., Friml, J. & Benková, E. 2006. Canalization of auxin flow by Aux/IAA-ARF-dependent feedback regulation of PIN polarity. *Genes & Development,* 20**,** 2902-2911.

Sawchuk, M. G., Donner, T. J., Head, P. & Scarpella, E. 2008. Unique and overlapping expression patterns among members of photosynthesis-associated nuclear gene families in Arabidopsis. *Plant Physiology,* 148**,** 1908-1924.

Sawchuk, M. G., Edgar, A. & Scarpella, E. 2013. Patterning of leaf vein networks by convergent auxin transport pathways. *PLoS Genetics,* 9**,** e1003294.

Sawchuk, M. G., Head, P., Donner, T. J. & Scarpella, E. 2007. Time-lapse imaging of Arabidopsis leaf development shows dynamic patterns of procambium formation. *New Phytologist,* 176**,** 560-571.

Scarpella, E., Francis, P. & Berleth, T. 2004. Stage-specific markers define early steps of procambium development in Arabidopsis leaves and correlate termination of vein formation with mesophyll differentiation. *Development,* 131**,** 3445-3455.

Scarpella, E. and Helariutta, Y., 2010. Vascular pattern formation in plants. In *Current Topics in Developmental Biology,* 91**,** 221-265. Academic Press.

Scarpella, E., Marcos, D., Friml, J. & Berleth, T. 2006. Control of leaf vascular patterning by polar auxin transport. *Genes & Development,* 20**,** 1015-1027.

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S. & Schmid, B. 2012. Fiji: an open-source platform for biological-image analysis. *Nature Methods,* 9**,** 676.

Schindelin, J., Rueden, C. T., Hiner, M. C. & Eliceiri, K. W. 2015. The ImageJ ecosystem: An open platform for biomedical image analysis. *Molecular Reproduction and Development,* 82**,** 518-529.

Schneider, C. A., Rasband, W. S. & Eliceiri, K. W. 2012. NIH Image to ImageJ: 25 years of image analysis. *Nature Methods,* 9**,** 671-675.

Sessions, A., Weigel, D. & Yanofsky, M. F. 1999. The *Arabidopsis thaliana* MERISTEM LAYER 1 promoter specifies epidermal expression in meristems and young primordia. *The Plant Journal,* 20**,** 259-263.

Shevell, D. E., Kunkel, T. & Chua, N. H. 2000. Cell wall alterations in the Arabidopsis emb30 mutant. *The Plant Cell,* 12**,** 2047-2060.

Sieburth, L. E. 1999. Auxin is required for leaf vein pattern in Arabidopsis. *Plant Physiology,* 121**,** 1179-1190.

Smetana, O., Mäkilä, R., Lyu, M., Amiryousefi, A., Sánchez Rodríguez, F., Wu, M.-F., Solé-Gil, A., Leal Gavarrón, M., Siligato, R., Miyashima, S., Roszak, P., Blomster, T., Reed, J. W., Broholm, S. & Mähönen, A. P. 2019. High levels of auxin signalling define the stem-cell organizer of the vascular cambium. *Nature,* 565**,** 485-489.

Solereder, H. & Scott, D. H. 1908. *Systematic Anatomy of the Dicotyledons: a Handbook for Laboratories of Pure and Applied Botany*, Clarendon Press.

Steffens, N. O., Galuschka, C., Schindler, M., Buèlow, L. & Hehl, R. 2004. AthaMap: an online resource for in silico transcription factor binding sites in the *Arabidopsis thaliana* genome. *Nucleic Acids Research*, 32, D368-D372.

Stewart, R. N. 1978. Ontogeny of the primary body in chimeral forms of higher plants. In *The Clonal Basis of Development***,** 131-160, (ed. S. Subtelny and I. M. Sussex). New York: Academic Press.

Steynen, Q. J. & Schultz, E. A. 2003. The *FORKED* genes are essential for distal vein meeting in Arabidopsis. *Development,* 130**,** 4695-4708.

Strader, L. C., Monroe-Augustus, M. & Bartel, B. 2008. The IBR5 phosphatase promotes Arabidopsis auxin responses through a novel mechanism distinct from TIR1-mediated repressor degradation. *BMC Plant Biology,* 8**,** 41.

Takada, S. & Jürgens, G. 2007. Transcriptional regulation of epidermal cell fate in the Arabidopsis embryo. *Development*, 134**,** 1141-1150.

Tan, Q. K.G. & Irish, V. F. 2006. The Arabidopsis zinc finger-homeodomain genes encode proteins with unique biochemical properties that are coordinately expressed during floral development. *Plant Physiology,* 140**,** 1095-1108.

Telfer, A. & Poethig, R. 1994. Leaf development in Arabidopsis. In "Arabidopsis" (EM Meyerowitz and CR Somerville, Eds.). Cold Spring Harbor Laboratory Press, Plainview, NY.

Thompson, N.P. & Jacobs, W.P. 1966. Polarity of IAA effect on sieve-tube and xylem regeneration in Coleus and tomato stems. *Plant Physiology,* 41**,** 673-682.

Tran, L. S. P., Nakashima, K., Sakuma, Y., Osakabe, Y., Qin, F., Simpson, S. D., Maruyama, K., Fujita, Y., Shinozaki, K. & Yamaguchi-Shinozaki, K. 2007. Co-expression of the stress-inducible zinc finger homeodomain ZFHD1 and NAC transcription factors enhances expression of the *ERD1* gene in Arabidopsis. *The Plant Journal,* 49**,** 46-63.

Tilney-Bassett, R. A. 1986. Plant chimeras, Edward Arnold (Publishers) Ltd.

Troll, W. 1939. Vergleichende Morphologie der hoheren Planzen, Vol. 1 . (Berlin: Gebruder Borntraeger).

Verna, C., Ravichandran, S. J., Sawchuk, M. G., Linh, N. M. & Scarpella, E. 2019. Coordination of tissue cell polarity by auxin transport and signaling. *bioRxiv,* 680090.

Verna, C., Sawchuk, M. G., Linh, N. M. & Scarpella, E. 2015. Control of vein network topology by auxin transport. *BMC Biology,* 13**,** 94.

Weijers, D., Franke-Van Dijk, M., Vencken, R.J., Quint, A., Hooykaas, P. & Offringa, R. 2001. An Arabidopsis Minute-like phenotype caused by a semi-dominant mutation in a *RIBOSOMAL PROTEIN S5* gene. *Development,* 128**,** 4289-4299.

Weijers, D., Van Hamburg, J.-P., Van Rijn, E., Hooykaas, P. J. J. & Offringa, R. 2003. Diphtheria toxin-mediated cell ablation reveals interregional communication during Arabidopsis seed development. *Plant Physiology,* 133**,** 1882-1892.

Wenzel, C. L., Marrison, J., Mattsson, J., Haseloff, J. & Bougourd, S. M. 2012. Ectopic divisions in vascular and ground tissues of *Arabidopsis thaliana* result in distinct leaf venation defects. *Journal of Experimental Botany,* 63**,** 5351-5364.

Wenzel, C. L., Schuetz, M., Yu, Q. & Mattsson, J. 2007. Dynamics of MONOPTEROS and PIN-FORMED1 expression during leaf vein pattern formation in Arabidopsis thaliana. *The Plant Journal,* 49**,** 387-398.

Windhövel, A., Hein, I., Dabrowa, R. & Stockhaus, J. 2001. Characterization of a novel class of plant homeodomain proteins that bind to the C4 phosphoenolpyruvate carboxylase gene of *Flaveria trinervia*. *Plant Molecular Biology,* 45**,** 201-214.

Wiśniewska, J., Xu, J., Seifertová, D., Brewer, P. B., Růžička, K., Blilou, I., Rouquié, D., Benková, E., Scheres, B. & Friml, J. 2006. Polar PIN localization directs auxin flow in plants. *Science,* 312**,** 883-883.

Xu, J., Hofhuis, H., Heidstra, R., Sauer, M., Friml, J. & Scheres, B. 2006. A molecular framework for plant regeneration. *Science,* 311**,** 385-388.

Xue, Y., Gao, X., Lindsell, C. E., Norton, C. R., Chang, B., Hicks, C., Gendron-Maguire, M., Rand, E. B., Weinmaster, G. & Gridley, T. 1999. Embryonic lethality and vascular defects in mice lacking the Notch ligand Jagged1. *Human Molecular Genetics,* 8**,** 723-730.

Yamaguchi, M., Goué, N., Igarashi, H., Ohtani, M., Nakano, Y., Mortimer, J. C., Nishikubo, N., Kubo, M., Katayama, Y., Kakegawa, K., Dupree, P. & Demura, T. 2010. VASCULAR-RELATED NAC-DOMAIN6 and VASCULAR-RELATED NAC-DOMAIN7 effectively induce transdifferentiation into xylem vessel elements under control of an induction system. *Plant Physiology,* 153**,** 906-914.

Zhang, C., Gong, F. C., Lambert, G. M. & Galbraith, D. W. 2005. Cell type-specific characterization of nuclear DNA contents within complex tissues and organs. *Plant Methods*, 1**,** 7.

Zhang, Q., Wang, D., Lang, Z., He, L., Yang, L., Zeng, L., Li, Y., Zhao, C., Huang, H., Zhang, H., Zhang, H. & Zhu, J.-K. 2016. Methylation interactions in Arabidopsis

hybrids require RNA-directed DNA methylation and are influenced by genetic variation. *Proceedings of the National Academy of Sciences of the United States of America,* 113**,** E4248-E4256.

Zhao, Y. 2010. Auxin biosynthesis and its role in plant development. *Annual Review of Plant Biology,* 61**,** 49-64.

Zhou, R., Benavente, L. M., Stepanova, A. N. & Alonso, J. M. 2011. A recombineering-based gene tagging system for Arabidopsis. *The Plant Journal*, 66**,** 712-723.

Zourelidou, M., Absmanner, B., Weller, B., Barbosa, I. C., Willige, B. C., Fastner, A., Streit, V., Port, S. A., Colcombet, J. & Van Bentem, S. D. L. F. 2014. Auxin efflux by PIN-FORMED proteins is activated by two different protein kinases, D6 PROTEIN KINASE and PINOID. *Elife,* 3**,** e02860.