CHARACTERIZING *ARABIDOPSIS THALIANA* MUTANT LINES THAT AFFECT LEAF SHAPE AND VEIN PATTERN

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I dedicate this thesis to my supervisor Dr. Elizabeth Schultz and my family for their consistent support and encouragement

ABSTRACT

An optimum leaf shape and vein pattern is necessary for vascular plant success. The isolation of leaf morphological mutants of *Arabidopsis thaliana* has been used for leaf development studies. Auxin is a signal molecular that regulates plant development and growth processes. I have analyzed four mutations in detail, and I suggest that they all have defects in auxin regulated plant development processes. Mutant *115-11-21-4* is an allele of *FKD1* with frequent distal free-ending veins. Mutant *31-83-4-3* likely has increased auxin flux through secondary veins and increased downward auxin transport through the shoot, possibly due to increased *PIN1* expression and/or altered PIN1 localization. Mutant *32-2-4* likely has increased auxin activity at marginal auxin convergence points resulting in the formation of increased leaf serrations. Mutant *33-7-5(4)* may have a global reduction in auxin biosynthesis or response, thus the whole plant is weak and pale, with slower shoot and root growth rate and gravitropic response.

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LIST OF ABBREVIATIONS

Genes and Protein Nomenclature

UPPERCASE ITALICS	wild type gene
lowercase italics	mutant allele
UPPERCASE NO ITALICS	wild type protein

Genes

ABCB	*ATP BINDING CASSETTE SUBFAMILY B*
APG10	*ALBINO AND PALE GREEN10*
ARE	*AUXIN RESPONSE ELEMENT*
ARF	*AUXIN RESPONSE FACTOR*
ARF-GAP	*ADP RIBOSYLATION FACTOR GTPASE ACTIVATION PROTEIN*
ATHB8	*ARABIDOPSIS THALIANA HOMEOBOX8*
AUX1/LAX	*AUXIN RESISTANT1/LIKE AUX1*
BDL	*BODENLOS*
CUC2	*CUP-SHAPED COTYLEDON2*
CVP2	*COTYLEDON VASCULAR PATTERN2*
DR5	*5 DIRECT REPEATS OF AN AUXIN RESPONSE ELEMENT*
FKD1	*FORKED1*
GL1	*GLABROUS1*
GSY	*GRASSY*
LCD1	*LOWER CELL DENSITY 1*
MP	*MONOPTEROS*
PAC	*PALE CRESS*
PIN	*PIN-FORMED*
SFC	*SCARFACE*
SSM	*SHORT STEM AND MIDRIB*
TAA	*TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS*
TAR2	*TRYPTOPHAN AMINOTRANSFERASE RELATED 2*
UNH	*UNHINGED*
VAM	*VACUOLAR MORPHOLOGY*
WEI8	*WEAK ETHYLENE INSENSITIV 8*
YUC	*YUCCA*

Chemicals

2,4-D	2,4-dichlorophenoxyacetic acid
DMF	Dimethylformamide
EDTA	Ethylenediaminetetraacetic acid
EMS	ethyl methane sulfonate
GFP	green flurescent protein
GUS	β-GLUCURONIDASE
IAA	indole-3-acetic acid
IPA	indole-3-pyruvic acid
NPA	1-Naphthylphtalamic acid
TBE	Tris/Borate/EDTA
Triton X-100	polyoxyethylene octyl phenyl ether
Trp	Tryptophan
X-gluc	5-bromo-4-chloro-3-indolyglucuronide

Terms

AGI	*Arabidopsis* Genome Initiative
AT	*Arabidopsis thaliana*
BAC	bacterial artificial chromosome
bc1	backcross to wild type once
bc2	backcross to wild type twice
bp	base pair
Col	Columbia ecotype
cM	Centimorgan
DAG	Days After Germination
DZ	differentiation zone
ER	endoplasmic reticulum
EZ	elongation zone
F1	First filial generation
F2	Second filial generation
GM	ground meristem
Het	heterozygous
Ler	Lansberg *erecta* ecotype

M1	seed mutagenized with a mutagen
M2	self-pollinated seed from M1
MZ	meristematic zone
PCR	polymerase chain reaction
PM	plasma membrane
RAM	root apical meristem
SAM	shoot apical meristem
s.e.m	standard error of the mean
SSLP	simple sequence length polymorphism
TAIR	The *Arabidopsis* Information Resource

INTRODUCTION

Optimum leaf shape and size are very important to plant success, as leaves are the most important photosynthetic organs of vascular plants. Leaf physiological functions are supported by several specialized cell types, such as paired guard cells in the epidermis for gas exchange, mesophyll cells for photosynthesis, and vascular cells for internal fluid and nutrient transport (Esau, 1965a). As a fundamental component of the plant body, the continuous vascular network provides not only mechanical strength but also the key role of transport: the vascular tissue xylem transports water and minerals, and phloem translocates dissolved photoassimilates efficiently (Esau, 1965b). Leaf morphogenesis corresponds closely with genetic controls and environmental factors (Tsukaya, 2005) and leaf morphology is often used to distinguish different plant species. Over the past two decades, the isolation of leaf morphological mutants of *Arabidopsis thaliana* has been commonly used to further genetic studies of leaf development (Scarpella *et al.*, 2010).

The leaf form of *Arabidopsis thaliana* is called a simple leaf (Figure 1A) and may have serrations (tooth-like projections; Figure 1B) and lobes (marginal segmentation; Figure 1C) to different extents along the leaf marginal area. In contrast, compound leaves have gaps that separate the single leaf blade into distinct units which are called lateral leaflets (Figure 1D) (Scarpella *et al.*, 2010). The wild type *Arabidopsis thaliana* has a relatively complex vein pattern with closed vein loops (Nelson and Dengler, 1997): in the middle of the leaf blade is the midvein (primary vein) which is generally thicker than lateral veins; the veins connected to the midvein are called secondary veins, similarly the veins connected to secondary veins but not the midvein are called tertiary veins. Veins which end freely in the leaf marginal area are sometimes connected to hydathodes (Figure 1E). A hydathode is an epidermal structure that is connected to a vein ending and has probably evolved from modified stomata. Hydathodes are specialized for secreting water, and are typically located at the leaf tip or the serration tip (Esau, 1965a).

Although there are detailed descriptions and consistent theoretical models to account for the ontogeny of leaf vascular patterns (Meinhardt, 1976, Mitchison, 1980), not all mechanisms of the patterning processes required to build such structures are known at the molecular level (Scarpella *et al.*, 2004, Sack and Scoffoni, 2013). The auxin canalization hypothesis (Sachs, 1981) seems to best account for the continuous vascular strands and the final vein pattern. A positive feedback mechanism causes cell to cell auxin transport (both influx to and efflux from cells) to become more efficient; this results in stable "auxin canals". The increased conductivity of these cells is proposed to not only lead to their vascular differentiation (caused by the high level of auxin flux), but also to deplete neighboring cells of auxin preventing them from taking on a vascular cell fate (Sachs, 1981).

General Information about Auxin

Plant hormones are signaling molecules that act as growth regulators to control complex growth and developmental processes and often allow plants to respond to environmental changes (Berleth *et al.*, 2004). Auxin is one of the most important plant hormones and plays a key role in many aspects of plant growth and development, including cell division and elongation, differentiation, patterning, tropisms, apical dominance, and flowering (Berleth *et al.*, 2004). To understand the mechanism by which auxin controls these processes, it is important to understand auxin regulation at three levels: auxin biosynthesis, auxin transport and auxin response.

1. Auxin biosynthesis sites and pathways

Auxin is synthesized in young tissues of both shoots and roots, but most auxin is synthesized in the shoot apex and young leaves (Ljung *et al.*, 2001). The first identified plant hormone, indole-3-acetic acid (IAA), has been recognized as the major auxin for more than seventy years (Mashiguchi *et al.*, 2011). Several auxin biosynthetic pathways have been reported recently, although the pathway by which auxin is synthesized in plants is still unclear at the genetic level (Mano and Nemoto, 2012). Tryptophan (Trp)-dependent and Trp-independent pathways have been proposed as the two major IAA biosynthetic pathways. There are four presumptive pathways in Trp-dependent IAA biosynthesis (Mano and Nemoto, 2012), and the indole-3-pyruvic acid (IPA) pathway is the one considered to be the main IAA biosynthesis pathway in *Arabidopsis thaliana* (Mashiguchi *et al*., 2011). Mashiguchi *et al* (2011) demonstrated that two enzymes, Tryptophan Aminotransferase of *Arabidopsis* (TAA) and YUCCA (YUC) flavin monooxygenase-like protein, are both required as they function in the same IPA pathway: the TAA family mainly produces IPA from Trp and the *YUC* family is mainly implicated in the conversion of IPA to IAA; thus YUC proteins catalyze a rate-limiting step of the IPA pathway that produces IAA that is essential for plant development.

The *Arabidopsis thaliana* genome contains 11 *YUC* gene members (*YUC1*-*YUC11*) that are mainly expressed in meristems, young primordia, vascular tissues, and reproductive organs. Within leaves, the expression of these genes is associated with leaf margin and hydathode development (Wang *et al.*, 2011). The YUC proteins appear to have overlapping functions; overexpression of each *YUC* gene leads to auxin overproduction, but disruption of a single *YUC* gene does not cause obvious developmental defects. Removal at least four genes (*YUC1*, *YUC2*, *YUC4* and *YUC6*) results in a narrow leaf with leaf margin defects (Cheng *et al.*, 2006, Cheng *et al.*, 2007). The *YUC* and the *TAA* gene families have been proposed to play essential roles in auxin biosynthesis, and it has been suggested that they participate in two independent pathways. Study of *taa* mutant phenotypes has allowed assessment of the genetic interaction between *YUC* and *TAA* (Won *et al*., 2011). Mutations in *TAA* genes mimicked the phenotypes of *yuc* mutants and the *yuc1 yuc4 wei8 tar2* quadruple mutants did not make any hypocotyls and roots (*wei8* and *tar2* are alleles of *taa1*), a phenotype that was not observed in either yuc1 yuc4 or wei8 tar2-1 double mutants (Won *et al.*, 2011). Together, these phenotypes suggest that the YUC and TAA act in independent and redundant pathways of auxin biosynthesis.

2. Auxin transport pathways

Since most auxin is synthesized in the shoot apex and young leaves, auxin transport from the sites of synthesis (source tissue) to the sites of action (sink tissue such as the roots) is very important for development of the whole plant (Ljung *et al.*, 2001). Generally auxin is transported in two major pathways. Most auxin is transported by bulk flow from source (young leaves) to sink (roots) through the mature phloem which plays the role of transporting metabolites from source (leaves) to the other parts of plant; this pathway is considered long distance transport (Petrášek and Friml, 2009). Another pathway is auxin transport from cell to cell in polarized streams via auxin influx and efflux carriers; this pathway is considered short distance transport, and is facilitated by chemiosmotic gradients (Peer *et al.*, 2011). When the extracellular auxin level is high, auxin can enter cells via lipophilic diffusion. In contrast, when the auxin level is low in the extracellular environment, auxin uptake from the environment to cells is mediated by the transmembrane proteins AUXIN RESISTANT1/LIKE AUX1 (AUX1/LAX) family which act as auxin influx carriers to create an auxin sink in cells (Bennett *et al.*, 1996, Swarup *et al.*, 2001). However, auxin is anionic in the cytosol (neutral pH) and cannot go passively through the membrane; instead it requires transporters to exit cells (Peer *et al.*, 2011). Auxin efflux carriers, PIN-FORMED (PIN) proteins, mediate auxin export, and the ATP Binding Cassette subfamily B (ABCB) transporters can coordinate with PIN proteins in exporting auxin through the cell membrane (Titapiwatanakun *et al.*, 2009, Zhang *et al.*, 2011).

3. PIN polarity determines the direction of auxin polar transport

The *Arabidopsis thaliana PIN* family consists of eight gene members. Five of them encode full length plasma membrane (PM) localized PINs (PIN1, 2, 3, 4, and 7) that act as auxin efflux carriers (Petrasek *et al.*, 2006), the other three genes encode short length endoplasmic reticulum (ER) localized PINs (PIN5, 6, and 8) that function in homeostatic auxin compartmentalization (Mravec *et al.*, 2009). The PIN proteins have been identified and characterized as key regulators of auxin dependent processes, including axis formation in pre-and post-embryogenesis (PIN1, 4, and 7), root meristem maintenance (PIN1, 3, 4, and 7) (Feraru and Friml, 2008) and vascular tissue differentiation and regeneration (PIN1, 5, 6, and 8) (Sawchuk *et al.*, 2013). PIN1 and PIN2 exhibit primarily polar localization on the PM. The polar localization at the single cell level determines the direction of intercellular auxin transport and the direction of auxin transport between neighboring cells. For example, the apical localization of PIN1 results in upward auxin transport, while the basal localization of PIN1 results in downward auxin transport (Wisniewska *et al.*, 2006). The fusion protein PIN1:GFP (green fluorescent protein) has been used widely to monitor the expression and localization of PIN1 in leaf cells. PIN1 localization can define the formation sites of not only serrations (or lobes), but also the veins, and the marker PIN1:GFP allows a dynamic observation of leaf shape and vein pattern developments to occur (Wenzel *et al.*, 2007). In the wild type *Arabidopsis thaliana* PIN1:GFP expression extends from the epidermal cells of leaf primordia into the ground tissue. Within the first leaves, from 2 DAG (Days After Germination) old primordia onwards, PIN1 expression narrows from successive regions (several cells wide) within the ground meristem (GM) to files of cells (one to three cells wide) that form successive vein orders (Scarpella *et al.*, 2006, Wenzel *et al.*, 2007). The narrowing of PIN1:GFP provides visual evidence of the auxin canalization process (Wenzel *et al.*, 2007).

4. Auxin response

Together with auxin synthesis and transport, proper auxin response is also important in plant development. Auxin Response Factors (ARFs) are DNA binding proteins that bind to Auxin Response Elements (AREs) and function as either activators or repressors that can either positively or negatively influence gene expression (Ulmasov *et al.*, 1999, Tiwari *et al.*, 2003). For example, *ARF5*, also known as *MONOPTEROS* (*MP*), is activated by auxin and induces the expression of auxin inducible genes such as *PIN1* and *ARABIDOPSIS THALIANA HOMEOBOX8* (*ATHB8*). By using *in situ* RNA hybridization, *MP* expression can be analyzed at the transcript level. This technique has revealed that during leaf development, *MP* is initially expressed in broad domains that gradually become confined within the vascular tissues (Wenzel *et al.*, 2007). MP can directly and positively regulate the expression of *ATHB8* through an Auxin Response Element in the *ATHB8* promoter (Donner *et al.*, 2010). *ATHB8* is expressed in both shoot and root vascular bundles, showing that it is correlated with vascularization throughout the plant, and the effects of *ATHB8* on vein formation depend on the activity of *MP* (Baima *et al.*, 2001). Within the broad field of cells expressing the Auxin Response Factor *MP*, only a subset of cells initiate *ATHB8* expression (Baima *et al.*, 1995).

5. Auxin canalization hypothesis

The auxin dependent vascular patterning model that is called the auxin canalization hypothesis is the most accepted model of vascular tissue formation: auxin creates a pathway for efficient auxin flow much like water eroding river beds; the auxin flow causes columns of cells to differentiate into procambium (vascular meristematic tissue) and eventually vascular tissue (Sachs, 1981). It has been proposed that auxin is initially produced in a broad region, then narrows to a file of cells; the presence of auxin in undifferentiated cells promotes cellular changes that allow auxin movement to change from slow diffusion (environment to cell, cell to cell via influx and efflux carriers) to rapid auxin flow (source to sink via mature phloem). The procambial cells appear as narrow, cytoplasmic-dense cells that emerge from the subepidermal tissue of the leaf primordium, termed the ground meristem (GM) (Foster, 1952, Esau, 1965b). Procambial cells are characteristically arranged in continuous strands and acquire their narrow shape through coordinated, oriented divisions that are parallel to the axis of the emerging strand.

Recent studies provide more evidence that auxin flow is the primary regulator of vascular tissue formation. For example, either genetic inhibition (mutants) or chemical inhibition of auxin transport by 1-Naphthylphtalamic acid (NPA, a specific inhibitor of the auxin efflux) can result in various vein defects (Mattsson *et al.*, 1999, Taiz and Zeiger, 2002). Based on the study of Mattsson *et al.* (1999), *Arabidopsis thaliana pin1* mutants have additional primary and secondary veins, as well as abundant continuous veins along the leaf margin area. Inhibition of auxin transport by low concentration of NPA in wild type *Arabidopsis thaliana* can produce very similar vein features to that of *pin1*. The phenotype suggested that the leaf margin is a source of auxin for the GM. In the absence of auxin transport, auxin accumulates at the margin, resulting in vascular proliferation (Mattsson *et al.*, 1999). This idea has been validated by the finding that PIN1:GFP is initially expressed in the marginal epidermis, where it generates auxin maxima that initiate PIN1 expression and vein formation within the GM (Scarpella *et al.*, 2006, Wenzel *et al.*, 2007).

Additional evidence for the importance of auxin distribution to vein formation comes from insensitive auxin response *Arabidopsis thaliana* mutants such as *mp*. The *mp* mutants are rootless and form fewer secondary veins with no veins along the leaf margin in both cotyledon and later leaves (Hardtke and Berleth, 1998). Following treatment with synthetic auxin 2,4-dichlorophenoxyacetic acid (2,4-D), *MP* transcription is induced by auxin and its expression pattern is similar to the pattern of auxin responsive reporter gene expression (Wenzel *et al.*, 2007). Whereas early in *mp* mutant leaf development, the *MP* expression is similar to wild type, in more advanced primordia *MP* expression is much reduced when compared to wild type accounting for the lack of higher-order veins in *mp* mutants leaves (Wenzel *et al.*, 2007). In addition, by introducing the reporter transgene *pPIN1::PIN1:GFP* into the *mp* mutant background, it is validated that *PIN1* expression is regulated by *MP*: in contrast to the normal *MP* expression in early *mp* mutant leaf development, PIN1:GFP expression is much reduced in *mp* mutants (Wenzel *et al.*, 2007). Together, these data suggest a feedback loop consistent with the original canalization hypothesis: auxin induces *MP*, resulting in higher auxin response, which in turn induces *PIN1*, resulting in higher auxin transport and higher auxin in cells (Wenzel *et al.*, 2007).

Processes of Leaf Shape Establishment

The interaction between the developing leaf primordia and the shoot apical meristem (SAM) is important in axis specification and phyllotaxis establishment, and is correlated with auxin transport direction and auxin activity (Figure 2 A). During the early stages of leaf development, apical PIN1 polarity in the SAM epidermal cells directs auxin flow toward the flanks of the SAM (Scarpella *et al.*, 2010). A region of high auxin activity (epidermal PIN1 convergence point) is created that defines the site of the initial leaf primordium (Figure 2 B). During leaf formation, the establishment of three axes plays an important role in the shape change of the initial leaf primordia and appears to be coupled with the flat structure of leaf blade or leaf lamina (Waites and Hudson, 1995). These three axes are the proximal-distal axis, the medial-lateral axis and the adaxial (upper side which faces the SAM)-abaxial (lower side which opposes the adaxial side) axis (Scarpella *et al.*, 2010).

The basic leaf shape is formed during primary morphogenesis, a stage in which cell division is predominantly responsible for growth. In the process of leaf lamina expansion, a short-lived marginal meristem is activated along the edge of the adaxial/abaxial boundary of the initial leaf primordium; leaf lamina expansion proliferates from the marginal meristem and converts the leaf primordium from rod shaped to spatula shaped (Donnelly *et al.*, 1999). Following this process, in the inner area of leaf primordia, the internal meristem is activated to establish the inside structure of the leaf blade: the cells at the adaxial side differentiate into palisade cells and the cells at the abaxial side differentiate into spongy cells in most plant species (Donnelly *et al.*, 1999). During the expansion of the leaf blade, cell proliferation is also controlled along proximal-distal and medial-lateral axes (Tsuge *et al.*, 1996). In *Arabidopsis thaliana* leaves, the proximal-distal and medial-lateral polarities are important in determining leaf length and width respectively, and the proximal-distal and medial-lateral axes are correlated with the final shape: the distal part of leaf primordium differentiates into the leaf blade while the proximal part of the leaf primordium develops into the leaf petiole (Tsukaya, 2005).

Serrations and lobes are formed along the leaf margin during secondary morphogenesis, a stage in which cell expansion is predominately responsible for growth (Scarpella *et al.*, 2010). Very similar to the initiation sites of leaf primordia on the SAM, elevated auxin activities in the leaf marginal area correspond to epidermal PIN1 convergence points that predict initiation of serrations (or lobes) at later stages of leaf development (Figure 2 C) (Hay *et al.*, 2006, Scarpella *et al.*, 2006). The operation of auxin activity maxima requires three processes. The first process is the polar (apical or basal) localization of PIN1 that determines the direction of auxin transport (red arrows in Figure

2 D); the second process is auxin flow that enhances PIN1 localization with the same polarity (black arrows in Figure 2 D). Together, these two processes create the maxima and minima of auxin concentration. The third process is the expression of *CUP-SHAPED COTYLEDON2* (*CUC2*) transcription factor in the sinuses between serrations, where it is proposed to repress growth (Nikovics *et al.*, 2006). CUC2 also enables reorientation of PIN1 (dashed black arrows in Figure 2 D) and, in turn, auxin inhibits CUC2, thus stabilizing the position of auxin maxima/CUC2 minima (predicting serrations) and auxin minima/CUC2 maxima (predicting sinuses) (Bilsborough *et al.*, 2011).

Processes of Vein Pattern Development

The basic leaf vasculature pattern is also formed during the primary morphogenesis process. During leaf lamina expansion, the concentrated auxin at the tip of the primordium is transported from the tip into the GM through basal PIN1 polarity in subepidermal cells (Benkova *et al.*, 2003, Scarpella *et al.*, 2006); the auxin flow through the leaf centre marks the position of the primary midvein (Figure 2B). In addition, the basal PIN1 polarity directs the lateral veins (secondary veins) to differentiate from the leaf margin toward the midvein (Figure 2C); subsequently, the bipolarity of PIN1 within specific cells can establish the closed vein loop later in vasculature development process (Scarpella *et al.*, 2006, Wenzel *et al.*, 2007, Scarpella *et al.*, 2010). Dual PIN1 polarity is required in the lateral vein formation to establish closed vein loops: the basal PIN1 localization directs auxin transport from the leaf marginal area to the proximal midvein which induces the lateral veins to form toward the proximal midvein; once the veins become connected, the PIN1 localization becomes apical and directs auxin transport from the lower part of lateral veins to the distal midvein. Thus the upper part of lateral veins extend from the lower part toward the distal midvein and finally form closed vein loops (Figure 2G) (Wenzel *et al.*, 2007, Scarpella *et al.*, 2010). Open-ended marginal vein precursors grow in both directions toward the pre-existing lateral veins, either forming closed vein loops or ending freely (Scarpella *et al.*, 2010). During secondary morphogenesis, similar to marginal veins, higher order veins (quaternary veins) appear in continuity within the expanding lamina and either end freely or connect to the pre-existing veins (Scarpella *et al.*, 2010).

It has frequently been observed that the branched venation pattern largely reflects the leaf shape, such as veins extending into the marginal area at the leaf serrations in *Arabidopsis thaliana* leaves (Nelson and Dengler, 1997, Dengler and Kang, 2001). One explanation is that the formation sites of veins from the leaf marginal area and the positions of the serrations are both correlated with PIN1 convergence within the marginal epidermis (Bilsborough *et al.*, 2011, Pahari *et al.*, 2014). *Arabidopsis thaliana* mutants with altered PIN1 marginal expression have altered leaf vein and serration phenotypes (Shirakawa *et al.*, 2009, Pahari *et al.*, 2014). For example, *unhinged-1* (*unh1*) leaves have serrated leaf margins combined with a simpler leaf vein pattern and frequent distal free-ending veins (Figure 2 H) (Pahari *et al.*, 2014). This phenotype results from expanded PIN1 expression in the marginal area; the predicted elevated auxin activities are proposed to lead to marginal out-growth (here represented as serrations) with free-ending lateral veins (Pahari *et al.*, 2014).

Several other mutant *Arabidopsis thaliana* have abnormal vein patterns which result from defects related to PIN1 expression and/or polar localization. The *Arabidopsis thaliana* mutant *forked1* (*fkd1*) leaves have normal shape but frequent distal free-ending veins (Steynen and Schultz, 2003). Previous studies have determined that *FKD1* encodes a protein that is required for PIN1 localization in developing leaf veins (Hou *et al.*, 2010). The absence of FKD1 delays the narrowing process of PIN1:GFP expression within incipient veins. As well, PIN1:GFP localization at the apical cell face is infrequent, which correlates with the failure of newly forming veins to connect to the previously formed veins. The *scarface* (*sfc*) mutant plants also have normal leaf shape but like *fkd1*, *sfc* has frequent free-ending veins as well as small vein segments called vascular islands, that do not connect to other veins (Deyholos *et al.*, 2000). Previous studies have determined that *SFC* encodes an ADP ribosylation factor GTPase activation protein (ARF-GAP) that is required for normal auxin efflux and vein pattering (Sieburth *et al.*, 2006) through appropriate PIN1:GFP localization (Scarpella *et al.*, 2006). Although proper PIN1 expression and polar localization are essential in auxin dependent vascular formation, *Arabidopsis thaliana pin1* mutant leaves have relatively mild vein-pattern defects when compared with *fkd1*, *sfc* and *unh*, indicating that the redundancy of other PIN proteins (PIN5, 6, and 8) in vein patterning underlies the developmental processes. Thus, when there is defect to one or two PIN protein expression or localization, the mutant plant may still be able to generate a relatively normal phenotype because the other PIN family members allow proper growth and development processes (Sawchuk *et al.*, 2013).

Root Development and Gravitropism

Auxin plays an important role in the embryonic root formation and root growth regulation. Different auxin concentrations have bimodal effects on primary root length (Eliasson *et al.*, 1989), and also control the root response to gravity (gravitropism)

(Rahman *et al.,* 2010). Auxin is synthesized in shoots (young leaves and cotyledons) and can be transported in a polar fashion from source to the sink such as roots (Ljung *et al.*, 2002). The downward auxin transport from shoot tissues to the root and auxin redistribution in the root tip are essential for root development and gravitropism. In addition to being synthesized in the shoots, auxin is also synthesized in the root, and root-generated auxin contributes to the maintenance of the auxin gradients which is required for normal root development (Ljung *et al.*, 2005). The local auxin concentration gradients and maximum auxin activity sites are crucial for primary root cell division and elongation.

During root embryogenesis, the root apical meristem (RAM) is established that will later provide new cells for the growing root. After the patterning process of RAM embryogenesis, the post-germination primary root matures through three distinct developmental zones. In the meristematic zone (MZ), a pool of cells is generated and will elongate and differentiate; once cells enter the elongation zone (EZ), cell division rate slows and cell expansion begins, the length of the cells will increase by many times their width; when cells in EZ reach their final size, they enter the differentiation zone (DZ), where the cells acquire their specialized characteristics and functions such as the formation of root hairs (Petricka *et al.*, 2012). The effect of auxin on primary root growth is based on both the cell division rate that occurs in the meristematic zone, and the cell expansion and elongation that occurs in the elongation zone (Overvoorde *et al.*, 2010).

Auxin is one of the key regulators of cell division in the MZ and elongation in the EZ that together contribute to primary root growth. Recent studies have suggested an auxin reflux loop that generates an auxin gradient in the root tip and maintains an auxin maximum in the RAM: auxin is first transported by PIN1, PIN2, PIN3 and PIN4 from the shoot through root vasculature and then redistributed in the root cap to the external root cells; auxin is then transported back toward the shoot by PIN2, PIN3 and PIN7 in the external root layers (Petricka *et al.*, 2012, Geisler *et al.*, 2013).

Root gravitropism is a complex process that allows the root to grow downward into the soil. The Cholodny-Went hypothesis suggested the root bending is caused by altered redistribution of auxin during root gravitropism (Went, 1974). During the last three decades, evidence has grown that cell-to-cell or polar auxin transport is sufficient to generate differential intercellular auxin gradients that guide root growth (Rashotte *et al.*, 2000). Following a 90° turn of the vertically grown roots, the auxin is redistributed to the lower side of the root tip within minutes due to the relocalization of PIN3; this results in asymmetric accumulation of auxin on the lower side of the root which inhibits cell elongation; meanwhile the cells at the upper side of the root continue to elongate which bends the root tip to growth downward again (Petricka *et al.*, 2012). As auxin is normally required to maintain cell elongation in the EZ, the redistribution of auxin toward the lower side of the root tip maintains these cells in this area in a proper dividing and elongating status (Petricka *et al.*, 2012).

Objectives

Although a vast inventory of morphological mutants of *Arabidopsis thaliana* is available, only some have been used for genetic studies of leaf development (Serrano-Cartagena *et al.*, 1999). In this thesis, with the aim of contributing to the genetic analysis of leaf development and in particular the coordination of leaf shape and vein pattern by auxin, I focused on mutants that appeared to have abnormal shape (elongated narrow leaves with/without obvious serrations) and leaf venation defects (frequent free-ending veins, lower vein density). Twenty-four ethyl methane sulfonate (EMS, which induces random mutations by nucleotide substitution) mutagenized mutant lines of *Arabidopsis thaliana* in Columbia ecotype were chosen as candidates for mutations in genes that are required for leaf morphogenesis, and these mutant lines were further characterized by assessing the first leaf phenotypes.

I hypothesized that a mutation in a single gene with a critical role in auxin regulation could change the whole plant development and alter the whole plant phenotype. With this hypothesis, the first leaf phenotype, the cotyledon phenotype, other shoot and root characters such as flowering time, stem branching, number of rosette leaves, root elongation, and gravitropism were also quantified to determine if global changes in auxin regulation were occurring in the mutants.

MATERIALS AND METHODS

Plant Materials

Wild type *Arabidopsis thaliana* seeds (Columbia ecotype) mutagenized with ethyl methane sulfonate (EMS) were purchased from Lehle Seed (Round Rock, TX). The *cup-shaped cotyledon* (*cuc2-3*) seeds of Columbia ecotype (Col) were generously provided by Masao Tasaka and Mitsuhiro Aida (Graduate School of Biological Sciences, Nara Institute of Science and Technology, Nara, Japan), and *DR5::GUS* by Jane Murfett (University of Missouri, Columbia, MO). All other seed material was generated in the lab. Wild type *Arabidopsis thaliana* of Col ecotype was used as a control for all the experiments.

Growth Conditions

Seeds were planted either on a mixture of ¾ Potting Mix and ¼ vermiculite (both from Coaldale Nurseries, Coaldale, AB) in 100 cm^2 pots or on *Arabidopsis thaliana* (AT) growth medium (Ruegger *et al.*, 1998) in Petri dishes. Pots were covered with plastic wrap and both pots and plates were incubated at 4°C in the dark for 3 days to allow most seeds germinate synchronously, after which they were transferred to growth chambers (Percival Scientific, Perry, IA) with 24 hours of continuous light at a photon flux density of approximately 130 $\mu mol\ s^{-1}\ m^{-2}$ from Sylvania Cool White, Grow Lux, and incandescent bulbs (Osram Sylvania Inc, Danvers, MA). Chambers were set at 22°C and 60% relative humidity. The day of transfer to the growth chambers was considered to be the day of germination or 0 Days After Germination (DAG). Plastic wrap was removed at 7 DAG and plants were maintained under constant growth conditions.

Mutant Phenotype Identification and Screening, Backcrossing and Segregation Analysis

Seeds mutagenized with EMS (Lehle Seed, Round Rock, TX) in Col ecotype are known as the M1 generation, its self-pollinated progeny, is the M2 generation, and so on. The mutant lines that are used in this thesis had been previously identified at the M2, M3, and M4 generations; the M4 seeds were planted and screened for abnormal first leaf defects by using wild type Columbia ecotype as a control for phenotypic comparisons. Mutations in M4 generation were backcrossed to the wild type, the F2 generation of backcrossing were examined for plants with first leaf shape and/or vein pattern defects, as well as the segregation ratio. For this study, either self-pollinated progeny from the EMS mutagenized mutants (e.g. M4) or progeny selected in the segregating population of the mutants which had been crossed to wild type (Col) (e.g. bc1F2) were screened. To determine the segregation ratio, these seeds were sown at a density of about 25 seeds per pot and 21 DAG first rosette leaves were cleared in cytoseal for identification of mutant phenotypes. In total, twenty-four mutant lines with either leaf shape defects or leaf vein pattern defects, or both, have been selected for segregation analysis and further phenotype analysis (see Appendix 1 for details). The Pearson's chi-squared test was used to establish that the mutant alleles segregated as expected for a single recessive mutation (3:1).

Complementation Test

Plants with selected single recessive mutations identified in this study were crossed to the previously identified mutants (e.g. *fkd1*, *sfc*, *unh*, *gsy*) that were available in our laboratory for complementation tests based on the phenotype. To be certain that the F1 seeds were from outcrossing, not from self-fertilization, a female *glabrous1* (*gl1*, hairless) plant of a mutant selected in this study was crossed to a male *GLABROUS1* (*GL1*, with hair) plant of a previously identified mutant (Larkin *et al.*, 1994). Since *GL1* is dominant, all the outcross F1 population plants will have hairs. If the plants of the F1 generation are all mutant phenotype, then the female mutant line and the male mutant line are allelic. In contrast, if the F1 generation are all wild type phenotype, then the female mutant line and the male mutant line are not allelic.

Phenotypic Analysis of Several Mutant Lines (Cotyledon, First and Fifth leaf, Shoot and Root)

To analyze and compare the cotyledon, first and fifth rosette leaves of all genotypes, homozygous mutant seeds (*31-83-4-3* from bc3F3, *32-2-4* from bc4F3, and *33-7-5(4)* from bc4F3) and Col seeds were sown on soil at a density of 16 seeds per pot; from the two leaved stage the density was maintained at 12 healthy plants per pot. For all genotypes except *33-7-5(4)* (seven days later than the normal DAG), cotyledons were removed at 14 DAG, first leaves were removed at 21 DAG, and fifth leaves were removed at 35 DAG, then treated with 70% ethanol for 1 day and cleared in chloral hydrate (Sigma): water: glycerol (8:2:1v/v/v) (Koizumi *et al.*, 2000) for one week prior to mounting in glycerol: water (2:1).

In the analysis of cotyledon and first leaf vein patterns, the mid-vein (primary vein) was considered to be the linear vascular strand approximately along the midline, secondary veins were considered to be those vascular strands connected to the mid-vein, tertiary veins were the veins connected to secondary veins (but not the mid-vein) and quaternary veins are the veins connected to tertiary veins (but not the mid-vein or secondary veins). These vascular strands were identified based on differentiated xylem. Branch points (junction of 2 veins meeting), areoles (area of leaf completely enclosed by veins), vascular islands (fragments of discontiguous vasculature), free-ending veins (veins connected at one end but disconnected at the other end), midvein extension (midvein ends freely at the tip and is not connected to secondary veins) and marginal venation gaps (a vascular discontinuity in the peripheral venation that creates a vein-free path to the mid-vein) were scored (Figure 1E).

In the analysis of first leaf shape, the leaf width (the longest line perpendicular to the mid-vein that extends from leaf margin to leaf margin), length and length/width, marginal early hydathodes, and serrations were also scored.

To analyze plant shoot morphology, plants were scored for flowering time when the inflorescence stem was 2 cm long, and the number of rosette leaves and stem branches were also recorded at 35 DAG.

To analyze root growth, seeds were sown on AT medium (Ruegger *et al.*, 1998) at a density of 30 seedlings per plate and grown vertically. Primary root lengths were measured at 4 DAG and 5 DAG to determine the root elongation within 24 hours. Root gravitropic response was determined by rotating vertically grown 5 DAG seedlings 90°. Images of the root tip were captured at 2, 4, and 6 hours after rotation, and the angles of root tip bending were measured by using ImageJ (http://imagej.nih.gov/ij).

Generation of Double Mutants

To generate double mutants between each leaf shape or vein pattern mutant and *cuc2*-3, homozygous mutant plants were crossed to *cuc2-3* plants to generate the F1 seeds. F1 plants were allowed to self-pollinate for F2 progeny. Seeds from F2 plants with the mutant leaf vein phenotype were harvested as F3. F3 populations were screened for segregation of *cuc2-3* (expecting lack of serrations). When the double mutant plants were fertile, seeds of double mutants segregating in the F3 were planted and the F4 plants were characterized. If double mutants were sterile (*31-83-4-3*), analysis was done in the segregating F3 population. All the double mutants were scored for total number of serrations on the fifth leaf of plants that were 35 DAG.

Introduction of *DR5::GUS* Transgene and Histochemical Staining for GUS

The *DR5::GUS* transgene was introduced into each mutant by crossing mutant plants with homozygous *DR5::GUS* plants in the Col ecotype to generate the F1 seeds. F1 plants were allowed to self-pollinate for F2 progeny. Seeds from F2 plants with the mutant leaf vein phenotype were harvested as F3. Expression of *DR5::GUS* was screened in F3 populations; individual F3 populations that were all expressing indicate homozygosity for *DR5::GUS* and were used for characterization. Mutants and wild type seed expressing *DR5::GUS* were planted at a density of 30 seeds per plate on AT plates. GUS staining was performed after Kang and Dengler (2002). Briefly, seedlings were removed from the medium, placed in acetone on ice for 20 minutes and washed twice with 50 µM NaPO4 Buffer (pH 7). The seedlings were vacuum infiltrated for 10 minutes with GUS staining buffer [final concentration: 0.2% Triton X-100; 50mM $NaHPO_4$ Buffer (pH7.2); 2mM Potassium Ferrocyanide; 2mM Potassium Ferricyanide; 2mM X-gluc, from 100mM stock solution in DMF)] (Vitha *et al.,* 1995) and then left for 16 hours before the GUS buffer was removed. Seedlings were decolourized with four rinses of 70% ethanol and finally cleared with chloral hydrate.

Mapping of Mutant Genes

Mapping was carried out through the use of ecotype-specific markers visible through PCR (simple sequence length polymorphisms, SSLPs) as described by Lukowitz *et al.* (2000). Once the phenotypes were determined to result from single nuclear recessive mutations, the homozygous mutant plants (*32-2-4* from bc4F2 and *33-7-5(4)* from bc3F2) were crossed to the Landsberg *erecta* (Ler) ecotype wild type and F2 seed was collected for the mapping population. DNA was extracted from the leaves of F2 plants showing the mutant phenotype (Dellaporta *et al.*, 1983). Previously identified primers were obtained from *Arabidopsis* website TAIR (http://www.arabidopsis.org/index.jsp). Useful small insertions/deletions (INDELs for SSLPs) between the publicly available Col sequence and Ler sequence were generated through Monsanto Company (http://www.arabidopsis.org/browse/Cereon/index.jsp) (Jander *et al.*, 2002). The web-based programs Primer3 Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) and IDT OligoAnalyzer 3.1 (http://www.idtdna.com/analyzer/Applications/OligoAnalyzer) were used to locate and design primers around each polymorphism. Successful marker primers are listed in Table 1, as well as the amplified DNA product lengths for both Col and Ler ecotypes. PCR was done using standard protocol (Bell and Ecker, 1994) [final concentration: Standars *Taq* Reaction Buffer, 1X; Mg^{2+}, 2.5 mM (or as indicated in Table 1); dNTPs, 160 µM each;

primer, 0.05 µM each; DNA Template, extracted from young leaves, 1 to 2 µl per 20 µl reaction; Taq DNA Polymerase 0.025 U] for 32-40 cycles at the specific annealing temperature for each primer pair (Table 1). PCR products were resolved by gel electrophoresis using a 2% to 4% agarose gel in Tris/Borate/EDTA (TBE) Buffer at 70~80 V depending on the product size.

Materials

5-bromo-4-chloro-3-indolyglucuronide (X-gluc), purchased from Rose Scientific, Edmonton, AB. Ethidium bromide was purchased from Sigma Chemical Co (St. Louis, MO); dNTPs were purchased from Invitrogen (Burlington, ON); primers were synthesized by either Invitrogen (Carlsbad, CA) or Integrated DNA Technologies (Coralville, IA); Taq DNA Polymerase was purchased from Truin Scientific (Edmonton, AB). Agarose was purchased from Bioshop Canada Inc. (Burlington ON).

Microscopy, Imaging and Statistics

A Leica MZ8 dissecting light microscope (Leica Microsystems, Wetzlar, Germany) was used for leaf shape and venation analysis of mature cotyledons and leaves. Tissues were either photographed using a Nikon Coolpix 990 camera (Nikon, Mississauga, ON) or an AxioCam ICc1 digital microscope camera (for the tissues that were stained with GUS); and the pictures were scored using Paint.NET (http://www.getpaint.net) and ImageJ (http://imagej.nih.gov/ij).

Measurements were recorded in Microsoft Excel (Redmond, WA) for subsequent determinations of average and standard error of the mean (s.e.m). The Pearson's chi-squared test was used to determine the significance ($p < 0.05$) of segregation from backcrosses and the gene linkage from mapping. The value of the test-statistic is

$$\chi^2 = \sum \frac{(O - E)^2}{E}$$

where χ^2 = Pearson's cumulative test statistic, O = an observed frequency; E = an expected (theoretical) frequency; the approximate p value of χ^2 was calculated using the online tool (http://www.socscistatistics.com/pvalues/chidistribution.aspx). The two-tailed Student's *t*-test assuming equal variance was applied in Microsoft Excel to determine the statistical differences for data sets with equal variance as determined by the *F*-test ($p > 0.05$). Data sets with unequal variance as determined by the *F*-test ($p < 0.05$) were analyzed by using two-tailed *t*-test assuming unequal variances. When a data set had no variation, the data points were converted from numerical to categorical to apply the two-tailed Fisher’s exact test using the online data analysis tool (http://graphpad.com/quickcalcs/contingency1).

RESULTS

The first leaves of wild type *Arabidopsis thaliana* (Col) have a spoon-shaped leaf blade, smooth leaf margins and a relatively closed vein pattern without many secondary free ending veins (Candela *et al.*, 1999). Some mutant *Arabidopsis thaliana* can generate abnormal leaves, such as narrow or curved leaf shapes, leaves with serrated leaf margins and leaves with simplified or frequent non-meeting vein patterns (Berná *et al.*, 1999). A total of twenty-four EMS mutagenized lines that had been previously identified as having defects of leaf shape and vein pattern were selected for further analysis (See Appendix 1 for details). After further backcrossing, only four mutant lines (*31-83-4-3*, *32-2-4*, *33-7-5(4)* and *115-11-21-4*) showed stable phenotypes that segregated approximately 3:1 in the F2 generation. These mutant lines were subjected to more detailed genetic and phenotypic analysis.

Genetic Analysis of *115-11-21-4, 31-83-4-3, 32-2-4* and *33-7-5(4)*

To determine the segregation ratio, self-fertilized seed as well as the F2 seeds of a backcross to each mutant line were sown on soil and the first leaves were cleared for phenotypic observation. For these four mutant lines, no wild type phenotype appeared within the self-fertilized populations, which means these four recessive mutations were all completely penetrant. For *32-2-4* and *115-11-21-4*, segregation ratios of approximately 3:1 were observed in the F2 of the fourth and second backcross respectively (Table 2), indicating that these two mutant phenotypes result from single recessive mutations. For *31-83-4-3* and *33-7-5(4)*, segregation ratios of approximately 9:1 and 7:1 were observed in the F2 generation of the third and fourth backcross respectively (Table 2). The most likely explanation for these lines with non 3:1 segregation ratios was that these two mutant phenotypes result from single recessive mutations (expect 15:1 ratio for recessive mutations in two genes), but that the mutations cause germination and seedling development defects. In addition, *31-83-4-3* may also cause gametophytic lethality, since the plants with the mutant phenotype were male sterile with infrequent pollen (data not shown). Line *33-7-5(4)* may also cause embryonic lethality, since aborted seeds within developing siliques of backcross F1 plants were observed (data not shown).

To determine the germination rate and observe the seedling development at 4 DAG, homozygous seed were sown on AT plates. For the homozygous *31-83-4-3* population, the germination rate was 81% (n=57) and the seedling lethality rate was 48% (n=46). For the homozygous *33-7-5(4)* population, the germination rate was 96% (n=55) and the seedling lethality rate was 85% (n=53). In contrast, the wild type germination rate is 100% (n=56) and no dead seedlings were observed. Thus, it seems possible that the deviation from a 3:1 ratio results from reduced germination and seedling lethality.

Complementation Test of *115-11-21-4* to *fkd1* and *sfc*

From the initial screen, mutant *115-11-21-4* was observed to have elongated first leaf shape and frequently exhibited distal free ends of secondary and tertiary veins (Figure 3 A). Based on the similarity of first leaf phenotype to *forked1* (*fkd1*) (Figure 3 B) and *scarface* (*sfc*) (Figure 3 C) which have frequent distal free ending veins (Steynen and Schultz, 2003, Sieburth *et al.*, 2006), crosses between mutant *115-11-21-4* and *fkd1* or *sfc* were made for a complementation test (Table 3). The F1 generation of mutant *115-11-21-4* and *fkd1* were all mutant with the phenotype of frequent free ending veins in the first leaf

(Figure 3 E, n=62). The F1 generation of mutant *115-11-21-4* and *sfc* were all wild type phenotype (Figure 3 F, n=51). These data showed that mutant *115-11-21-4* was an allele of *fkd1*. Because the phenotypes of six *fkd1* alleles have been previously published (Hou *et al.*, 2010), and the phenotype of *115-11-21-4* was not obviously different to the previously identified *fkd1*, no further phenotypic analysis was done for this mutant line.

First Leaf Shape Analysis of *31-83-4-3*, *32-2-4* and *33-7-5(4)*

In order to determine the specific quantitative differences between mutant and wild type *Arabidopsis thaliana* first leaves, several leaf shape and vascular patterning characters were measured in mature first leaves. The first leaves of wild type, mutant *31-83-4-3* and *32-2-4* were removed at 21 DAG, while the first leaves of mutant *33-7-5(4)* were removed at 28 DAG due to the slower growth rate.

Wild type *Arabidopsis* first leaves were shaped like spoons (leaf length/width=1.10±0.01, n=30) with a smooth leaf margin (no serrations), and generally have three hydathodes (Figure 4A, Table3). Mutant *31-83-4-3* first leaves were quite narrow and elongated (leaf length/width=1.52±0.03, n=30) with an obviously pointed leaf tip (Figure 4B, C), more pronounced serrations (1.60±0.16, n=30), and less pronounced hydathodes (1.63±0.14, n=30) (Table 4). Mutant *32-2-4* first leaves were slightly more elongated (leaf length/width=1.20±0.01, n=30) but a bit smaller than wild type (Figure 4D), with more pronounced serrations (1.70±0.15, n=30) and hydathodes (3.27±0.08, n=30) (Table3). Mutant *33-7-5(4)* first leaves were more elongated (leaf length/width=1.31±0.04, n=30) but significantly smaller than wild type, and were flipper shaped with a very pointed leaf tip (Figure 4E, F) with more pronounced serrations (1.87±0.16, n=30) and less pronounced hydathodes (2.60±0.14, n=30) (Table 4).

First Leaf Vein Pattern Analysis of *31-83-4-3, 32-2-4* and *33-7-5(4)*

The venation pattern of wild type *Arabidopsis thaliana* first leaves is mostly predictable: a single primary vein (the midvein) runs through the middle of the leaf lamina from the stem to the distal leaf tip; the secondary veins branch from the distal part of the midvein on both sides and extend toward the margin, and either end freely (free-ending veins), or ultimately connect to other veins (the proximal part of the midvein, another secondary vein or higher-order veins); tertiary veins branch from secondary veins and quaternary veins branch from tertiary veins (Wenzel *et al.*, 2007). In wild type most veins are connected to each other to form regions of the lamina that are completely enclosed by vein loops called areoles, and the junction of two veins is called a branch point (Dengler and Kang, 2001).

In *31-83-4-3* first leaves, the midvein frequently extends to the leaf tip and terminates in a hydathode which is termed a midvein extension (as shown in Figure 1 E, and Figure 4 C), while in wild type first leaves, the midvein never reaches the leaf tip, although a hydathode forms at the leaf tip (Table 5). Mutant *31-83-4-3* has a strong reduction in the number of secondary, tertiary and quaternary veins and a higher percentage of free-ending secondary veins, therefore the total number of branch points and areoles are reduced significantly. Vascular islands (a vein fragment that is disconnected to other veins) and marginal venation gaps (a vein-free path from leaf margin area to midvein) are observed frequently in *31-83-4-3* first leaves (Table 5, Figure 4 C).

Mutant *32-2-4* first leaves mimic the wild type vein pattern and are relatively predictable. The number of secondary, tertiary and quaternary veins is reduced relative to wild type, resulting in a reduction in the total number of areoles and branch points. The percentage of free-ending veins is quite similar to wild type. Like *31-83-4-3*, some of the mutant *32-2-4* first leaves have vascular islands (Table 5).

Like *31-83-4-3*, mutant *33-7-5(4)* first leaves frequently have a midvein extension and marginal venation gaps. In addition a significant reduction in the number of secondary, tertiary and quaternary veins, and a higher percentage of free-ending secondary and tertiary veins occurs in this mutant. As a result of these characteristics, the total numbers of areoles and branch points are significantly reduced (Table 5).

Cotyledon Vein Pattern Analysis of *31-83-4-3, 32-2-4* and *33-7-5(4)*

In addition to the first leaf vascular patterning characters, the vein pattern phenotype of wild type and three mutant lines was scored in mature cotyledons using the same rules as for first leaves. A variety of vascular pattern defects were found in mutant cotyledons, which were not seen in wild type cotyledons.

Wild type *Arabidopsis thaliana* cotyledons usually have two to four areoles formed by the closed secondary and tertiary vein loops (Figure 5 A-D). No free-ending proximal secondary veins were observed in wild type cotyledons, and quaternary veins were observed rarely and only in wild type (Table 6).

Generally, mutant *31-83-4-3* cotyledons have an increased percentage of distal and proximal free-ending secondary veins compared to wild type, resulting in a reduced number of areoles (Table 6). The frequent observation of midvein extension in *31-83-4-3* cotyledon (Figure 5 E, F, red arrows) is similar to that in the first leaves. In addition, some cotyledons have extremely abnormal vein patterns, with altered midvein location (Figure 5 G, H, dashed yellow lines indicate the normal midvein location).

Mutant *32-2-4* cotyledons have a vein pattern quite similar to wild type (Figure 5 I-L), with the numbers of areoles, branch points, and free-ending veins not significantly different from wild type (Table 6). In addition, 8.33% (n=48) of cotyledons have closed tertiary vein loop (tertiary vein branches from secondary vein and connected to the same secondary vein, indicated by the red arrows in Figure 5 J), instead of the normally closed secondary vein loop (secondary vein branches from secondary vein but connected to the midvein) in wild type.

Mutant *33-7-5(4)* cotyledons have a significant reduction in the number of secondary and tertiary veins, and an increased percentage of distal and proximal free-ending secondary veins (Table 6). Therefore the total number of areoles and branch points is reduced in *33-7-5(4)* cotyledons. Some cotyledons have a very simple vein pattern, such as only a midvein (Figure 5 P).

Shoot Phenotype Analysis of *31-83-4-3, 32-2-4* and *33-7-5(4)*

The range of defects to first leaf shape and vein pattern, as well as the cotyledon vein pattern described above are often correlated with defects to auxin biosynthesis, transport and response. To determine if the mutations affected auxin regulated shoot developmental processes, shoot characters such as number of rosette leaves, number of stem branches, and flowering time were measured and compared among wild type and the three mutant lines.

The average flowering time of wild type *Arabidopsis thaliana* was about 21 DAG, and at 35 DAG the wild type plants had about seven rosette leaves and three to four lateral inflorescence stems; the main stem was longer than lateral inflorescence stems (Figure 6 A, Table 7).

The average flowering time of mutant *31-83-4-3* plants was about 29 DAG which was significantly delayed compared to wild type (Table 7). At 35 DAG, the *31-83-4-3* plants had about nine rosette leaves and seven inflorescence stems, but the rosette leaves were smaller and main inflorescence stem was shorter than wild type. The inflorescence changes resulted in *31-83-4-3* plants having a bushy appearance. A unique shoot character, fused leaf and stem (Figure 6 B, white squares) was frequently seen in this mutant (54.17%, n=24), as well as fused leaves, fused sepals, curved petals and improperly developed pistils and stamens (data not shown).

The flowering time of *32-2-4* was about two days later than wild type, and *32-2-4* plants had more leaves and fewer branches (Figure 6 C, Table 7).

Mutant *33-7-5(4)* plants were pale and grew significantly more slowly than the wild type and other two mutant lines (Figure 6). They produced very small weak leaves and inflorescence stems; the average flowering time was more than 30 DAG.

Root Phenotype Analysis of *31-83-4-3, 32-2-4* and *33-7-5(4)*

A certain auxin concentration, proper auxin transport and response are required for the normal root elongation and gravity response. To determine if the mutations affected auxin-regulated root developmental processes, root characters such as growth rate and gravitropism were measured and compared among wild type and three mutant lines.

Within 24 hours, the average elongation of wild type roots was about 8.86 mm. The average elongation of *32-2-4* roots was slightly less than wild type. However, the average elongation of *31-83-4-3* and *33-7-5(4)* roots were about 5.74 mm and 1.50 mm respectively, both significantly less than wild type (Table 8).

To determine the gravitropism of wild type and three mutant lines, the 5 DAG seedlings were rotated 90° for horizontal angle measurements after 2, 4, 6 hours. Based on the angle measurement, there was no significant difference in gravitropic response between *32-2-4* and wild type. In contrast, the responses of *31-83-4-3* and *33-7-5(4)* were smaller than wild type at all the time points and never reached the expected rotation of 90º (Table 8, Figure 7).

DR5::GUS* Expression is Reduced in *33-7-5(4)

The *DR5::GUS* reporter gene enables relative levels of active auxin to be compared qualitatively (Ulmasov *et al.*, 1999). To test if *33-7-5(4)* leaves show altered auxin response, the homozygous population of *33-7-5(4)* that expressed *DR5::GUS* were screened and a developmental series of roots and leaves of *33-7-5(4):DR5::GUS* and wild type:*DR5::GUS* from 2 DAG to 5 DAG was compared (Figure 8). Within the 5 DAG root tips, the *DR5::GUS* expression was strongly reduced both in level (the intensity of color) and extent (the pattern of expression) in the mutant *33-7-5(4)* compared to wild type (Figure 8 A, B). Within the 5 DAG wild type first leaves, *DR5::GUS* expression was intense and predicted the leaf vein pattern development (Figure 8 C); in contrast, no *DR5::GUS* expression was seen in *33-7-5-(4)* (Figure 8 D), and the first leaf size was about the same size as 2 DAG to 3 DAG wild type first leaves (Figure 8 E, F).

To analyze auxin response level in *32-2-4*, the mutants were crossed to *DR5::GUS*. In the F2 generation, 61 plants with *32-2-4* phenotypes were harvested and screened for homozygous GUS expression (expecting a quarter of 61). However, no homozygous plants were found which suggested that *32-2-4* was linked to *DR5::GUS*. The heterozygous seeds were saved for further screening but no further study of them was completed.

Double Mutants *cuc2-3 32-2-4* has Smooth Leaf Margin

In order to determine if serrated leaf mutants can be affected by the leaf margin mutant *cuc2-3* (smooth leaf margin with no serrations), crosses were made between *cuc2-3* and mutant lines (*cuc2-3×32-2-4* and *cuc2-3×33-7-5(4)* to generate double mutants.

The wild type fifth leaves had an average of 4.76 serrations (n=17), the *32-2-4* homozygous fifth leaves had an average of 8.80 serrations (n=15) which was significantly more than wild type. The fifth leaves of double mutant *cuc2-3 32-2-4* had no serrations like the single mutant *cuc2-3* (Table 9, Figure 9). Thus, *cuc2-3* was epistatic to the serration phenotype of *32-2-4*.

Two hundred and sixteen (216) F2 seeds were planted to screen for the plants with *33-7-5(4)* phenotype. Of these, fourteen (14) plants had the *33-7-5(4)* phenotype. None of the F3 populations generated by these self-fertilizing plants segregated for *cuc2-3*. One possible explanation for this observation was that *33-7-5(4)* was linked to *cuc2-3*. A second possible explanation was that *33-7-5(4)* was epistatic to *cuc2*-3. Thus, no *cuc2-3 33-7-5(4)* double mutant was confirmed.

Mapping of *32-2-4* and *33-7-5(4)*

Mutants *33-7-5(4)* and *32-2-4* (Col ecotype) were crossed to the Landsberg *erecta* (Ler) ecotype wild type plants and the F2 plants were later planted as the mapping population. DNA was extracted from the leaves of F2 plants showing a clear mutant phenotype and used as PCR template (Dellaporta *et al.*, 1983). To determine the chromosomal location of the mutation, three to five primers on each chromosome were tested and used for amplifying the isolated mutant DNA (Table 1).

The genetic linkage was established by looking at the length of the PCR products on the agarose gel (Figure 10). For each pair of the SSLP markers, DNA samples from three controls (Col, Het (F1 of cross between wild type Col and Ler), Ler), and the mapping population were selected and amplified through the PCR reaction. Following gel electrophoresis of the PCR products, the genotypes of the mapping population were obtained by comparing the DNA band location of the mapping samples and controls. If the Col and Ler genotypic ratio was significantly different from 1:1 as determined by Pearson's chi-squared test, I considered that this marker was linked with the mutant allele.

With a mapping population of 108 and 106 respectively, the *32-2-4* gene was mapped to chromosome II between markers 2-61 and 2-82, approximately 9.72 and 5.19 map units from markers 2-61 and 2-82 respectively. With a mapping population of 135, the *33-7-5(4)* gene mapped to chromosome V between markers 5-85 and 5-116, approximately 16.30 and 9.63 map units from the markers 5-85 and 5-116 respectively (Table 10). From this mapping data, *33-7-5(4)* is linked with *cuc2-3* (located on chromosome 5, AGI position 102.9 cM), thus explaining the lack of *cuc2-3 33-7-5(4)* double mutants.

DISCUSSION

Auxin is required for a number of key processes during plant development (Scarpella *et al.*, 2010). Studies of a variety of *Arabidopsis thaliana* mutants indicate that defects in auxin biosynthesis, transport or response often result in aberrant plant development (Berná *et al.*, 1999). I have identified four genetically stable mutants based on the first leaf phenotype. Mutant *115-21-4* had first leaves with frequent free-ending veins, and was determined to be an allele of *FKD1* by complementation testing. Mutant *31-83-4-3* had elongated first leaves with pointed leaf tips, frequent serrations and free-ending veins; mutant *32-2-4* had first leaves with small serrations and a simplified vein pattern; mutant *33-7-5(4)* had small, pale first leaves with pointed leaf tips, frequent serrations, extremely simplified vein pattern and frequent free-ending secondary veins. These last three mutant lines (*31-83-4-3*, *32-2-4* and *33-7-5(4)*) were further studied for shoot and root phenotypes. In addition, models to explain their first leaf development were proposed based on the phenotypic defects. Two mutant lines (*32-2-4* and *33-7-5(4)*) were mapped to the specific chromosomes using molecular markers.

31-83-4-3 Phenotype was Consistent with Defects in Auxin Transport

The first leaf of *31-83-4-3* had a pointed leaf tip and frequent serrations, and a simplified leaf vein pattern with frequent distal free ends in secondary veins. The first leaf phenotype suggested the *31-83-4-3* phenotype may have, like the *unh* and *vam3-4* (*vacuolar morphology*) phenotype, resulted from expanded PIN1 within the leaf margin (Figure 2 F, H) (Shirakawa *et al.*, 2009, Pahari *et al.*, 2014). Alternatively (or additionally), the *31-83-4-3* phenotype may have resulted from defective auxin transport (lack of upward auxin flux in the margin or in developing veins) possibly correlated with changes to PIN1 polarity; as compensation, auxin concentration maxima at the leaf marginal area form and induce serration outgrowth.

A series of shoot and root phenotypic defects in *31-83-4-3*, such as increased numbers of rosette leaves, decreased internode elongation and increased branching (axillary bud growth) in the shoot, and reduced primary root growth and length, were consistent with an auxin transport defect. Auxin transport is required for cell division and elongation, which are correlated with the shoot and root elongation. Increased downward auxin transport reduces the auxin level in the shoot (resulting in a slower shoot growth rate) and increases the auxin level in root (inhibiting root growth) (Peer *et al.*, 2011). The bushy appearance of mature *31-83-4-3* plants suggested that the lateral buds were released from the apical dominance; the increased lateral bud outgrowth either resulted from increased downward auxin transport (Prusinkiewicz *et al.*, 2009) as suggested above for root growth or from the lack of upward auxin transport in the stem (Leyser, 2003). *31-83-4-3* developed more rosette leaves than wild-type and formed a bushy rosette with wavy leaf blades, these characters were also similar to the mutant *short stem and midrib* (*ssm*), an allele of *VAM3/SYP22* which has been implicated in vesicle transport to the vacuole (Ohtomo et al., 2005).

The fact that *31-83-4-3* shared many phenotypic characters with both *unh* and *vam3-4*, suggested that *31-83-4-3* may, like *unh* and *vam3* (Shirakawa *et al.*, 2009, Pahari *et al.*, 2014), result in higher levels of PIN1, thus increasing auxin flux through secondary veins and the shoot. A model of the first leaf of *31-83-4-3* with increased auxin level in the margin, as well as increased auxin flux through the secondary veins was proposed (Figure

11 A). I suggest that the increased auxin flux through veins might result in additional basal localized PIN1 and lack of apical localized PIN1, and increased serration and distal non-meeting veins as the consequences, similar to what happens in *unh* mutants (Pahari *et al.*, 2014).

Other phenotypic defects such as delayed flowering time, reduced fertility and reduced gravitropic response, suggested the *31-83-4-3* mutant may also have defects in auxin response that were correlated with changes to auxin transport. The ARF family are auxin response factors and individual ARFs have unique and overlapping functions, and positively or negatively regulate gene expression. Delayed flowering and reduced fertility have been found in *arf2* mutants (Ellis *et al.*, 2005) which have auxin response defects, and these phenomena are also observed in *31-83-4-3*. In addition, organ fusion was frequently observed in *31-83-4-3*, which has been suggested to occur because of a defect in auxin regulated cell division (Peer *et al.*, 2011). Crosses between *31-83-4-3* and *Ler*, as well as plants transgenic for PIN1:GFP or *DR5::GUS* were completed, however no seed was successfully generated due to the sterility of *31-83-4-3*. My inability to generate a mapping population of *31-83-4-3* or integrate *DR5::GUS* into *31-83-4-3* limited further study of this mutant line. Additional back crosses may remove the sterility from *31-83-4-3*.

Serrated Leaf of *32-2-4* May Result from Higher Auxin in the Leaf Margin

Relative to wild type, the *32-2-4* first leaf had serrations and a simplified vein pattern with closed vein loops. Since the mature plant phenotype of *32-2-4* was similar to wild type except for the serrated leaves and slightly pale color, and there was no significant difference in the gravity response of the root tip, I propose that there was no significant defect in global auxin biosynthesis, transport or response levels in *32-2-4*. The later rosette leaves of *32-2-4* had more serrations and the serrations were bigger than wild type; this phenotype suggested that *32-2-4* may have, like *BODENLOS* (*BDL*) mutants, increased auxin activity in the marginal area especially in the positions of the serrations (Bilsborough *et al.*, 2011). I proposed a model of the *32-2-4* first leaf shape and vein pattern to show that the increased leaf serrations were correlated with increased marginal auxin maxima (Figure 11 B).

The *CUC2* gene directs PIN1 re-localization within the leaf margin which determines the auxin transport direction, a process necessary for the formation of auxin convergence points at the leaf margin area where the serrations are forming (Bilsborough *et al.*, 2011). A feedback loop among PIN1, auxin and CUC2 has been proposed to maintain a certain auxin concentration at the auxin convergence points for the serration growth (Bilsborough *et al.*, 2011). The *cuc2-3* mutant failed to develop serrations due to the lack of CUC2. The *cuc2-3* phenotype was epistatic to *32-2-4* (Figure 9 D) which suggested the *32-2-4* was functioning in the same pathway as CUC2. The lack of serrations when *32-2-4* was combined with *cuc2-3* suggested that relocalization of PIN1 by CUC2 was required for *32-2-4* to generate enlarged serrations, consistent with the idea that *32-2-4* increased auxin activity at auxin convergence points.

The delayed development of *32-2-4* (late flowering time which corresponds with small rosette leaves and increased leaf number) may be due to the pale color, and the pale color may result from lower chlorophyll content or lower cell density in the leaf blade. Thus, further study of mutant *32-2-4* should focus on the gene function in serration formation and the pale color. Mutant *32-2-4* mapped to the position between flanking markers 2-61 and 2-82 on chromosome 2, approximately at 74.7cM on AGI Map. Based on the map position and the pale color phenotype, *32-2-4* could be an allele of some known genes such as *ALBINO AND PALE GREEN10* (*APG10*) and *LOWER CELL DENSITY1* (*LCD1*). The *apg10* mutant has pale green cotyledons and rosette leaves, leaf development is delayed and the leaves are small, narrow, with some serrations (Noutoshi *et al.*, 2005). Similar to *32-2-4*, the *lcd1* mutant plants are pale-green compared to wild type under optimal light intensity, and even paler under higher light intensities (Barth and Conklin, 2003).

Auxin Response is Reduced in *33-7-5(4)*

33-7-5(4) had a flipper shaped first leaf, and the rosette leaves were small, pale and serrated. Both the shoot growth and root growth were slow, resulting in a weak plant with the developmental progress delayed about 4-5 days compared to wild type. At 5DAG, the *DR5::GUS* expression in the mutant *33-7-5(4)* root tip was significantly reduced compared to the wild type root tip. This result suggested that the auxin response in *33-7-5(4)* was lower than wild type which was consistent with the slower root gravitropic response. Thus, the gene *33-7-5(4)* may not only function in leaf shape and vein patterning, but also in primary development or physiological processes which could alter overall auxin levels.

Due to the whole plant phenotype and the very low *DR5::GUS* expression in both roots and leaves, I suggest that overall low auxin level results in reduced cell division and expansion in *33-7-5(4)* mutant. A possible explanation for the leaf phenotype is that, auxin levels in the leaf margin were above a threshold value that allowed serration outgrowth and the development of secondary veins, while auxin levels in the leaf blade were too low to support sufficient cell division and/or expansion, as well as development of higher-order veins. Supporting the idea that *33-7-5(4*) had reduced auxin response, its highly simplified vein pattern resembled *mp* mutants which produced fewer secondary veins and no veins along the leaf margin (Mattsson *et al.*, 1999).

In contrast to the other two models I have proposed for the leaf serration formation, I propose that the serrations of *33-7-5(4)* were due to the reduced growth of the leaf blade relative to the leaf margin. Thus, in the model of *33-7-5(4)* first leaf shape and vein pattern formation, the auxin maxima that indicate the sites of serration on the margin were reduced relative to wild type and the other two mutants (*31-83-4-3* and *32-2-4*) (Figure 11 C).

Mutant *33-7-5(4)* mapped to the position between flanking marker 5-85 and 5-116 on chromosome 5, approximately at 104.5 cM on AGI Map. Based on the mapping position of *33-7-5(4)* and the identified position of *CUC2* (102.9 cM on AGI Map), these two genes were linked which was consistent with my inability to obtain plants homozygous for *cuc2-3* and *33-7-5(4)*.

Conclusions

In this study, four mutant lines were analyzed in detail: mutant *115-11-21-4* was an allele of *FKD1* with frequent distal free-ending veins, and the other three mutants were likely defective in auxin biosynthesis, transport or response. Mutant *31-83-4-3* likely had increased auxin flux through secondary veins and increased downward auxin transport through the shoot, possibly due to the increased *PIN1* expression and/or altered PIN1 localization. Mutant *32-2-4* likely had no significant defect in global auxin biosynthesis, transport or response levels, but instead had increased auxin activity at marginal auxin convergence points to enable the formation of increased leaf serrations. Mutant *33-7-5(4)* may have had global reduction in auxin biosynthesis or response levels, thus the whole plant was weak and pale, with slower shoot and root growth rate and root gravitropism.

REFERENCES

Baima, S., F. Nobili, G. Sessa, S. Lucchetti, I. Ruberti and G. Morelli (1995). "The expression of the *Athb-8* homeobox gene is restricted to provascular cells in *Arabidopsis thaliana*." Development **121**(12): 4171-4182.

Baima, S., M. Possenti, A. Matteucci, E. Wisman, M. M. Altamura, I. Ruberti and G. Morelli (2001). "The *Arabidopsis* ATHB-8 HD-zip protein acts as a differentiation-promoting transcription factor of the vascular meristems." Plant Physiol **126**(2): 643-655.

Barth, C. and P. L. Conklin (2003). "The lower cell density of leaf parenchyma in the *Arabidopsis thaliana* mutant lcd1-1 is associated with increased sensitivity to ozone and virulent Pseudomonas syringae." Plant J **35**(2): 206-218.

Bell, C. J. and J. R. Ecker (1994). "Assignment of 30 microsatellite loci to the linkage map of Arabidopsis." Genomics **19**(1): 137-144.

Benkova, E., M. Michniewicz, M. Sauer, T. Teichmann, D. Seifertova, G. Jurgens and J. Friml (2003). "Local, efflux-dependent auxin gradients as a common module for plant organ formation." Cell **115**(5): 591-602.

Bennett, M. J., A. Marchant, H. G. Green, S. T. May, S. P. Ward, P. A. Millner, A. R. Walker, B. Schulz and K. A. Feldmann (1996). "*Arabidopsis* AUX1 gene: a permease-like regulator of root gravitropism." Science **273**(5277): 948-950.

Berleth, T., N. T. Krogan and E. Scarpella (2004). "Auxin signals--turning genes on and turning cells around." Current Opinion in Plant Biology **7**(5): 553-563.

Berná, G., P. Robles and J. L. Micol (1999). "A mutational analysis of leaf morphogenesis in *Arabidopsis thaliana*." Genetics **152**(2): 729-742.

Bilsborough, G. D., A. Runions, M. Barkoulas, H. W. Jenkins, A. Hasson, C. Galinha, P. Laufs, A. Hay, P. Prusinkiewicz and M. Tsiantis (2011). "Model for the regulation of *Arabidopsis thaliana* leaf margin development." Proceedings of the National Academy of Sciences **108**(8): 3424-3429.

Candela, H., A. Martinez-Laborda and J. L. Micol (1999). "Venation pattern formation in *Arabidopsis thaliana* vegetative leaves." Dev Biol **205**(1): 205-216.

Cheng, Y., X. Dai and Y. Zhao (2006). "Auxin biosynthesis by the YUCCA flavin monooxygenases controls the formation of floral organs and vascular tissues in *Arabidopsis*." Genes Dev **20**(13): 1790-1799.

Cheng, Y., X. Dai and Y. Zhao (2007). "Auxin synthesized by the YUCCA flavin monooxygenases is essential for embryogenesis and leaf formation in Arabidopsis." Plant Cell **19**(8): 2430-2439.

Dellaporta, S., J. Wood and J. Hicks (1983). "A plant DNA minipreparation: Version II." Plant Molecular Biology Reporter **1**(4): 19-21.

Dengler, N. and J. Kang (2001). "Vascular patterning and leaf shape." Curr Opin Plant Biol **4**(1): 50-56.

Deyholos, M. K., G. Cordner, D. Beebe and L. E. Sieburth (2000). "The SCARFACE gene is required for cotyledon and leaf vein patterning." Development **127**(15): 3205-3213.

Donnelly, P. M., D. Bonetta, H. Tsukaya, R. E. Dengler and N. G. Dengler (1999). "Cell cycling and cell enlargement in developing leaves of *Arabidopsis*." Dev Biol **215**(2): 407-419.

Donner, T. J., I. Sherr and E. Scarpella (2010). "Auxin signal transduction in *Arabidopsis* vein formation." Plant Signal Behav **5**(1): 70-72.

Eliasson, L., G. Bertell and E. Bolander (1989). "Inhibitory action of auxin on root elongation not mediated by ethylene." Plant Physiol **91**(1): 310-314.

Ellis, C. M., P. Nagpal, J. C. Young, G. Hagen, T. J. Guilfoyle and J. W. Reed (2005). "AUXIN RESPONSE FACTOR1 and AUXIN RESPONSE FACTOR2 regulate senescence and floral organ abscission in *Arabidopsis thaliana*." Development **132**(20): 4563-4574.

Esau, K. (1965a). Plant anatomy. New York, NY, USA, John Wiley and Sons.

Esau, K. (1965b). Vascular differentiation in plants. New York, NY, USA, Holt Rinehart and Winston Inc.

Feraru, E. and J. Friml (2008). "PIN polar targeting." Plant Physiology **147**(4): 1553-1559.

Foster, A. S. (1952). Foliar venation in angiosperms from an ontogenetic standpoint. Am, J. Bot.

Geisler, M., B. Wang and J. Zhu (2013). "Auxin transport during root gravitropism: transporters and techniques." Plant Biology: 50-57.

Hardtke, C. S. and T. Berleth (1998). "The *Arabidopsis* gene *MONOPTEROS* encodes a transcription factor mediating embryo axis formation and vascular development." The EMBO Journal **17**(5): 1405-1411.

Hay, A., M. Barkoulas and M. Tsiantis (2006). "ASYMMETRIC LEAVES1 and auxin activities converge to repress BREVIPEDICELLUS expression and promote leaf development in *Arabidopsis*." Development **133**(20): 3955-3961.

Hou, H., J. Erickson, J. Meservy and E. A. Schultz (2010). "*FORKED1* encodes a PH domain protein that is required for PIN1 localization in developing leaf veins." The Plant Journal **63**(6): 960-973.

Kang, J. and N. Dengler (2002). "Cell cycling frequency and expression of the homeobox gene *ATHB-8* during leaf vein development in *Arabidopsis*." Planta **216**(2): 212-219.

Koizumi, K., M. Sugiyama and H. Fukuda (2000). "A series of novel mutants of Arabidopsis thaliana that are defective in the formation of continuous vascular network: calling the auxin signal flow canalization hypothesis into question." Development **127**(15): 3197-3204.

Larkin, J. C., D. G. Oppenheimer, A. M. Lloyd, E. T. Paparozzi and M. D. Marks (1994). "Roles of the GLABROUS1 and TRANSPARENT TESTA GLABRA genes in *Arabidopsis* trichome development." The Plant Cell Online **6**(8): 1065-1076.

Leyser, O. (2003). "Regulation of shoot branching by auxin." Trends Plant Sci **8**(11): 541-545.

Ljung, K., R. P. Bhalerao and G. Sandberg (2001). "Sites and homeostatic control of auxin biosynthesis in *Arabidopsis* during vegetative growth." The Plant Journal **28**(4): 465-474.

Ljung, K., A. K. Hull, J. Celenza, M. Yamada, M. Estelle, J. Normanly and G. Sandberg (2005). "Sites and regulation of auxin biosynthesis in *Arabidopsis* roots." Plant Cell **17**(4): 1090-1104.

Ljung, K., A. K. Hull, M. Kowalczyk, A. Marchant, J. Celenza, J. D. Cohen and G. Sandberg (2002). "Biosynthesis, conjugation, catabolism and homeostasis of indole-3-acetic acid in *Arabidopsis thaliana*." Plant Molecular Biology **49**(3): 249-272.

Mano, Y. and K. Nemoto (2012). "The pathway of auxin biosynthesis in plants." J Exp Bot **63**(8): 2853-2872.

Mashiguchi, K., K. Tanaka, T. Sakai, S. Sugawara, H. Kawaide, M. Natsume, A. Hanada, T. Yaeno, K. Shirasu, H. Yao, P. McSteen, Y. Zhao, K.-i. Hayashi, Y. Kamiya and H. Kasahara (2011). "The main auxin biosynthesis pathway in *Arabidopsis*." Proceedings of the National Academy of Sciences **108**(45): 18512-18517.

Mattsson, J., Z. R. Sung and T. Berleth (1999). "Responses of plant vascular systems to auxin transport inhibition." Development **126**(13): 2979-2991.

Meinhardt, H. (1976). "Morphogenesis of lines and nets." Differentiation **6**(2): 117-123.

Mitchison, G. (1980). "A model for vein formation in higher plants." Proceedings of the Royal Society of London. Series B. Biological Sciences **207**(1166): 79-109.

Mravec, J., P. Skupa, A. Bailly, K. Hoyerova, P. Krecek, A. Bielach, J. Petrasek, J. Zhang, V. Gaykova, Y. D. Stierhof, P. I. Dobrev, K. Schwarzerova, J. Rolcik, D. Seifertova, C. Luschnig, E. Benkova, E. Zazimalova, M. Geisler and J. Friml (2009). "Subcellular homeostasis of phytohormone auxin is mediated by the ER-localized PIN5 transporter." Nature **459**(7250): 1136-1140.

Nelson, T. and N. Dengler (1997). "Leaf vascular pattern formation." Plant Cell **9**(7): 1121-1135.

Nikovics, K., T. Blein, A. Peaucelle, T. Ishida, H. Morin, M. Aida and P. Laufs (2006). "The balance between the MIR164A and CUC2 genes controls leaf margin serration in *Arabidopsis*." The Plant Cell Online **18**(11): 2929-2945.

Noutoshi, Y., T. Ito and K. Shinozaki (2005). "ALBINO AND PALE GREEN 10 encodes BBMII isomerase involved in histidine biosynthesis in *Arabidopsis thaliana*." Plant Cell Physiol **46**(7): 1165-1172.

Ohtomo, I., H. Ueda, T. Shimada, C. Nishiyama, Y. Komoto, I. Hara-Nishimura and T. Takahashi (2005). "Identification of an allele of VAM3/SYP22 that confers a semi-dwarf phenotype in *Arabidopsis thaliana*." Plant Cell Physiol **46**(8): 1358-1365.

Overvoorde, P., H. Fukaki and T. Beeckman (2010). "Auxin control of root development." Cold Spring Harbor Perspectives in Biology **2**(6).

Pahari, S., R. D. Cormark, M. T. Blackshaw, C. Liu, J. L. Erickson and E. A. Schultz (2014). "*Arabidopsis* UNHINGED encodes a VPS51 homolog and reveals a role for the GARP complex in leaf shape and vein patterning." Development **141**(9): 1894-1905.

Peer, W. A., J. J. Blakeslee, H. Yang and A. S. Murphy (2011). "Seven things we think we know about auxin transport." Mol Plant **4**(3): 487-504.

Petrášek, J. and J. Friml (2009). "Auxin transport routes in plant development." Development **136**(16): 2675-2688.

Petrasek, J., J. Mravec, R. Bouchard, J. J. Blakeslee, M. Abas, D. Seifertova, J. Wisniewska, Z. Tadele, M. Kubes, M. Covanova, P. Dhonukshe, P. Skupa, E. Benkova, L. Perry, P. Krecek, O. R. Lee, G. R. Fink, M. Geisler, A. S. Murphy, C. Luschnig, E. Zazimalova and J. Friml (2006). "PIN proteins perform a rate-limiting function in cellular auxin efflux." Science **312**(5775): 914-918.

Petricka, J. J., C. M. Winter and P. N. Benfey (2012). "Control of *Arabidopsis* root development." Annu Rev Plant Biol **63**: 563-590.

Prusinkiewicz, P., S. Crawford, R. S. Smith, K. Ljung, T. Bennett, V. Ongaro and O. Leyser (2009). "Control of bud activation by an auxin transport switch." Proc Natl Acad Sci U S A **106**(41): 17431-17436.

Rahman, A., M. Takahashi, K. Shibasaki, S. A. Wu, T. Inaba, S. Tsurumi and T. I. Baskin (2010). "Gravitropism of *Arabidopsis thaliana* roots requires the polarization of PIN2 toward the root tip in meristematic cortical cells." Plant Cell **22**(6): 1762-1776.

Rashotte, A. M., S. R. Brady, R. C. Reed, S. J. Ante and G. K. Muday (2000). "Basipetal auxin transport is required for gravitropism in roots of *Arabidopsis*." Plant Physiology **122**(2): 481-490.

Ruegger, M., E. Dewey, W. M. Gray, L. Hobbie, J. Turner and M. Estelle (1998). "The TIR1 protein of *Arabidopsis* functions in auxin response and is related to human SKP2 and yeast grr1p." Genes Dev **12**(2): 198-207.

Sachs, T. (1981). The control of the patterned differentiation of vascular tissues, Academic Press.

Sack, L. and C. Scoffoni (2013). "Leaf venation: structure, function, development, evolution, ecology and applications in the past, present and future." New Phytol **198**(4): 983-1000.

Sawchuk, M. G., A. Edgar and E. Scarpella (2013). "Patterning of leaf vein networks by convergent auxin transport pathways." PLoS Genet **9**(2): e1003294.

Scarpella, E., M. Barkoulas and M. Tsiantis (2010). "Control of leaf and vein development by auxin." Cold Spring Harbor Perspectives in Biology **2**(1): 1-17.

Scarpella, E., P. Francis and T. Berleth (2004). "Stage-specific markers define early steps of procambium development in *Arabidopsis* leaves and correlate termination of vein formation with mesophyll differentiation." Development **131**(14): 3445-3455.

Scarpella, E., D. Marcos, J. Friml and T. Berleth (2006). "Control of leaf vascular patterning by polar auxin transport." Genes & Development **20**(8): 1015-1027.

Serrano-Cartagena, J., P. Robles, M. R. Ponce and J. L. Micol (1999). "Genetic analysis of leaf form mutants from the *Arabidopsis* Information Service collection." Molecular and General Genetics **261**(4-5): 725-739.

Shirakawa, M., H. Ueda, T. Shimada, C. Nishiyama and I. Hara-Nishimura (2009). "Vacuolar SNAREs function in the formation of the leaf vascular network by regulating auxin distribution." Plant Cell Physiol **50**(7): 1319-1328.

Sieburth, L. E., G. K. Muday, E. J. King, G. Benton, S. Kim, K. E. Metcalf, L. Meyers, E. Seamen and J. M. Van Norman (2006). "SCARFACE encodes an ARF-GAP that is required for normal auxin efflux and vein patterning in *Arabidopsis*." Plant Cell **18**(6): 1396-1411.

Steynen, Q. J. and E. A. Schultz (2003). "The *FORKED* genes are essential for distal vein meeting in *Arabidopsis*." Development **130**(19): 4695-4708.

Swarup, R., J. Friml, A. Marchant, K. Ljung, G. Sandberg, K. Palme and M. Bennett (2001). "Localization of the auxin permease AUX1 suggests two functionally distinct hormone transport pathways operate in the *Arabidopsis* root apex." Genes Dev **15**(20): 2648-2653.

Taiz, L. and E. Zeiger (2002). Plant physiology. Third Edition. Sunderland, Massachusetts., Sinauer Associates, Inc.

Titapiwatanakun, B., J. J. Blakeslee, A. Bandyopadhyay, H. Yang, J. Mravec, M. Sauer, Y. Cheng, J. Adamec, A. Nagashima, M. Geisler, T. Sakai, J. Friml, W. A. Peer and A. S. Murphy (2009). "ABCB19/PGP19 stabilises PIN1 in membrane microdomains in *Arabidopsis*." Plant J **57**(1): 27-44.

Tiwari, S. B., G. Hagen and T. Guilfoyle (2003). "The roles of auxin response factor domains in auxin-responsive transcription." Plant Cell **15**(2): 533-543.

Tsuge, T., H. Tsukaya and H. Uchimiya (1996). "Two independent and polarized processes of cell elongation regulate leaf blade expansion in *Arabidopsis thaliana* (L.) Heynh." Development **122**(5): 1589-1600.

Tsukaya, H. (2005). "Leaf shape: genetic controls and environmental factors." International Journal of Developmental Biology **49**(5/6): 547.

Ulmasov, T., G. Hagen and T. J. Guilfoyle (1999). "Activation and repression of transcription by auxin-response factors." Proc Natl Acad Sci U S A **96**(10): 5844-5849.

Vitha, S., K. Beneš, J. Phillips and K. A. Gartland (1995). Histochemical GUS Analysis. Agrobacterium Protocols. K. A. Gartland and M. Davey, Springer New York. **44:** 185-193.

Waites, R. and A. Hudson (1995). "phantastica: a gene required for dorsoventrality of leaves in Antirrhinum majus." Development **121**(7): 2143-2154.

Wang, W., B. Xu, H. Wang, J. Li, H. Huang and L. Xu (2011). "YUCCA genes are expressed in response to leaf adaxial-abaxial juxtaposition and are required for leaf margin development." Plant Physiol **157**(4): 1805-1819.

Went, F. W. (1974). "Reflections and speculations." Annual Review of Plant Physiology **25**(1): 1-27.

Wenzel, C. L., M. Schuetz, Q. Yu and J. Mattsson (2007). "Dynamics of *MONOPTEROS* and *PIN-FORMED1* expression during leaf vein pattern formation in *Arabidopsis thaliana*." The Plant Journal **49**(3): 387-398.

Wisniewska, J., J. Xu, D. Seifertova, P. B. Brewer, K. Ruzicka, I. Blilou, D. Rouquie, E. Benkova, B. Scheres and J. Friml (2006). "Polar PIN localization directs auxin flow in plants." Science **312**(5775): 883.

Won, C., X. Shen, K. Mashiguchi, Z. Zheng, X. Dai, Y. Cheng, H. Kasahara, Y. Kamiya, J. Chory and Y. Zhao (2011). "Conversion of tryptophan to indole-3-acetic acid by TRYPTOPHAN AMINOTRANSFERASES OF ARABIDOPSIS and YUCCAs in Arabidopsis." Proc Natl Acad Sci U S A **108**(45): 18518-18523.

Zhang, J., S. Vanneste, P. B. Brewer, M. Michniewicz, P. Grones, J. Kleine-Vehn, C. Lofke, T. Teichmann, A. Bielach, B. Cannoot, K. Hoyerova, X. Chen, H. W. Xue, E. Benkova, E. Zazimalova and J. Friml (2011). "Inositol trisphosphate-induced Ca2+ signaling modulates auxin transport and PIN polarity." Developmental Cell **20**(6): 855-866.

Table 1. SSLP markers designed and used in mapping *32-2-4* and *33-7-5(4)*

Primer Name	Sequence(5’-3’)	AGI BAC	Annealing Temp. (℃)	Mg^{2+} (mM)	Col* (bp)	Ler* (bp)
1-11 F	ACC CAA GTG ATC GCC ACC	F21M12	56.5	2.0	111	89
1-11 R	AAC CAA GGC ACA GAA GCG					
1-15 F	GCA GCA TAG AGG CGA GGT TA	F7A19	54.2	2.5	210	169
1-15 R	GGT GGA TCG ACT GTG AGG TT					
1-19 F	TTG GGA AAT CAA AAC GCA GT	F20D23	53.8	2.5	156	141
1-19 R	TGC TTC AAT CAG GGA TTT CG					
1-52 F	TGA AAC GCG ACT TGA TCT TG	F28J9	55.0	2.5	255	211
1-52 R	TAG CCG AGA AGG GAT TGA TG					
1-82 F	TGG ATT CAA GAC CTA AAG CTG A	F11F12	55.5	2.5	327	273
1-82 R	TGG ATC ATC GAG GGA CTC AT					
1-116 F	TCA CGA TAC GTT CAA AAT TAG GG	F20P5	55.2	2.5	196	164
1-116 R	CCG ATG TAT TTT GCT AAT TGA GG					
2-9 F	GCA CAT ACC CAC AAC CAG AA	T8K22	50.6	2.5	213	217
2-9 R	CCT TCA CAT CCA AAA CCC AC					
2-11 F	CCC AAA AGT TAA TTA TAC TGT	T18C20	51.8	2.5	105	90
2-11 R	CCG GGT TAA TAA TAA ATG T					
2-23 F	CCT CCA AAC CAG CTA TCG TC	F23M2	53.2	2.5	384	316
2-23 R	TCG GAG ATA CCC GAG CTA AA					
2-45 F	CAA AAG CCA CAC TAA GGG ACA	F3K23	52.0	2.5	396	328
2-45 R	TGC ACC ATT GAG ACA AGC TC					
2-50 F	GCA CAG TCC AAG TCA CAA CC	F3C11	53.4	2.0	189	213
2-50 R	CGC TAC GCT TTT CGG TAA AG					
2-61 F	GTT CAT GGA CGG ATG TGT ATG C	T6B20	55.4	2.5	75	83
2-61 R	CTA GTG GTG GTT AAA ATA TAC GC					
2-82 F[a]	CTC ATC TTG TTT ACT AAT ATC AAC TCG	F6E13	51.0	2.5	196	168
2-82 R[a]	TTT AAT CAT TGT TTT GTG CGA AAA T					
3-23 F	TTG GAA CTG CAA TTG ATG GA	T2O4	48.3	2.5	340	244
3-23 R	TAT AGG GCA AGC CCA AGA TG					
3-42 F	CCC CGA GTT GAG GTA TT	MFE16	56.5	2.5	180	230
3-42 R	GAA GAA ATT CCT AAA GCA TTC					
3-50.5 F[a]	GTA TCG ATC GGC ACC TTT GT	MSJ3	54.2	2.5	156	176
3-50.5 R[a]	GCT GCA ATG CCA TCT TGA TA					
3-68.5 F	CGG AAT TGA TGT TTT GGA CC	T10D17	53.8	2.5	850	1000

3-68.5 R	CCC TAC ATT CTA CAA CCA TGT AGC C					
3-74 F	TGC ATT GGT TTC TCT GCT TG	T6H20	55.0	2.5	370	294
3-74 R	TGC ACA CTC ATG GTT TCC TC					
3-86 F	ATG GAG AAG CTT ACA CTG ATC	T17J13	55.5	2.5	143	128
3-86 R	TGG ATT TCT TCC TCT CTT CAC					
3-88 F	TGC CAG GGG AAT AGA TGA AA	T20O10	55.2	2.5	235	210
3-88 R	TCT TCC AAA CCA TGC AAT GA					
4-11 F	GAC AAT AAC CTT GCG TGG TAC A	T7B11	50.6	2.5	274	297
4-11 R	TGG TTA CTG TAT GCC AAA TGA A					
4-31 F	ACA TTT GGT GGG CGA GTA AC	F24G24	51.8	2.5	333	253
4-31 R	TAG AGG GAA CAA TCG GAT GC					
4-60 F	ACT TGC TTT CGC TTT GCA GT	F27B13	53.2	2.5	353	265
4-60 R	TGG CCT TTT GCT CTC TGT TT					
5-13 F	TTT CTC TGT TGG GGC AAA AC	MBK20	52.0	2.5	384	322
5-13 R	AGG CAG CGG CAT CTT TAA TA					
5-51 F	TGG ATC TCG GCT ATT GAT TG	F2P16	53.0	2.5	345	269
5-51 R	TCC CAA ATC AAT TCA AGG AAA					
5-85 F	GAT TTC GCT CTC TGC CAA AA	K23L20	55.4	2.5	345	278
5-85 R	ACA AAA TAC AAG CCC AAC AAG T					
5-115 F	CCA CAT TTT CCT TCT TTC ATA	MSL3	51.0	2.0	137	133
5-115 R	CAA CAT TTA GCA AAT CAA CTT					
5-116 F	CTT GGA TCT CTG AGT AGC GTT G	MAC9	48.3	2.5	300	255
5-116 R	GAA CAC ATG GAA TCT TCA CAT CA					

The AGI BAC indicates the bacterial artificial chromosome where the primer pair is located on the *Arabidopsis* Genome Initiative map.

The annealing temperature and Mg^{2+} concentration indicate the variations from the standard PCR reaction.

[*] Indicates the length of final DNA fragment of each ecotype.

[a] New markers designed for this study.

Table 2. Segregation analysis of various mutant families

F2 Population	WT Count	Mutant Count	Approximate Ratio (WT: Mutant)	Pearson's Chi-squared Statistics
31-83-4-3 bc3F2	178	20	8.90 : 1	23.44, $p < 0.001^*$
32-2-4 bc4F2	153	54	2.83 : 1	0.13, $p = 0.718$
33-7-5(4) bc4F2	149	21	7.10 : 1	14.50, $p < 0.001^*$
115-11-21-4 bc2F2	183	65	2.82 : 1	0.19, $p = 0.660$

All the plants were scored at 21 DAG, except 33-7-5(4), which were 28 DAG due to the slower growth.
Pearson's chi-squared test (one-tailed) was applied based on the expected 3: 1 ratio.
* Indicates this value is significantly different from the expectation ($p < 0.05$).

Table 3. Complementation data of *115-11-21-4*

Cross (Female × Male)	F1 Progeny Phenotype
115-11-21-4×*fkd1*	Mutant (62)
115-11-21-4×*sfc*	Wild Type (51)

Number in bracket represents number of plants scored.

Table 4. First leaf shape characters of various genotypes at 21 DAG[a]

Genotype	Wild Type (30)	*31-83-4-3* (30)	*32-2-4* (30)	*33-7-5(4)* (30)
Serrations	0	1.60±0.16 $p < 0.001^{\$}$	1.70±0.15 $p < 0.001^{\$}$	1.87±0.16 $p < 0.001^{\$}$
Hydathodes	3.00±0.07	1.63±0.14 $p < 0.001^{*}$	3.27±0.08 $p = 0.015^{*}$	2.60±0.14 $p = 0.014^{*}$
Width (W, mm)	6.55±0.07	4.19±0.16 $p < 0.001^{*}$	4.94±0.06 $p < 0.001^{*}$	3.06±0.12 $p < 0.001^{*}$
Length (L, mm)	7.21±0.10	6.24±0.18 $p < 0.001^{*}$	5.94±0.07 $p < 0.001^{*}$	3.93±0.12 $p < 0.001^{*}$
L/W	1.10±0.01	1.52±0.04 $p < 0.001^{*}$	1.20±0.01 $p < 0.001^{*}$	1.31±0.04 $p < 0.001^{*}$

Values present means ± s.e.m.
Number in bracket represents number of plants scored.
[a] 33-7-5(4) were scored at 28 DAG due to the slower growth.
[$] Indicates significant difference from wild type based on two-tailed Fisher's exact test.
[*] Indicates significant difference from wild type based on two-tailed Student's *t*-test.
Significance level: $p < 0.05$.

Table 5. First leaf vascular pattern characters of various genotypes at 21 DAG[a]

Genotype		Wild Type (30)	*31-83-4-3* (30)	*32-2-4* (30)	*33-7-5(4)* (30)
Areoles		21.03±0.72	9.27±0.76, p < 0.001[*]	14.47±0.51, p < 0.001[*]	4.63±0.48, p < 0.001[*]
Secondary Veins		9.03±0.27	6.93±0.26, p < 0.001[*]	8.17±0.18, p = 0.009[*]	3.93±0.16, p < 0.001[*]
Tertiary Veins		25.20±0.87	10.10±0.86, p < 0.001[*]	17.53±0.46, p < 0.001[*]	6.03±0.56, p < 0.001[*]
Quaternary Veins		7.00±0.50	1.73±0.40, p < 0.001[*]	4.57±0.35, p < 0.001[*]	1.07±0.24, p < 0.001[*]
Free Ends	Secondaries (%)	1.10±0.19 (12.18%)	1.93±0.24 (27.88%) p = 0.008[*]	1.13±0.15 (13.50%) p = 0.892	0.77±0.14 (19.49%) p = 0.170
	Tertiaries (%)	12.73±0.60 (50.53%)	5.87±0.53 (58.09%) p < 0.001[*]	10.40±0.48 (59.17%) p = 0.004[*]	4.70±0.35 (77.90%) p < 0.001[*]
	Quaternaries (%)	6.43±0.43 (91.90%)	1.57±0.35 (90.38%) p < 0.001[*]	4.23±0.30 (92.48%) p < 0.001[*]	0.93±0.19 (87.50%) p < 0.001[*]
Midvein Extension		0	0.40±0.09, p < 0.001[$]	0	0.40±0.09, p < 0.001[$]
Branch Points		62.33±1.83	28.30±1.93, p < 0.001[*]	44.70±0.88, p < 0.001[*]	16.07±1.24, p < 0.001[*]
Vascular Islands		0	0.37±0.12, p = 0.005 [$]	0.17±0.07, p = 0.052	0
Marginal Venation Gaps		0	0.97±0.24, p < 0.001[$]	0	1.17±0.18, p < 0.001[$]

Values present means ± s.e.m.
The number in brackets beside the genotype represents the number of plants scored.
The number in brackets beside the free ends represents the percentage of free-ending veins.
[a] 33-7-5(4) were scored at 28 DAG due to the slower growth.
[*] Indicates significant difference from wild type based on two-tailed Student's t-test.
[$] Indicates significant difference from wild type based on two-tailed Fisher's exact test.
Significance level: p < 0.05.

Table 6. Cotyledon vascular pattern characters of various genotypes at 14 DAG[a]

Genotype		Wild Type (30)	*31-83-4-3* (30)	*32-2-4* (30)	*33-7-5(4)* (30)
Areoles		3.07±0.12	2.10±0.13, $p < 0.001$[*]	3.00±0.13, $p = 0.700$	0.53±0.16, $p < 0.001$[*]
Secondary Veins		3.13±0.11	3.27±0.16, $p = 0.499$	2.97±0.14, $p = 0.360$	2.13±0.18, $p < 0.001$[*]
Tertiary Veins		1.13±0.16	0.87±0.15, $p = 0.235$	1.3±0.17, $p = 0.489$	0.03±0.03, $p < 0.001$[*]
Quaternary Veins		0.10±0.06	0, $p = 0.237$	0, $p = 0.237$	0, $p= 0.237$
Free Ends	Distal Secondaries (%)	0.07±0.07 (2.13%)	0.97±0.16 (29.59%) $p < 0.001$[*]	0.10±0.06 (4.44%) $p = 0.703$	1.37±0.16 (64.06%) $p < 0.001$[*]
	Proximal Secondaries (%)	0	0.23±0.10 (7.14%) $p = 0.052$	0 $p = 1$	0.23±0.09 (10.94%) $p = 0.024$[$]
	Tertiaries (%)	1.10±0.17 (97.06%)	0.83±0.14 (96.15%) $p = 0.234$	1.13±0.17 (85.71%) $p = 0.890$	0.03±0.03 (100%) $p < 0.001$[*]
	Quaternaries (%)	0.10±0.06 (100%)	0, $p = 0.237$	0, $p= 0.237$	0, $p= 0.237$
Midvein Extension		0.07±0.05	0.80±0.07, $p < 0.001$[*]	0.07±0.05, $p = 1$	0.20±0.07, $p = 0.134$
Branch Points		7.47±0.17	7.03±0.29, $p = 0.204$	7.30±0.18, $p = 0.505$	2.90±0.32, $p < 0.001$[*]

Values present means ± s.e.m. For the row of Genotype, number in bracket represents the number of plants scored.
For the rows of Free Ends, the number in bracket represents the percentage of free-ending veins.

[a] 33-7-5(4) were scored at 21 DAG due to the slower growth.

[*] Indicates significant difference from wild type based on two-tailed Student's *t*-test.

[$] Indicates significant difference from wild type based on two-tailed Fisher's exact test.

Significance level: $p < 0.05$.

Table 7. Adult plant shoot characters of various genotypes at 35 DAG [a]

Genotype	Wild Type (36)	*31-83-4-3* (37)	*32-2-4* (39)	*33-7-5(4)* (37)
Flowering Time (DAG)	20.94±0.31	28.68±0.31 $p < 0.001^*$	22.56±0.42 $p = 0.003^*$	30.30±0.41 $p < 0.001^*$
Stem Branches	3.44±0.09	7.27±0.35 $p < 0.001^*$	3.15±0.11 $p = 0.053$	4.27±0.17 $p < 0.001^*$
Rosette Leaves	6.56±0.18	8.97±0.24 $p < 0.001^*$	7.79±0.27 $p < 0.001^*$	8.08±0.24 $p < 0.001^*$
Fused Leaf and Stem	0	0.54±0.10 (24) $p < 0.001^\$$	0 $p = 1$	0 $p = 1$

Values present means ± s.e.m.
Number in bracket represents number of plants scored.
[a] Excludes flowering time.
[*] Indicates significant difference from wild type based on two-tailed Student’s *t*-test.
[$] Indicates significant difference from wild type based on two-tailed Fisher’s exact test.
Significance level: $p < 0.05$.

Table 8. Root characters of various genotypes from 4 DAG to 5 DAG [a]

Genotype			Wild Type (30)	31-83-4-3 (25)	32-2-4 (30)	33-7-5(4) (30)
Root Elongation within 24 hours (from 4 DAG to 5 DAG, mm)			8.86±0.29	5.74±0.37 $p < 0.001^{*}$	8.14±0.21 $p = 0.050^{*}$	1.50±0.16 $p < 0.001^{*}$
Gravitropism	Horizontal Angle (°) of Root Tip after Rotated 90°	2hr	46.26±1.85	16.91±3.87 $p < 0.001^{*}$	44.78±2.72 $p = 0.655$	5.08±2.12 $p < 0.001^{*}$
		4hr	74.40±1.58	43.50±4.36 $p < 0.001^{*}$	75.49±2.62 $p = 0.725$	15.67±2.67 $p < 0.001^{*}$
		6hr	86.00±1.42	60.31±4.05 $p < 0.001^{*}$	86.92±2.18 $p = 0.726$	25.43±3.52 $p < 0.001^{*}$

Values present means ± s.e.m, number in bracket represents number of plants scored.

[a] Roots were rotated 90° at 5 DAG and captured for gravitropism measurements after 2, 4, 6 hours.

[*] Indicates significant difference from wild type based on two-tailed Student's *t*-test ($p < 0.05$).

Table 9. Fifth leaf serrations numbers of various genotypes at 35 DAG

Genotype	Wild Type (17)	*cuc2-3* (15)	*32-2-4* (15)	*32-2-4×cuc2-3* (15)
Serrations	4.76±0.34	0 $p < 0.001^{\$}$	8.80±0.30 $p < 0.001^{*}$	0 $p < 0.001^{\$}$

Values present means ± s.e.m.
Number in bracket represents number of plants scored.
$^{\$}$ Indicates significant difference from wild type based on two-tailed Fisher’s exact test.
* Indicates significant difference from wild type based on two-tailed Student’s t-test.
Significance level: $p < 0.05$.

Table 10. Mapping distances between the mutations and the primer pairs

Genotype	*32-2-4*		*33-7-5(4)*		
Primer Pair	2-61 F, 2-61 R	2-82 F, 2-82 R	5-85 F, 5-85 R	5-115 F, 5-115 R	5-116 F, 5-116 R
Number of Plants Scored	108	106	135	76	135
Col : Ler Ratio	195:21	201:11	226:44	141:11	244:26
Pearson's chi-squared Test	140.17 $p < 0.001^*$	170.28 $p < 0.001^*$	122.68 $p < 0.001^*$	111.18 $p < 0.001^*$	176.01 $p < 0.001^*$
Map Distance (cM)	9.72	5.19	16.30	7.24	9.63

Pearson's chi-squared test (one-tailed) was applied based on the expected 1: 1 ratio.
[*] Indicates this value is significantly different from the expectation ($p < 0.05$).

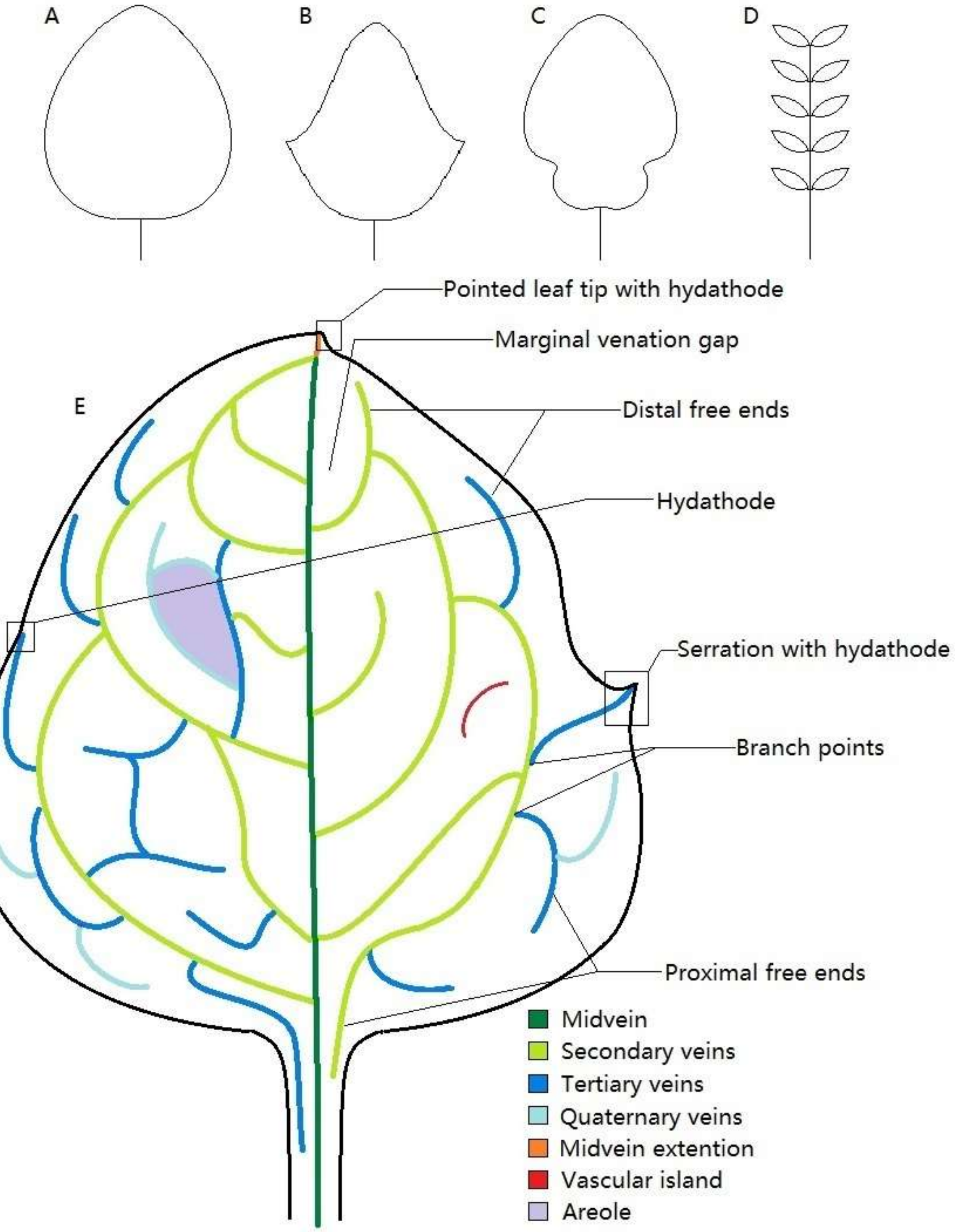

Figure 1. Diagrams of different leaf shape and leaf characters.
(A) Simple leaf with smooth margin, (B) Simple leaf with serrations, (C) Simple leaf with lobes, (D) Compound leaf, (E) *Arabidopsis thaliana* first leaf that shows the various vein pattern and leaf shape characters: the left half is presenting the wild type; the right half is presenting the potential mutant.

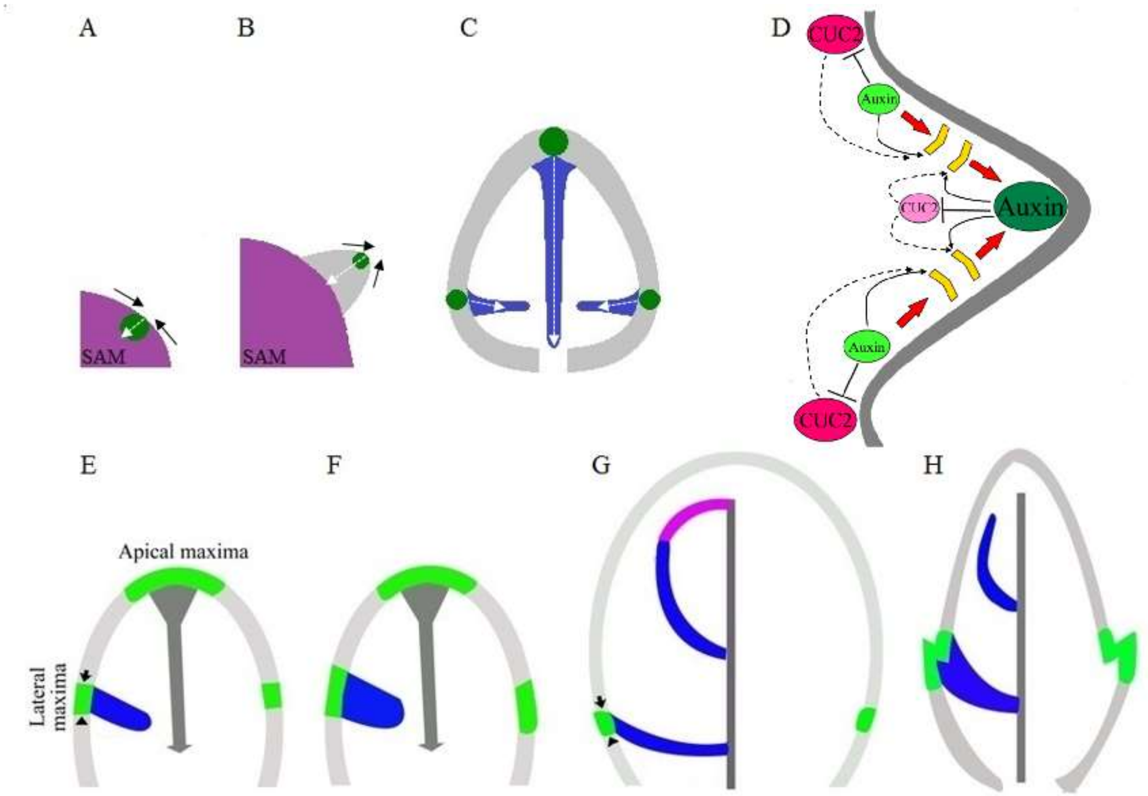

Figure 2. Models of leaf shape and vein pattern formation.
(A) Leaf initials at the flanks of SAM (purple) correspond to the site of elevated auxin activity (green), the white arrow indicates the auxin transport direction resulting from PIN1 polarity (black arrows). (B) Early leaf primordium (grey): a maximum of auxin activity forms at the tip of the primordium; and auxin is drained through the centre of the primordium, marking the position of midvein (white arrows). (C) Primary morphogenesis: lateral vein (blue) formation and positions of serration development are correlated with auxin activity maxima at the margin. (D) The feedback loop among PIN1, auxin and CUC2: the polar (apical or basal) localization of PIN1 (yellow) determines auxin transport (red arrows), auxin flow enhances PIN1 localization with the same polarity (black arrows); *CUC2* enables reorientation of PIN1 (dashed black arrows) and, in turn, auxin inhibits CUC2, thus stabilizing the position of auxin maxima (dark green)/CUC2 minima (pink) (predicting serrations) and auxin minima (bright green)/CUC2 maxima (magenta) (predicting sinuses). (E) In wild type first leaf, the apical maximum and lateral maxima (green) initiate the formation of midvein (dark grey) and first pair of secondary veins (blue). (F) In *unh* first leaf, the lateral maxima (green) is stronger than wild type. (G) At a later stage of wild type first leaf, the switch of PIN1 polarity leads the auxin transport direction toward to the distal part of midvein and gives rise to closed vein loop (purple). (H) At a later stage of *unh* first leaf, the expanded PIN1 expression (green) in the margin results stronger downward auxin flux and prevents the upward auxin flux, results the serration formation and free-ending secondary veins (Scarpella *et al.*, 2010, Bilsborough *et al.*, 2011, Pahari *et al.*, 2014).

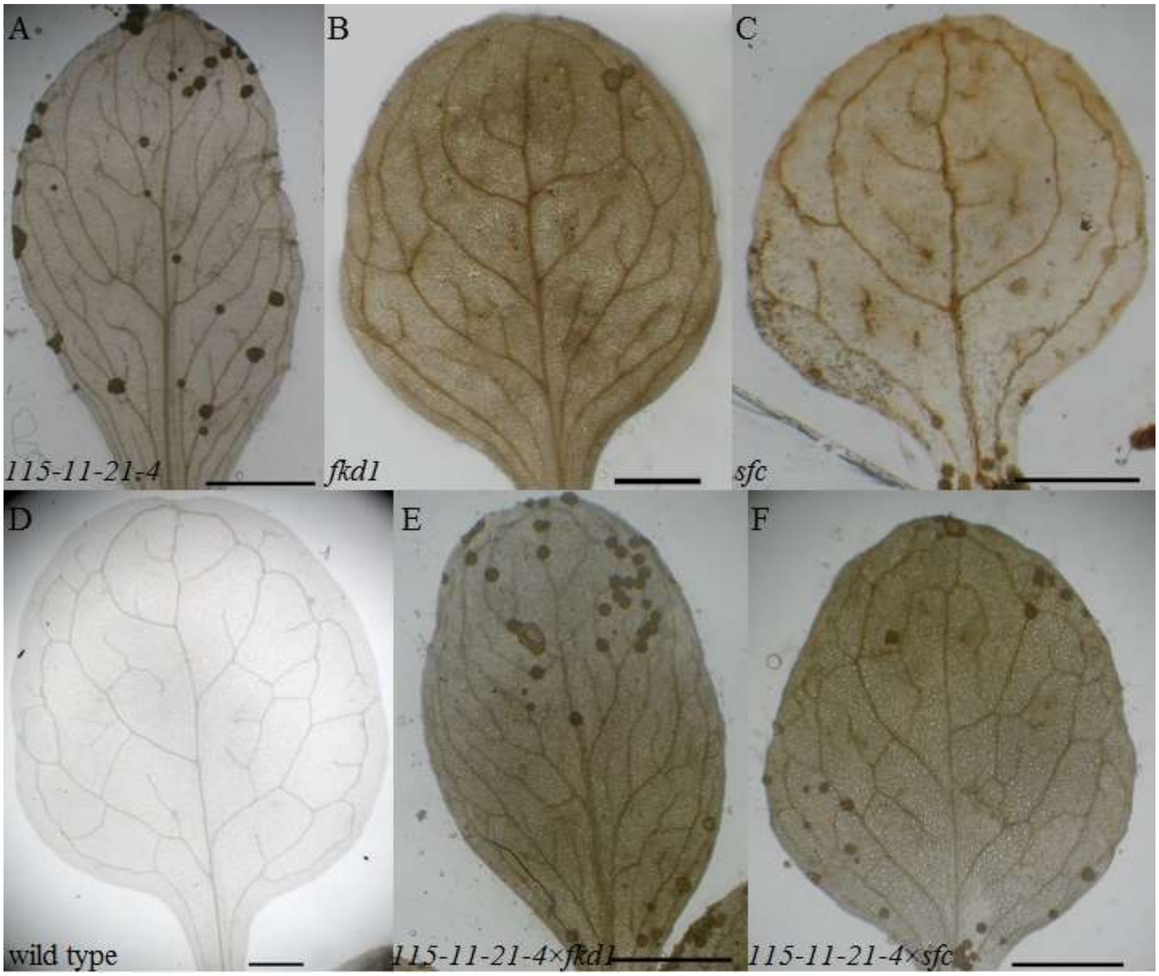

Figure 3. Complementation test of mutant *115-11-21-4* to *fkd1*and *sfc*.
(A) *115-11-21-4* has elongated first leaf shape and frequently exhibits distal free ends of secondary and tertiary veins, (B) *fkd1* first leaf, (C) *sfc* first leaf, (D) wild type first leaf, (E) F1 generation from crossing of *115-11-21-4* and *fkd1* was mutant phenotype with frequent free ending veins in first leaf, thus *115-11-21-4* was an allele of *fkd1*, (F) F1 generation from crossing of *115-11-21-4* and *sfc* was wild type phenotype, thus *115-11-21-4* was not an allele of *sfc*. Scale bars = 1 mm.

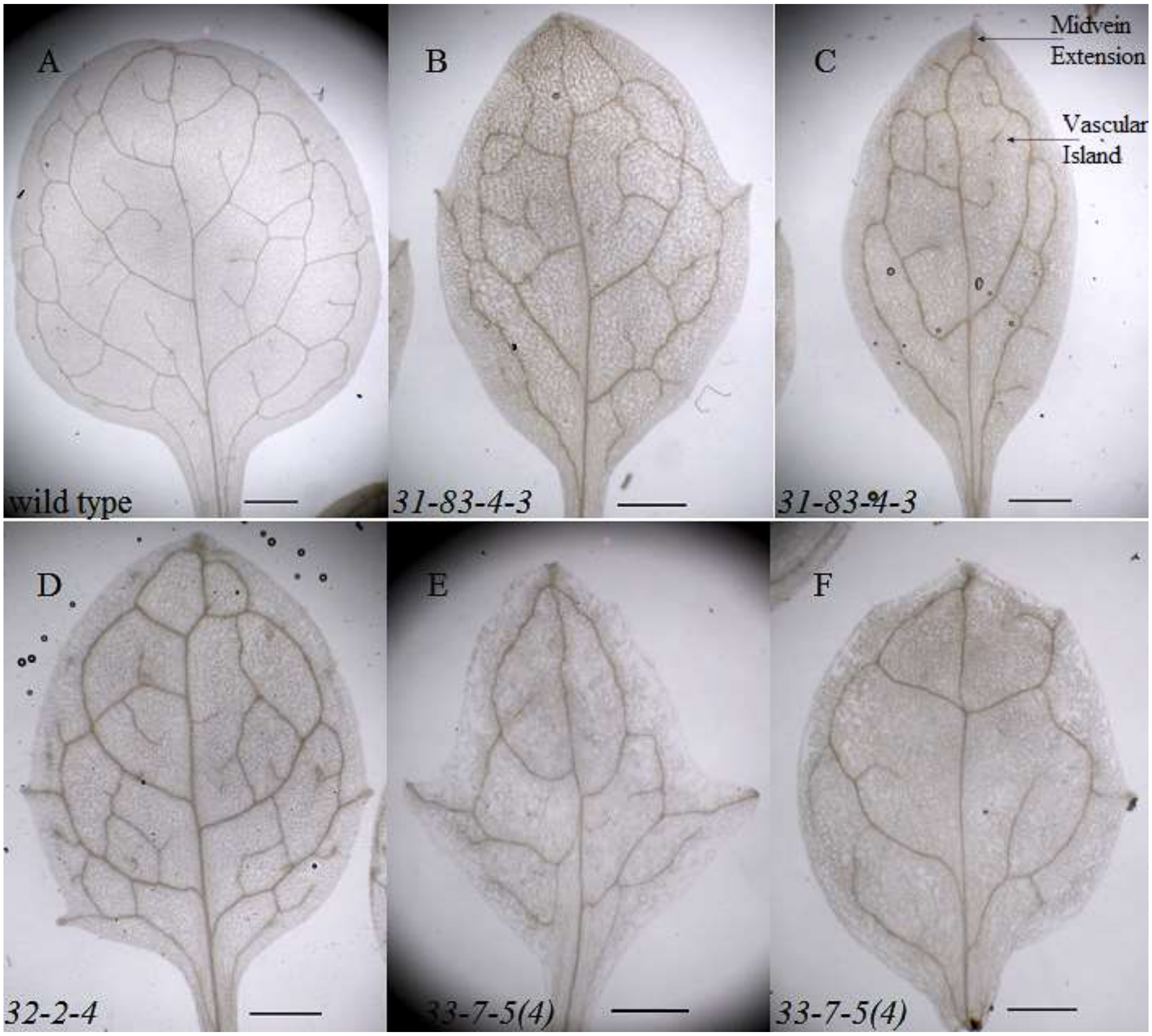

Figure 4. Cleared first leaves of various genotypes viewed under dissecting light microscope.
(A) wild type, (B,C) *31-83-4-3*, (D) *32-2-4*, (E,F) *33-7-5(4)*. All first leaves were removed at 21 DAG, except *33-7-5(4)*, which were removed at 28 DAG. Black arrows indicate the midvein extension and vascular island. Scale bars = 1 mm.

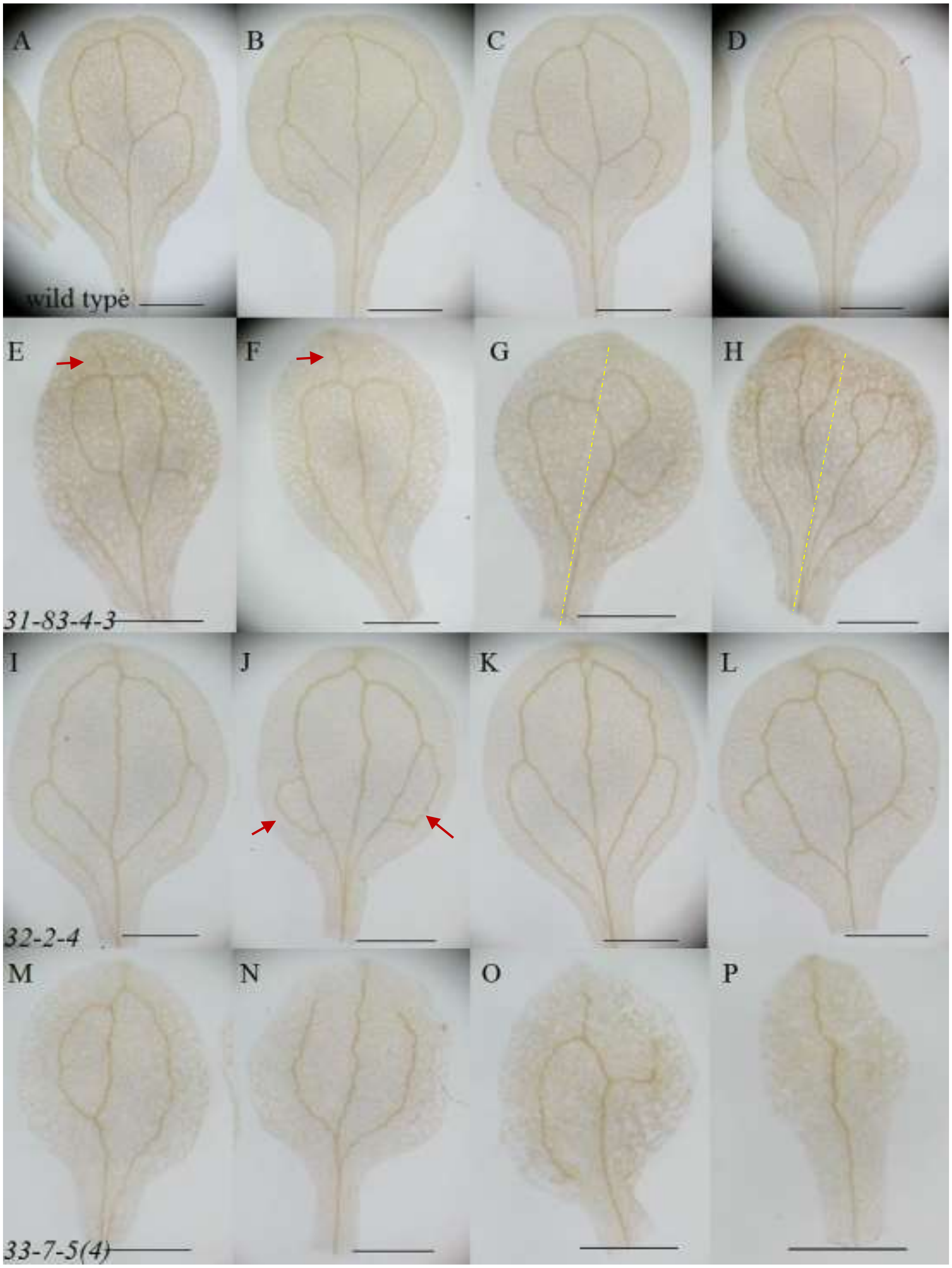

Figure 5. Cleared cotyledons of various genotypes viewed under dissecting light microscope.

(A-D) wild type, (E-H) *31-83-4-3*, red arrows indicate the midvein extension, dashed yellow lines marked the normal midvein location, (I-L) *32-2-4*, red arrows indicate the areoles formed by tertiary veins, (M-P) *33-7-5(4)*. All cotyledons were removed at 14 DAG, except *33-7-5(4)*, which were removed at 21 DAG. Scale bars = 1 mm.

Figure 6. Shoot phenotype of various genotypes at 35 DAG.
(A) wild type, (B) *31-83-4-3*, white square magnified the fused leaf and stem, (C) *32-2-4*, (D) *33-7-5(4).* Plants were grown in soil. Scale bars = 1 cm.

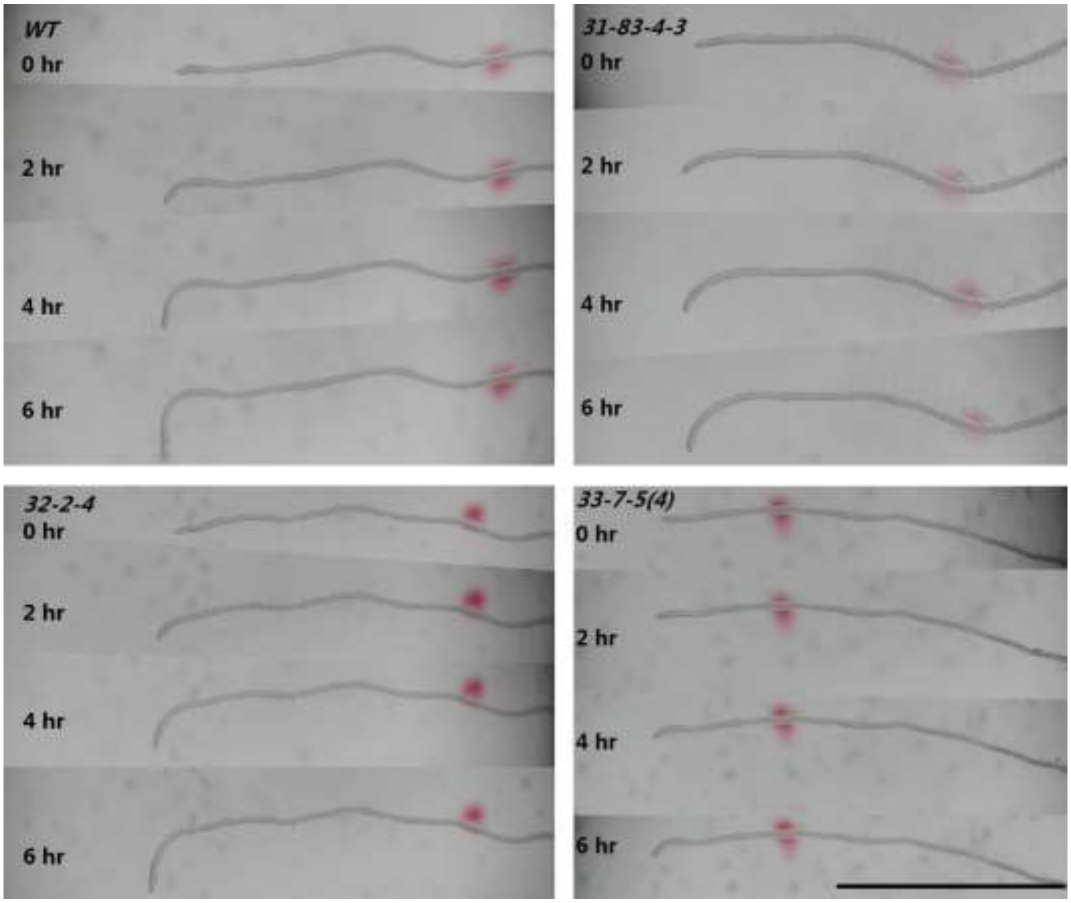

Figure 7. Root tip bend after being rotated 90° for 2, 4, 6 hours of various genotypes: wild type, *31-83-4-3*, *32-2-4* and *33-7-5(4)* as viewed under dissecting light microscope. The red dots in each picture indicated the root tip position at 4 DAG. All the roots were rotated at 5 DAG. Scale bar = 5 mm.

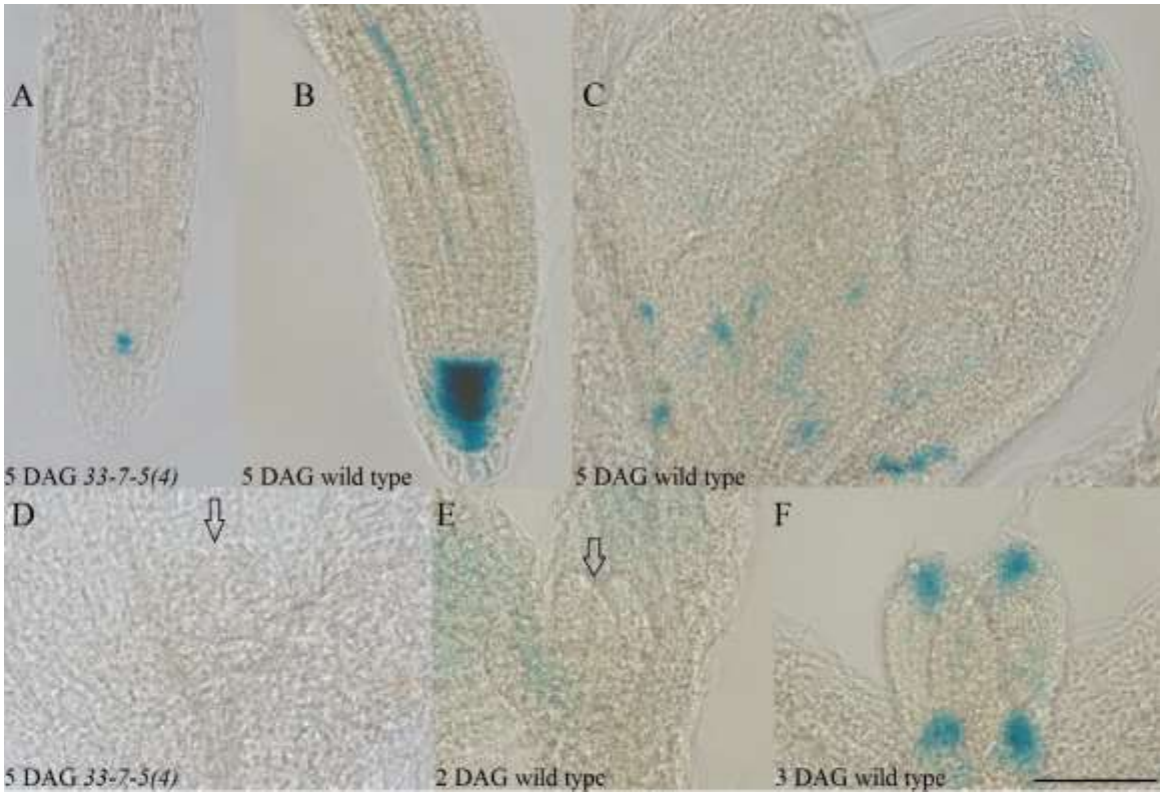

Figure 8. *DR5::GUS* expression in *33-7-5(4)* and wild type root tips and first leaves. At 5 DAG, *DR5::GUS* expression in (A) *33-7-5(4)* root tip is reduced compared to (B) wild type. (C) *DR5::GUS* expression in the 5 DAG first leaves of wild type is presented and predicts the position of leaf vein formation. (D) No *DR5::GUS* expression is seen in *33-7-5-(4)* 5 DAG first leaves, and the first leaf size is about the same size as 2 DAG (E) to 3 DAG (F) wild type first leaves. Scale bar = 100 µm.

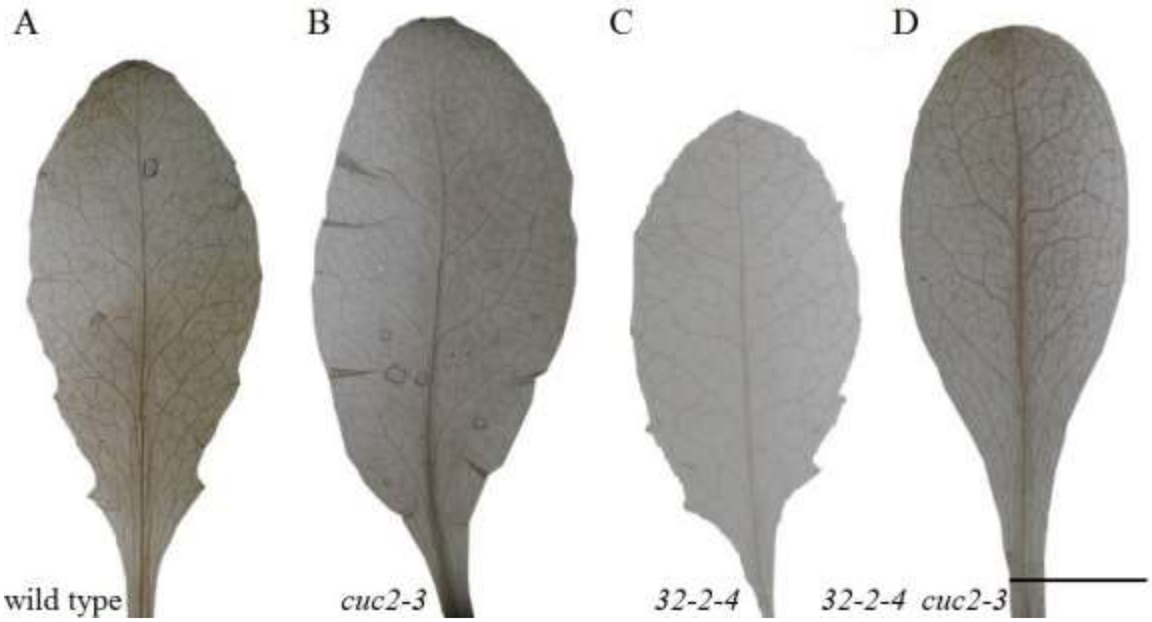

Figure 9. Fifth leaf phenotypes of various genotypes at 35 DAG.
(A) wild type, (B) *cuc2-3*, (C) *32-2-4*, more serration on the distal part of the leaf blade as compared to wild type (D) double mutant *cuc2-3 32-2-4*. The serrations of *32-2-4* are supressed by *cuc2-3*. Scale bar = 5 mm.

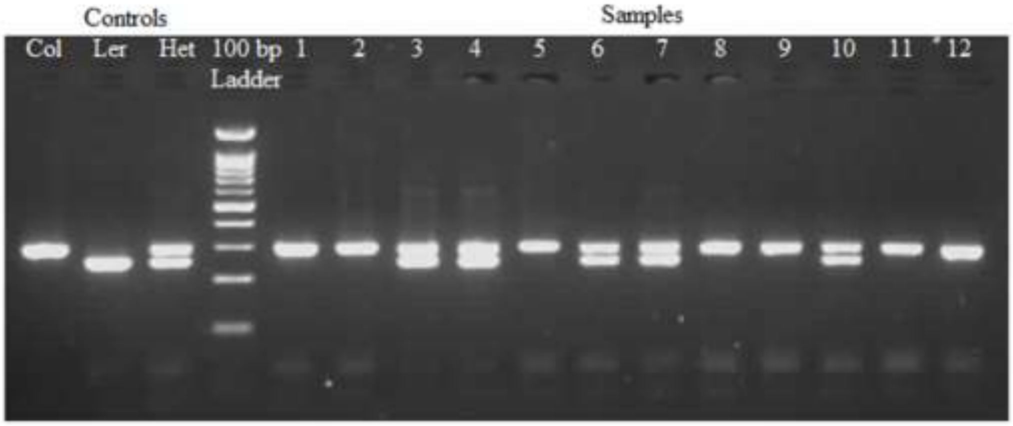

Figure 10. PCR products defining molecular markers used in mapping.
SSLP marker linked to tested mutant showing the banding pattern of controls (Col, Het (Col×Ler F1), and Ler) and mutant×Ler F2 mapping samples.

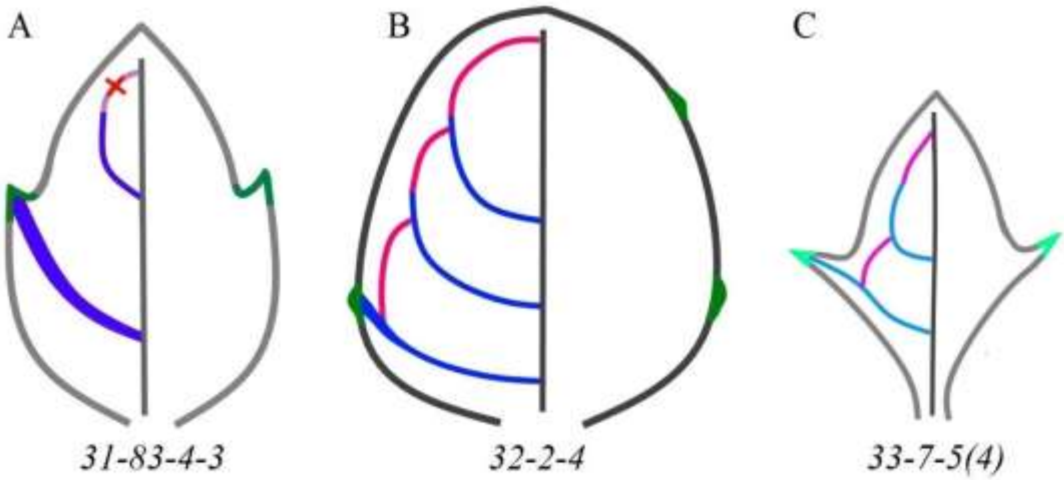

Figure 11. Models for first leaf shape and vein pattern formation associated with defective auxin regulation in three mutant lines.
(A) The serrations of *31-83-4-3* first leaf may result from higher PIN1 expression in the leaf margin, thus the increased auxin maxima on the leaf margin (green) induces the serration outgrowth; and the increased the auxin flux through secondary veins prevents the upward auxin transport (pink with a red cross mark), result the frequent distal free-ending veins. (B) No defects were determined in overall auxin level of *32-2-4*, the serrated leaf shape results from increased auxin activity at the auxin convergence points in the leaf margin. (C) Extremely reduced auxin level in *33-7-5(4)* limits the growth of leaf blade, and the auxin maxima on the leaf margin (light green) is reduced relative to wild type and other two mutants (*31-83-4-3* and *32-2-4*). Thus the serration may not result from the outgrowth of the margin, instead it may result from slow cell division and expansion in the central lamina.

APPENDIX 1. Selected mutant lines with leaf shape and/or vein pattern defects

Mutant Lines	Previous Generation	Current Generation	Complementation Test	Notes
31-83-4-3	**bc1F3**	**bc4F2**	**Not an allele of *fkd1*, *unh*, *gsy***	**Serrated first leaf with non-meeting secondary veins**
32-1-4(1)	bc2F2	bc2F3	Allele of *gsy*	Seeds from mutants were harvested and saved as bc2F3
32-2-4	**bc3F4**	**bc5F2**	**Not completed**	**Serrated first leaf, simplified vain pattern**
32-26-1(3)	bc1F2	bc2F3	Not an allele of *fkd1*, *unh*, *gsy*	Small round thick purple leaf; nearly only one stem; short silique
32-28	bc2F2	bc2F3	**Not completed**	No phenotype in bc2F2, M4 seeds did not germinate
32-28-1	bc1F2	bc2F3	Not an allele of *fkd1*, *unh*, *gsy*	No abnormal phenotype in bc2F2
33-7-5(4)	**bc3F2**	**bc5F2**	**Not completed**	**Serrated first leaf with non-meeting secondary veins**
33-17-9(2)	bc1F2	bc2F3	**Not completed**	No abnormal phenotype in bc2F2
115-11-21-4	**M4**	**bc2F3**	**Allele of *fkd1***	**Frequent distal free-ending veins**
226(7)	M5	bc1F2	**Not completed**	Small rosette leaves, floristic defects
es30	bc2F1	bc4F3	Not an allele of *fkd1*, *unh*, *gsy*	Simple vein pattern, curved later rosette leaves, bushy
es31	M3	bc2F3	Not an allele of *fkd1*, *unh*, *gsy*	No abnormal phenotype in bc2F2
es32	M4	bc1F3	Not an allele of *fkd1*, *unh*, *gsy*	No abnormal phenotype in bc2F3
es33	bc1F2	bc2F3	Not an allele of *fkd1*, *unh*, *gsy*	(Looks like bc1F3)gl1:12,14, No.1 crossed to everything
es33-1	bc1F2	bc2F3	Not an allele of *fkd1*, *unh*, *gsy*	No phenotype in bc2F2
es39-4	M3	bc2F3	Not an allele of *fkd1*, *unh*, *gsy*	Parallel simple veins, late flowering
es39-4(9)	bc1F1	bc2F3	Not an allele of *unh*	Serrated leaf, simple vein, no abnormal phenotype in bc2F2
es39-6	M3	bc2F3	Not an allele of *fkd1*, *unh*, *gsy*	Needle shape first leaf/no first leaf, serrated later leaves, weak
es39-6(8)	bc1F1	bc3F2	Not an allele of *fkd1*, *unh*, *gsy*	F2 seeds collected, need to select phenotype and cross
es47-9	M4	bc1F3	**Not completed**	No abnormal phenotype in bc1F2
es48-15	M4	bc2F3	Not an allele of *fkd1*, *unh*, *gsy*	No.8 hydathodes
es51-20	bc1F2	bc2F3	Not an allele of *fkd1*	Simple vein pattern, round leaf shape, no-meeting distal vein
es74	M3	bc1F3	**Not completed**	No abnormal phenotype in bc1F2
es74-16	bc1F2	bc1F4	**Not completed**	No abnormal phenotype in leaves, but something in stem