THE *UNHINGED* GENE IS ESSENTIAL FOR VASCULAR COMPLEXITY IN THE LEAVES OF *ARABIDOPSIS*

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To Meok and Thomas with love.

Abstract

The complex vasculature seen in the vascular plants provides a scaffolding of structural support and is responsible for the movement of water, minerals, photosynthate and some hormones. The auxin canalization model proposes that a positive feedback mechanism causes auxin-transporting cells to become vascular cells. We have isolated a leaf-patterning mutant, *unhinged* (*unh*), which shows a simplified leaf vascular pattern with more freely ending veins and fewer secondary veins. Expression of the *ATHB8::GUS* reporter construct indicates that the *UNH* gene acts prior to procambial patterning of the first presumptive secondary veins. Expression of the auxin responsive reporter gene *DR5::GUS* is reduced in *unh* leaves and roots, indicating that *UNH* may be involved in auxin signaling. Increasing the level of auxin in *unh* leaves through the addition of auxin transport inhibitors, low concentrations of 2,4-dichlorophenoxyacetic acid, and through introducing *unh* into mutants in which auxin transport is defective partially rescues the *unh* phenotype, supporting this hypothesis. The *unh* mutation maps to a 60kb region near the top of chromosome IV. No other leaf vascular mutant or auxin-associated mutant have been reported in this area, thus *UNH* represents a novel component of leaf vascularization and auxin signaling.

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Gene and Protein Nomenclature

UNH	*UNHINGED* wildtype gene
unh	*UNHINGED* mutant allele
UNH	*UNHINGED* protein product

Genes

AAP	*AMINO ACID PERMEASE*
ARE	*AUXINE-RESPONSE ELEMENT*
ARF	*AUXIN-RESPONSE FACTOR*
AS	*ASYMMETRIC LEAF*
ATHB8	*ARABIDOPSIS THALIANA HOMEOBOX8*
AUX1	*AUXIN INSENSITIVE1*

AXR1	*AUXIN RESISTANT1*
AXR2	*AUXIN RESISTANT2*
CUL1	*CULLIN1*
CVP2	*COTYLEDON VASCULR PATTERN2*
EIR1	*ETHYLENE INSENSITIVE ROOT 1*
FKD1	*FORKED1*
GN	*GNOM*
GUS	*β-GLUCURONIDASE*
GL1-1	*GLABROUS1-1*
ILL	IAA-AMINO ACID HYDROLASE
IAR3	*IAA-ALANINE RESISTANT*
LAX	*LIKE AUX1*
MP	*MONOPTEROS*
PID	*PINOID*
PIN1	*PIN FORMED1*
PLT	*PLETHORA*
RTY1	*ROOTY1*
SAUR	*SMALL-AUXIN-UP-RNA*
SCF	*SKP1/CULLIN/F-BOX*
SFC	*SCARFACE*
SUR	*SUPERROOT*
TIR1	*TRANSPORT INHIBITOR RESPONSE 1*
TT4	*TRANSPARENT TESTA 4*

UNH	*UNHINGED*
VAN3	*VASCULAR NETWORK 3*

Chemicals

2,4-D	2,4-dichlorophenoxyacetic acid
EMS	ethyl methane sulfonate
DMSO	dimethyl sulfoxide
HFCA	2-chloro-9-hydroxyfluorene-9-carboxylic acid
IAA	indole-3-acetic acid
NAA	1-naphthaleneacetic acid
NOA	1-napthoxyacetic acid
NPA	naphthylphthalamic acid
TIBA	2,3,5-triiodobenzoic acid
X-gluc	5-bromo-4-chloro-3-indolyl glucuronide

Terms

AGI	*Arabidopsis* Genome Initiative
AT	*Arabidopsis thaliana*
ATI	auxin transport inhibitor
BAC	bacterial artificial chromosome
CAPS	cleaved amplified polymorphic sequence
DDAM	distal *DR5* auxin maximum
dCAPS	derived cleaved amplified polymorphic sequence

Col	Columbia ecotype
DAG	days after germination
Ler	Landsberg *erecta* ecotype
PCR	polymerase chain reaction
SNP	single nucleotide polymorphism
SSLP	simple sequence length polymorphism
TAIR	The Arabidopsis Information Resource
TE	tracheary element

Introduction

Most higher plants owe their evolutionary fitness in part to their continuous network of interconnected vascular cells (Roth-Nebelsick, 2001). This network not only provides structural scaffolding for the plant organs, but also permits efficient transport of water, minerals, hormones and photosynthate. The highly predictable and characteristic vascular patterns are in many cases species and tissue specific, suggesting a genetically based developmental process. The precise mechanism behind vein pattern formation in leaves, however is not well understood.

Vascular Development

In leaves, vascular tissue is derived from elongated precursor cells called procambium, which form from the undifferentiated ground meristem (GM) cell population and later differentiate into vascular elements (xylem and phloem) (Scarpella et al., 2004). During this process, the leaf is expanding through cell divisions, which slow down and stop first in the distal and then in the proximal part of the leaf (Donnelly et al., 1999; Steynen & Schultz, 2003), resulting in larger vascular bundles in the proximal regions of the leaf, even along the same strand (Kang & Dengler, 2004). Strands are often categorized into vein orders: the midvein (primary vein) is considered as the linear procambium or vascular strand approximately along the midline, secondaries are those vascular strands (or procambium) connected to the midvein, tertiaries are connected to secondaries (but not the midvein) and quaternaries are connected to tertiaries (but not the midvein nor secondaries).

In order to better understand the development of procambium and to more easily view procambium, several vascular specific reporter genes have been examined in developmental series. The *Et1335::GUS* and *AtHB-8::GUS* expression lines have been shown to be reliable markers of procambium and both preprocambium and procambium, respectively and have revealed patterns in procambium development and differentiation in the *Arabidopsis* first leaf (Scarpella et al., 2004). Based on *AtHB-8* expression, the formation of pre-procambia secondaries develops acropetally, that is, from the midvein to the tip. Based on *Et1335::GUS* expression and procambium cell diagnostic traits (i.e. cell elongation), preprocambial secondaries differentiate into procambium either very quickly or simultaneously relative to all other cells of the same strand (Scarpella et al., 2004). The fact that strands of *AtHB-8* expression include either procambium or GM, but never both in the same strand supports this conclusion (Scarpella et al., 2004).

In the first leaf, the formation of the vein orders follows a relatively consistent pattern based on the use of the above-mentioned marker lines and the appearance of the elongated procambium (Scarpella et al., 2004). Between day 2 and day 2.5 (from germination) the leaf primordium lacks any sign of procambial cells. At day 3, procambium appears along the midline, forming the presumptive mid-vein. As the leaf continues to elongate, these procambial cells continue to divide (Donnelly et al., 1999), thus ensuring vascular continuity in the growing organ. The first two secondary veins appear at day 5 as arches that join to the tip of the midline procambium and to a lower position of the midline procambium, forming presumptive areoles (intercostal areas). At day 5.5, two additional arches of secondary procambia appear forming presumptive areoles below the previous two. At day 7 the primordium is 0.7 mm long and secondary

procambia form within the first two areoles. At day 8 an additional pair of secondaries appear in the most proximal region. Tertiary procambium is first seen at day 7 within the central areoles and appears in the more newly formed proximal areoles at day 8. Similar observations have been made by Kang & Dengler (2004) and Baima et al. (2001).

In all vein orders, differentiation into xylem can be easily seen by lignification approximately two days after procambial strand formation (Scarpella et al., 2004) and generally occurs in the basipetal direction (Kang & Dengler, 2004). By day 21, all growth and vascular differentiation of the two first node leaves have been completed (Candela et al., 1999). Subsequent rosette leaves are larger and have an increasing amount of vasculature, however the venation density and the number of branch points per unit area remain relatively constant.

Auxin Response is Essential for Proper Vascular Development

For over 50 years it has been known that auxins, such as indole-3-acetic acid (IAA), can contribute to vascularization (Jacobs, 1952), yet the specific mechanism by which auxin forms complex and unique venation patterns in leaves has not been well understood. Several experiments involving *Arabidopsis* mutants defective in auxin synthesis, transport, or response, reporter genes linked to auxin inducible promoters, antibodies specific for auxin transport proteins and treatment with exogenous auxin and auxin transport inhibitors have provided a strong conceptual framework for the role of auxin in leaf vascular patterning. It becomes clear after careful examination of these experiments that vascular patterns are dependent on auxin response, auxin synthesis and

auxin transport systems and changes to any one of these processes can lead to changes in the final vein pattern observed.

In addition to being a consistent and predictable marker of procambium and pre-procambium, *AtHB-8::GUS* is auxin inducible (Baima et al., 1995) indicating an essential role of auxin in the vascular patterning process. Like many other homeobox genes, *AtHB-8* is believed to be important in patterning and developmental processes (Baima et al., 1995). Its expression profile, its induction by wounding of the stem and its presence in the root vascular bundles show that it is specific to vascularization throughout the plant (Baima et al., 2001). Furthermore, the precocious xylem differentiation seen in *35S::AtHB-8* transgenic plants indicates it likely has a role in xylem development.

MONOPTEROS (MP) is an auxin response factor (ARF), which functions to activate the expression of auxin inducible genes important in producing an auxin response (Hardtke & Berleth, 1998). Based on *AtHB-8::GUS* expression in *35S::MP* overexpression lines and Northern blot analysis in *35S::MP* and *mp* mutant plants, MP likely acts upstream of AtHB-8 (Mattson et al., 2003). Consistent with a role in vein formation, *MP* is expressed throughout young leaf primordia except in the midrib region and gradually becomes restricted to the procambium by day 7 (Hardtke & Berleth, 1998; Hardtke et al., 2004). Moreover, *mp* loss-of-function mutants show a reduction in vasculature in the cotyledons, leaves, as well as other organs if present (Przemeck et al., 1996).

The auxin response gene *AXR1* contributes to an auxin response by targeting AUX/IAA proteins for degradation through the ubiquitin pathway (del Pozo et al., 2002). In low auxin conditions, AUX/IAA proteins (such as BODENLOS and IAA7/AXR2)

inhibit the auxin response factors (ARFs, such as MP) preventing a transcription based auxin response. When AUX/IAA proteins are degraded by a process involving AXR1 in high auxin conditions, the ARFs are able to act to promote (and/or inhibit) transcription of the appropriate genes (del Pozo et al., 2002). Thus a loss-of-function *axr1-3* mutant produces a much lower auxin response. Like *MP* and *AtHB-8*, *AXR1* has been shown to be expressed in the vasculature of leaves and all other organs based on *in situ* hybridization, *AXR1::GUS* expression and immunolocalization (del Pozo et al., 2002). *axr1-3* has significantly fewer areoles and branch points compared with wild type (Steynen & Schultz, 2003). These results indicate a requirement for auxin response in vein formation.

Further spatial and temporal evidence that auxin response is essential in leaf vascular patterning has come from expression of the strong auxin response marker *DR5::GUS*. The *DR5::GUS* reporter line is a synthetic construct with 7 auxin responsive promoters in tandem coupled with a β-glucuronidase (*GUS*) reporter gene (Ulmasov et al., 1997). While not as specific as *AtHB-8*, the *DR5* expression line has been recognized as a pre-procambial and procambial cell marker (Mattson et al., 2003; Kang & Dengler, 2004) as well as an indicator of auxin concentrations in plants with intact auxin response (Avsian-Kretchmer et al., 2002). *DR5::GUS* lines exhibit very high *GUS* expression in young leaf primordia and the tips and edges of developing leaves and cotyledons (Avsian-Kretchmer et al., 2002). Close examination of leaf expression in developmental series of young first leaves shows dynamic *DR5::GUS* expression beginning with the appearance of an auxin maximum at the distal tip at 2 DAG (Mattson et al., 2003; Steynen & Schultz, 2003; Zgurski et al., 2005). At 4 DAG, this strong and thick *GUS*

expression expands in a basipetal direction from the distal tip along the margin and from the margin to the midvein, a pattern that predicts future secondary veins. Around 5 DAG, high expression is seen in the lamina. At 6 DAG expression is drastically reduced to only the distal tip, hydathodes and proximal lamina.

Auxin Production in the Leaf

In order to determine how the timing and pattern of auxin response seen in *AtHB-8*, *DR5*, *AXR1*, *FKD1* and *MP* expression lines corresponds temporally and spatially with auxin production, it is important to know where and when the hormone is produced in the leaf. Based on auxin transport inhibition experiments on leaves, it is believed that around day 4 and 5, the leaves begin producing their own auxin, thus switching from an auxin sink to an auxin source (Figure 1; Avsian-Kretchmer et al., 2002; Aloni et al., 2003). When developing seedlings containing the *DR5::GUS* construct were treated with low concentrations of the auxin efflux inhibitor, naphthylphthalamic acid (NPA), auxin response was eliminated in very young first leaves, but appeared at the tips of 5 to 6 DAG first leaves and gradually declined as the leaf matured (Avsian-Kretchmer et al., 2002). These results were confirmed with two other auxin transport inhibitors (2-chloro-9-hydroxyfluorene-9-carboxylic acid (HFCA) and 2,3,5-triiodobenzoic acid (TIBA), two other auxin reporter lines (*BA::GUS* and *SAUR::GUS*) and immunolocalization using anti-auxin antibodies. Under auxin efflux inhibition, procambial formation is delayed about one day (Sieberth, 1999). The delay in auxin response following NPA treatment has been interpreted to be the result of a lack of external auxin transport into the leaf; auxin response at 5-6 DAG in NPA treated plants may thus indicate the point at which

leaves respond to auxin produced within the leaf. These results would indicate that under normal conditions any procambial development prior to 4 DAG (ie. the midvein) is likely the result of external auxin, while procambial development after 5 DAG (ie. secondary, tertiary and quaternary procambia) is likely the result of auxin produced in the leaf itself. This interpretation is summarized in Figure 1.

Active auxin is produced in at least two ways in leaf primordia, *de novo* auxin synthesis and hydrolysis of auxin conjugates. The *NITRILASE* genes (*NIT1-3*), the products of which are essential in tryptophan-dependent auxin biosynthesis (Bartel & Fink, 1994) and the auxin conjugate hydrolase genes (*ILL2, ILL3* and *IAR3*) were both expressed equally in the leaf primordia margin and lamina based on RT-PCR analyses (Aloni et al., 2003). In *Arabidopsis*, *de novo* auxin synthesis was localized in transgenic seedlings with a *GUS*-based marker coupled with the promoter of each of the four *NITRILASE* genes. Collectively, expression occurred at the tips of the relatively small second node leaves and gradually increased to homogenous expression throughout the leaf (Bartel & Fink, 1994). Similar results were seen in tobacco; fully expanded leaves showed strong homogenous expression of *NIT::GUS*, while younger leaves showed expression primarily at the tips and leaf base (Hillebrand et al., 1998).

The expression of three of the auxin conjugate hydrolase genes *IAA-ALANINE RESISTANT 3* (*IAR3*) and *IAA-AMINO ACID HYDROLASE 2* and *3* (*ILL2*, *ILL3*) showed similar changes in expression patterns (Rampey et al., 2004). Expression of *ILL2*::*GUS*, *ILL3*::*GUS* and *IAR3*::*GUS* all appeared between 5 and 8 DAG, which is consistent with the source to sink transition mentioned earlier. Collectively, the expression pattern of auxin conjugate hydrolase genes corresponds with that of *NITRILASE* genes.

Auxin Transport in the Leaf is Essential for Proper Vascular Patterning

The localization profile of the *NIT1-3*, *ILL2-3* and *IAR3* marker lines corresponds with the auxin response profile (as seen by *DR5::GUS* expression) in the temporal sense, but is quite different in the spatial sense. While auxin response is focused at the distal tip and regions of presumptive venation and quickly disappears, auxin production in the young primordia generally starts at the tip and seems to expand or stay the same during development. A link between the spatially distinct auxin response and auxin production can be made through intercellular auxin transport. The importance of auxin transport in leaf vascularization can be appreciated by the leaf phenotype of plants grown on NPA. Unlike the ATI-free grown plants, first leaves of NPA treated plants exhibit increased vascularization at the margin near the distal tip and increased vasculature in the petiole and midvein region due to an increase in the number and size of vascular bundles (Mattson et al., 1999; Sieburth, 1999). Treated leaves also display an increase in the number of secondary and tertiary veins and veins are often more linear than those of the control. ATIs influence not only the final pattern of tracheary elements (TEs), but also the early pattern of procambium. When plants treated with 10 μM HFCA were examined in developmental series, the appearance of procambium was delayed about a day, while the differentiation to TEs is accelerated from 2 days to 1 (Sieberth, 1999). Consistent with the final TE pattern, there are morphological changes in the procambial pattern such as an increase in procambial cell files near the margin. The additional and often misshapen TEs at the distal margin appeared after either a very brief procambial stage or none at all. The defects become more severe as the concentration of NPA increased (Mattson et al., 1999). Examination of *DR5::GUS* transgenic plants germinated and grown 3 to 5 days on

various concentrations of NPA showed a shift in the expression profile, proving that auxin transport inhibitors alter the auxin response pattern of the leaf, which in turn alters the vascular pattern (Mattson et al., 2003). Exposing plants to NPA at later stages of development resulted in a decrease in marginal venation and an increase in higher order veins. These results show that auxin transport is important for all vein orders, normally limits vascular density, helps to define the regions of auxin response and is important relatively early in procambium development.

As we might expect, plant homozygous for the loss-of-function mutant of the auxin efflux carrier PIN1 has a leaf venation that resembles that of plants grown in low levels of ATIs or transferred to ATIs later in development (Mattson et al., 1999). The expression profile and subcellular localization of the PIN proteins has been examined in young leaf primordia through immunolocalization (Reinhardt et al., 2003). Expression of PIN1 is seen at the earliest stages of primordium development in the epidermis, apically placed such that the auxin is transported to the primordial apex. Internal PIN1 expression persists in the central region, including vascular regions. The apically placed PIN1 in the adaxial epidermis and the placement of PIN1 in the central region lead to the proposal that a PIN-mediated reverse fountain pattern of auxin may be occurring in the leaf as it does in cotyledons and flowers (Benkova et al., 2003).

Mutants of genes responsible for proper PIN placement also display vascular defects and show that PIN also has an important function in promoting vascular continuity and patterning. Based on immunolocalization studies, the PIN1 protein is polarly localized to the plasma membrane through rapid GNOM (GN) mediated, endosome to plasma membrane vesicular cycling (Muday et al., 2003), a process that

requires sterols for correct docking of PIN1 at the plasma membrane (Souter et al., 2004). The *gn* partial loss-of-function mutation results in disorganized PIN1 localization and misaligned and disorganized vasculature in the embryo (Geldner et al., 2004) while the sterol biosynthesis mutant *sterolmethyl transferase1* displays cotyledons with discontinuous secondary veins and vascular islands (Souter et al., 2004). PIN localization in the roots and shoots of *pinoid* loss of function mutants as well as *35S::PINOID* overexpression lines have shown that the expression of *PINOID*-like genes (or lack thereof) may be responsible for shifting PIN polarity, acting as a binary switch (Friml et al., 2004). It seems likely that a similar mechanism may occur in the leaf. Unfortunately, the leaf vascular phenotype of these lines has not yet been characterized.

Efficient transport of auxin requires not only efflux, but also influx carriers of auxin. As we might expect, auxin influx seems to be important in leaf vascularization. The subcellular localization of AUX1 using HA-AUX1 antibodies is seen primarily in the epidermis of leaf primordia (Reinhardt et al., 2003). The *aux1-7* mutant has previously been reported as having normal venation (Steynen & Schultz, 2003) but this is likely due to redundancy with other influx carriers in the shoot (Stieger et al., 2002) such as LAX1, LAX2, LAX3, or AAP1 (Swarup et al., 2004). One might expect that mutant plants lacking two or more functional influx carriers would result in a decrease in auxin being taken up by the cells and produce an altered leaf and cotyledon vascular phenotype.

A Model for Auxin-Dependent Vascular Patterning

Auxin efflux activity is important for a proper auxin response pattern, vascular continuity, vascular density and early procambium development of all vein orders. The

mechanism that best accounts for the continuous vascular strand and likely the final vascular pattern is the auxin canalization hypothesis (Figure 2; Sachs, 1981). The auxin canalization hypothesis proposes that a positive feedback mechanism causes auxin transporting cells to become more efficient in auxin transport (both influx and efflux) resulting in stable "auxin canals". The increased conductivity of these cells not only leads to their vascular differentiation (caused by the high levels of auxin flux), but also depletes neighboring cells of auxin preventing them from taking on a vascular cell fate (Sachs, 1981). Auxin canalization is based on assumptions of 1) shifting auxin transport towards an auxin maximum (the canal) 2) feedback loops in the auxin response and auxin transport systems (transport ability keeping up with demand) and 3) vascular differentiation occurring in cells with high auxin flux. Recent evidence has given considerable validity to the first two assumptions (Benkova et al., 2003; Friml et al., 2003; Reinhardt et al., 2003; Blilou et al., 2005), but there has been little support for the last assumption. Overcoming the challenges in measuring auxin flux in the leaf at the cellular level may validate the final assumption.

The first assumption, that auxin transport can shift towards an auxin maximum, is supported by extensive experimental evidence. The expression of *MP* in leaves and embryos shows that auxin response starts off broad and narrows during development (Hardtke & Berleth, 1998; Hardtke et al., 2004). This is not likely a result of changes in auxin production, as mentioned previously, but must rather be due to auxin transport focusing the auxin within this broad zone, perhaps by redirecting PIN localization. This notion of shifting PIN localization based on the location of the auxin maximum is not without precedent. When the shoot apical meristem (SAM) of *pin1-1* plants is treated

with IAA, or when the SAM of wild type plants are treated with the efflux independent auxin 2,4-D, ring shaped organs are produced. This suggests PIN1 normally functions in focusing the auxin maximum in the SAM. In the SAM of the *mp* mutant, PIN1 is expressed uniformly in the peripheral zone, rather than in a localized region. Furthermore, application of IAA to the SAM of auxin responsive *pinoid* plants increases the transcription and translation PIN1 at the site of application (Reinhardt et al., 2003). Shifts in PIN polarity have also been seen in root after root ablation. As auxin accumulates proximal to the ablated quiescent center and a new quiescent center forms, PIN4 expression shifts proximally (Xu et al., 2006).

Feedback loops linking auxin to the expression of specific auxin transporters are well established. In the root, translation, but not transcription of EIR1/PIN2 is reduced with auxin treatment in wild type plants but not *axr1-3* mutant plants. This demonstrates that a negative feedback loop involving auxin response and *EIR1/PIN2* translation controls auxin flow in the root. Additionally, a separate positive feedback loop is observed in the root. Ablation studies (Xu et al., 2006) and analysis of the *plethora1 plehtora2* (*plt1 plt2*) double mutant (Blilou et al., 2005) show that the *PLT1* and *2* genes are essential for *PIN4* transcription and important for enhancing *PIN3* and *PIN7* transcription. These results indicate that in roots, PIN expression is dependent on the PLT proteins. Moreover PLT1 protein expression in ablated wild type roots occurs as the new auxin maximum appears (Xu et al., 2006). Consistent with this, the *PLT1* and *2* genes were shown to be both auxin-inducible and likely to act downstream of the ARF's as the auxin inducibility of *PLT1* and *2* transcription is delayed about 6 hours following auxin application and *PLT1* and *2* transcription is absent in the *mp* globular to heart stage

embryo (Aida et al., 2004; Xu et al., 2006). This demonstrates the possibility of a complex positive feedback system where an auxin maximum results in expression of genes (*PLT1* and *2*) that regulate auxin efflux.

An additional positive feedback system has also been observed at the level of auxin efflux protein activity. PIN1 is constantly being cycled to and from the plasma membrane via endosomal vesicles. Recently it has been shown that IAA, NAA and 2,4-D inhibit PIN1 internalization, thus interrupting the cycle and causing an accumulation of PIN1 at the plasma membrane (Paciorek et al., 2005). This inhibition of internalization would transiently increase PIN1 activity and may help buffer fluctuations in auxin in a manner independent of transcription and translation.Taken together, these results provide strong evidence that the accumulation of auxin and subsequent auxin response can induce changes in both the direction and intensity of polar auxin flow, thus supporting two of the three assumptions of the auxin canalization hypothesis.

The evidence mentioned above has shown that auxin response, auxin production and auxin transport are interdependent and may strongly influence changes in the vascular complexity. I have characterized the novel mutant *unhinged* (*unh*), which has decreased vascular complexity in leaves and cotyledons. Here I demonstrate that *unh* represents a defect in auxin response, consistent with the auxin canalization hypothesis.

Materials and Methods

Plant Materials

Lines of *Arabidopsis* previously treated with ethyl methanesulfonate (EMS) were purchased from Lehle Seed (Round Rock, Tx). Glabrous (*gl1-1*), *axr2-1* and *axr1-3* seed of Columbia-0 ecotype (Col) was obtained courtesy of George Haughn (Department of Botany, University of British Columbia, Vancouver, BC). *AtHB-8::GUS*, *pin1-1*, mp^{G92} were generously provided by Thomas Berleth (University of Toronto, Toronto, ON). *DR5::GUS* was donated by Jane Murfett (University of Missouri, Columbia, MO). Isolation of *fkd1* (Steynen and Schultz, 2003) and construction *FKD1::GUS* ,(Hou, unpublished results), was previously done in this laboratory. The mutant *cvp2* was kindly given by Francine Carland and Timothy Nelson (Department of Molecular, Cellular and Developmental Biology, Yale University, New Haven, CT). All other seed material (*aux1-7*, *eir1/pin2*, *rty1*) was obtained from the *Arabidopsis* Biological Resource Center (Columbus, Ohio).

Growth Conditions

Seed were planted either on a mixture of ¾ Flora Compo compost (The Professional Gardener Co Ltd., Calgary AB) and ¼ vermiculite (Coaldale nurseries, Coaldale, AB) in 100 cm^2 pots or on Petri dishes containing *Arabidopsis thaliana* (AT) growth medium (Ruegger et. al., 1998). Pots were covered with a plastic cover and both pots and dishes were incubated at 4°C in the dark for 3 days, after which they were transferred to growth chambers (Percival Scientific, Perry, IA) with 24 hours of light at an intensity of approx. 130 $\mu mol \bullet sec^{-1} \bullet m^{-2}$ from Sylvania Cool White, Grow Lux and

incandescent bulbs (Osram Sylvania Inc, Danvers, MA). Chambers were set at 20°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).

Microscopy and Imaging and Statistics

A Leica MZ8 dissecting light microscope was used for analysis of mature cotyledons, leaves and flowers (Leica Microsystems, Wetzlar, Germany). Seedlings were dissected by hand using 23 gauge needles (Becton Dickinson, Oakville ON) and were mounted on slides with 50% glycerol. Analysis of leaf developmental series, *AtHB-8::GUS* leaves, *DR5::GUS* leaves and auxin transport inhibitor treated leaves was performed using an Eclipse E600 compound light microscope (Nikon, Mississauga, ON). Tissues were photographed using a Nikon Coolpix 990 camera (Nikon, Mississauga, ON) and scored using Adobe Photoshop 5.0 (Adobe Systems Inc., San Jose, CA) and NIH Image (http://rsb.info.nih.gov/nih-image/). Measurements were recorded in Microsoft (Redmond, WA) Excel for subsequent determination of averages, standard error and P values by F-test and Student's t tests. Data sets that had significantly different variances ($p<0.05$), as determined by the F-test, were analyzed using two tailed T-test assuming unequal variances, otherwise two tailed T-test assuming equal variances was used.

Mutant Isolation

Approximately 6000 seed of an M2 generation following EMS treatment of the Col ecotype (Lehle Seed) were sown at a density of 50 seeds per pot and screened for aberrations in venation patterning at 14 DAG. Putative vascular pattern mutants were

grown to maturity and M3 seed was harvested and subsequently re-screened. The *unh* mutant represents one of several mutants identified. The *unh* mutant line was back-crossed to Col four times prior to all analyses.

Mapping of *UNH*

Mapping was carried out through the use of ecotypic specific markers (Table 1) visible through PCR (SSLPs) or a combination of PCR and restriction endonucleases (CAPS) as described in Lukowitz et al. (2000). *unh* was crossed into the Landsberg erecta (Ler) background and F2 seed was collected for the mapping population. DNA was extracted from the leaves of F2 plants showing the *unh* mutant phenotype after Dellaporta et al. (1983). F3 seed was collected from each plant to confirm homozygosity of the *unh* allele. Useful SSLPs and SNPs (for CAPS) between Col and Ler were identified through the Cereon polymorphism database (http://www.arabidopsis.org/Cereon/) (Jander et al., 2002). The web-based programs Primer3 (http://frodo.wi.mit.edu/) (Rozen & Skaletsky, 2000), Blastdigester (http://bbc.botany.utoronto.ca/ntools/cgi-bin/ntools_blast_digester.cgi) (Ilic et al., 2005) and dCAPS finder 2.0 (http://helix.wustl.edu/dcaps/dcaps.html) (Neff et al., 2002) were used in locating or designing primers around each polymorphism. Generally large SSLP's were identified and used first, then SNPs (CAPS and dCAPS) were used in regions where SSLP's could not be found. Successful marker primers are listed in Table 1. PCR was done using standard conditions (Bell & Ecker, 1994) and PCR products were resolved by gel electrophoresis using a 4% agarose gel at 100 V.

Ethidium bromide was purchased from Sigma Chemical Co (St. Louis, MO) dNTP's 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 Fisher Scientific (Ottawa, ON) and New England Biolabs (NEB, Ipswich, MA); Restriction enzymes were obtained through NEB. Agarose was purchased from EM Science (Gibbstown, NJ).

Phenotypic Analysis of *unh*

To analyze and compare the first leaves of all genotypes, seed were sown on soil at a density of 25 seed per pot. After 21 DAG, first leaves were removed, treated with 70% ethanol for 1 day and cleared in chloral hydrate (Sigma):water:glycerol (8:2:1 v/v/v)(after Koizumi et al., 2000). For developmental series analysis, first leaves from wild type (Col ecotype) and *unh* seedlings grown on AT medium were removed and treated as above at 5, 6 and 7 DAG.

To examine the effects of 2,4-D and NPA on leaf vascular development, *unh* and wild type seed were sown on AT dishes and AT dishes supplemented with 2,4-D (Sigma, St. Louis, MO) or NPA (Chem Service, West Chester, PA). These chemicals were added to the autoclaved medium. The leaf phenotype was examined at 10 DAG.

In this thesis, the midvein (primary vein) is considered to be the linear vascular strand approximately along the midline, secondaries are considered to be those vascular strands connected to the midvein, tertiaries are veins connected to secondaries (but not the midvein) and quaternaries are veins connected to tertiaries (but not the midvein or secondaries). These vascular strands are 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), marginal venation gaps (a vascular discontinuity in the peripheral venation that creates a vein-free path to the midvein) and leaf width (the longest line perpendicular to the midvein that extends from leaf margin to leaf margin) were also scored.

To analyze whole plant morphology, *unh* and Col seed were sown on soil at a density of 25 seeds per pot. Plants were scored for root growth, internode length, days to bolting of 50% of plants, leaves at bolting and shoots at bolting. With the exception of time to bolting and root growth, these traits were examined at 30 DAG. Root growth was determined by transferring 4 DAG seedlings with roots between 8 and 15 mm long to fresh AT medium at a density of 30 seedlings per plate. Growth was then measured at 9 DAG. Root gravitropism was determined by rotating 90° 5 DAG *unh* seedlings on AT medium and measuring the response to gravity after 72 hours.

The *unh* mutation was introduced into *ATHB-8::GUS* transgenic plants by crossing of *unh* plants with homozygous *ATHB-8::GUS* plants in the Col background. Plants expressing both *ATHB-8::GUS* and *unh* in the F2 were allowed to set seed. Individual F3 populations that showed no segregation of *ATHB-8::GUS* were allowed to self and the F4 generation was used for characterization. A similar procedure was used for generating an *unh DR5::GUS* line (Ulmasov et al., 1997) and an *unh FKD1::GUS* line.

Seed from marker lines were planted at a density of 30 seeds per plate on AT plates. *unh* and wildtype plants containing either *AtHB-8::GUS* or *FKD1::GUS* were

stained for 6 hours, while those containing *DR5::GUS* were stained for 24 hours (leaves), or 30 minutes, 2 hours, or 4 hours (roots). *GUS* staining and clearing of 5 and 7 DAG first leaves were performed after Kang and Dengler (2002). Potassium ferricyanide and dimethyl formamide were purchased from Sigma. 5-bromo-4-chloro-3-indolyl glucuronide (X-gluc) was purchased from Rose Scientific (Edmonton, AB); $NaPO_4$ and EDTA were obtained from BDH Inc. (Toronto, ON); nonidet P40 was obtained from EM Science.

Generation of Double Mutants

Double mutants were generated between *unh* and *mp, pin1-1, axr2, axr1-3, eir1, cvp2, rty1, fkd-1* and *aux1-7.* All double mutant populations were screened in the F2 generation and ratios were analyzed using the chi-square goodness of fit statistic. With the exception of populations segregating for *axr2-1*, *mp, rty1* and *pin1-1*, seed from the F2 plants that were heterozygous for *unh* but homozygous for the other mutation was harvested. The subsequent F3 lines that showed segregation of the double mutant were allowed to self and double mutant F4 plants were characterized. In generating double mutant lines with *eir1*, *axr1-3* and *aux1-7*, the lack of gravitropism was used to confirm the presence of the other mutation in the double mutant. As *mp, rty1* and *pin1-1* are infertile, *unh* was selected in the F2 generation and double mutants segregating in the F3 population were used for characterization. The presence of a *pin1-1* allele was confirmed in the F1 generation and F2 *unh* line using a dCAPS marker: *pin1-1*dF 5'- CAAATCGT-TGTTCTTCAGTGTATCAcTT & *pin1-1*R 5'- GTTTCCAAAGGTTGTCTTCCA, DdeI digest gives 106+67 bp (wild type) or 106+42+25 bp (*pin1-1*) (dCAPS sequence courtesy

of Michael Prigge, Department of Molecular, Cellular and Developmental Biology, University of Michigan). For the dominant *axr2-1* mutant, seed from F2 plants resembling *axr2-1* but not the double mutant, was collected. F3 lines that were homozygous for *axr2-1* and segregating for the double mutant were allowed to set seed. Due to low seed set, the *unh axr2-1* double mutant plants of the F4 generation were used to make more seed and the plants of the F5 generation were scored.

Results

UNH Maps to Chromosome IV

The first leaves of wild type *Arabidopsis thaliana* (Col) have a relatively predictable and simple closed venation pattern (Candela et al., 1999). Using this as a comparison, an EMS mutagenized population was screened for mutants impaired in vascular pattern formation. From this initial screen, a mutant with a strong reduction in secondary and tertiary veins was identified. This mutant also frequently exhibits disconnected freely ending veins, hence the mutant was named *unhinged* (*unh*).

A segregation ratio of 3:1 was observed in the F2 backcrosses to wild type (Col), indicative of a monogenic recessive mutation. For mapping, *unh* was crossed to the polymorphic Ler ecotype. With a mapping population of 103, the *UNH* gene mapped to chromosome IV approximately 11 map units from the telomere, between markers 4-11-3 and 4-11-5b (Table 1). The approximate position on chromosome IV has been confirmed by linkage to the CUL1 mutant *axr6-1*. No other vascular patterning mutants have been reported in this region of 16 protein coding genes (Table 2), thus *UNH* represents a novel vascular patterning gene. In order to identify the *UNH* gene, 32 available SALK T-DNA insertion lines were screened. These SALK lines were comprised of exon insertions in 11 of the 16 genes and non-exon insertions in the remaining 5. Of these 32 SALK lines, only one line resembled the *unh* phenotype, however PCR revealed no T-DNA insertion and complementation tests indicate that it is not an allele of *unh*.

unh has a Simplified Cotyledon and First Leaf Phenotype

In order to determine the specific quantitative differences between *unh* and wild type, several vascular patterning traits were scored in mature first leaves and cotyledons (Figure 3 a –b; Table 3). A strong reduction in the number of areoles, branch points, secondary, tertiary and quaternary veins and a significant increase in free-ending veins was observed. In wild type, the midvein of the leaf was always completely enclosed by areoles. This was rarely the case in *unh*. The *unh* mutant often had 1 or more vascular discontinuities in the venation near the margin, which created a vein-free portion of the lamina extending from the margin to the midvein (hereafter called "marginal vein gaps"). More pronounced hydathodes, pointed leaf tips and leaf curling were also observed in *unh*. Regions of early leaf senescence within the relatively large areoles were frequently observed as well as anthocyanin accumulation on the leaf blade (Figure 4). The vascular pattern defect of *unh* cotyledons was more subtle than that of the leaves, showing a phenotype ranging from normal (4 areoles and no free ending veins) to severe (no areoles and 4 or more free ending veins) (Table 4).

In order to determine when the vascular defects first appear in *unh* leaves, the first leaves of 5, 6 and 7 DAG wild type and *unh* seedlings were dissected, cleared and scored (Figure 5; Table 5). As expected from the mature *unh* leaf phenotype, no differences were observed in midvein formation (Figure 5 a, b, g, h). By day 6, when xylem lignification is visible in secondary and tertiary veins, significant differences were seen in the number of areoles, free ending veins, secondaries, tertiaries and branch points (Figure 5 c-f, i-l; Table 5). This demonstrates that *unh* acts during or prior to the xylem differentiation in the leaf.

To ascertain whether *unh* represents a defect in procambial patterning or a defect in differentiating a correct procambial pattern into xylem, lines of the procambial marker *AtHB-8::GUS* with and without the *unh* mutation were examined (Figure 6; Table 6). The procambial tissue observed in wild type leaf primordia is arranged in an identical pattern as later differentiated xylem. In 7 DAG *unh* leaves, there is a twenty-fold increase in the number of free-ending strands of presumptive secondaries and a four-fold decrease in the number of presumptive tertiaries relative to wild type leaves. These defects show that *unh* is defective in procambial patterning and that *AtHB8* expression is partially controlled by *UNH*.

DR5::GUS Expression is Reduced in *unh* Leaves

To test if *unh* leaves show altered auxin response, a developmental series of leaves of *unh DR5::GUS* and wild type *DR5::GUS* from 3 to 7 DAG was compared (Figure 7). The *DR5::GUS* reporter line is a synthetic construct with 7 auxin response promoters in tandem coupled with a β-glucuronidase (*GUS*) reporter gene (Ulmasov et al., 1997). *DR5::GUS* expression shows a well characterized, dynamic pattern in young wild type first leaves and a disrupted pattern or reduced expression in some mutants lines (Figure 7; Avsian-Kretchmer et al., 2002; Carland & Nelson, 2004; Mattson et al., 2003; Steynen & Schultz, 2003; Koizumi et al., 2005; Zgurski et al., 2005). In *unh*, the overall pattern is very similar, however the intensity of *DR5* expression is clearly reduced throughout early leaf development. Similar changes in DR5 expression have been seen in the leaves of *van3*, *fkd1* and *cvp2*, which are mutants thought to be involved in auxin signaling (Steynen & Schultz, 2003; Carland & Nelson, 2004; Koizumi et al., 2005). A

reduced auxin response in the roots has also been seen previously in the auxin response mutants *axr1-3*, *axr1-12* and *axr3* (Sabatini et al., 1999). Consistent with the idea of global reduction in auxin response, the intensity of *DR5::GUS* expression is also reduced in the primary root tip of *unh* plants (Figure 8). Taken together, these observations provide strong evidence that *unh* is defective in auxin response.

Double Mutants with Leaf Vascular Patterning Mutants

In order to determine if *unh* acts in the same pathways as other known vascular patterning mutants, double mutants were generated (Figure 3 i,j,m,n; Table 3). Like the *unh* mutant, both *fkd1-1* and *cvp2* mutants alone show a higher than normal proportion of vascular non-meeting and free-ending veins as well as decreased *DR5::GUS* expression and, in the case of *cvp2*, vascular islands are also present (Figure 3 i,m; Table 3; Steynen & Schultz, 2003; Carland & Nelson, 2004). The *unh cvp2* double mutant produced a pattern that was simpler than either single mutant (Figure 3 j; Table 3). Areoles, secondary and tertiary veins and branch points were all reduced in number and this was coupled with an increase in marginal venation gaps. The number of vascular islands was essentially the same as *cvp2*. The *unh fkd1* double mutant was also simpler than either single mutant. The double mutant showed an increase in the number of vascular islands with a relatively simplified pattern. Additionally, *unh* plants display an altered pattern of *FKD1::GUS* expression in *unh* compared to wildtype (Figure 9). Taken together, the double mutant results suggest that the activity of both the *CVP2* and *FKD1* gene products are at least partially intact in the *unh* leaf while expression analysis suggests that *FKD1*

expression is partially controlled by UNH. Thus CVP2 and FKD1 function in overlapping, partially redundant pathways with UNH in the vascular patterning process.

Double Mutants with Auxin Response Mutants

In order to better understand how *unh* interferes with auxin response, I sought to identify possible relationships between *unh* and three auxin response mutants, *axr1-3*, *axr2-1* and *mp* by generating and analyzing double mutants (Figure 3 ;Table 3). The auxin response gene *AXR1* contributes to an auxin response by targeting AUX/IAA proteins for degradation through the ubiquitin pathway and has been shown to be expressed in the vasculature of leaves (del Pozo et al., 2002). The *axr1-3* mutant shows a strong reduction in areoles and secondary and tertiary veins and has increased free ends and marginal venation gaps relative to wild type (Figure 3 e; Table 3; Steynen & Schultz, 2003). The extremely simple venation pattern of the *unh axr1-3* double mutant is indicated by the low numbers of branch points and tertiary veins and increased number of marginal venation gaps (Figure 3 d; Table 3). Curiously, the number of vascular islands also increases considerably, a characteristic that is rare in both single mutants. Thus, the *unh* and *axr1-3* mutations have either an additive or synergistic interaction depending on the trait examined, suggesting the two genes function in overlapping pathways that are partially redundant and that the AXR1 pathway is at least partially intact in the *unh* leaf and vice versa.

In low auxin conditions, AUX/IAA proteins such as IAA7/AXR2 inhibits the auxin response factors (ARF) preventing a transcription based auxin response (Weijers et al., 2005). The dominant gain-of-function mutant *axr2-1* produces epinastic leaves and

agravitropic roots and shoots. The vascular pattern in *axr2-1* shows no significant difference in areoles and secondary veins compared to wild type, but a reduction in branch points, tertiary and quaternary veins, leaf width and free ending veins is observed (Figure 3 g; Table 3; Steynen & Schultz, 2003). The overall plant habit of the *axr2-1 unh* double mutant plants strongly resembles *axr2-1* although the leaves are significantly smaller. On the other hand, the vascular pattern in the leaves is most similar to *unh* except with fewer tertiary and quaternary veins (Figure 3 h; Table 3). When one considers the less extreme vascular phenotype of *axr2-1* relative to *axr1-3* and the specificity of AUX/IAA's on ARF's (Weijers et al., 2005) this additive phenotype likely reflects a minor influence of *axr2-1* on leaf vascular patterning. Put another way, the specific ARF inhibited by IAA7/AXR2 likely has a minor role in vascular patterning and UNH activity.

MP is an ARF, which functions to activate the expression of auxin inducible genes important in producing an auxin response (Hardtke & Berleth, 1998). *MP* is expressed broadly in young leaf primordia with the exception of the midrib region and gradually becomes restricted to the procambium by day 7 (Hardtke & Berleth, 1998; Hardtke et al., 2004). The *mp* partial loss-of-function mutants rarely produce roots or organs from the SAM. The *mp* mutant shows a reduction in vasculature in the cotyledons, leaves, as well as other organs if present (Figure 3 o; Table 3; Table 4; Przemeck et al., 1996). The vascular pattern in the double mutant cotyledons strongly resembles *mp* (Table 4). Surprisingly, a higher frequency of *unh mp* double mutants seedlings form one or more leaves (51% n=70) relative to *mp* (22%, n=144). A similar suppression of *mp* shoot defects has been seen by the *fkd1* mutation (Steynen & Schultz, 2003). With the exception of the free-ending veins and marginal venation gaps, the leaf characteristics of

the *mp unh* double mutant are less extreme than those produced by the *mp* single mutant (Figure 3 p; Table 3).

The *unh* Whole Plant Phenotype

To determine if *unh* is defective in auxin response pathways outside the leaves and cotyledons, I examined root growth, internode elongation, apical dominance, leaf formation and flower development (Table 7 & 8), which are all traits known to be influenced by auxin. Auxin response mutants often show defects in leaf vascular development, as well as other defects in shoot and root growth and organ development. For example, both *axr1-3* and *mp* mutants have a simplified vascular pattern and root defects (Hobbie & Estelle, 1995; Berleth et al., 2000) and plants expressing the 35S::*CUL1* construct produced a pin-like apex with no shoot organs (Hellman et al., 2003). Moreover *axr1-3* and *axr1-12* show decreased apical dominance and a reduction in height (Lincoln et al., 1990). In *unh*, root growth and average internode length are reduced compared to wild type and number of leaves on primary shoot at bolting is increased (Table 7 & 8), suggesting that *UNH* influences several aspects of development in a manner similar to characterized auxin response mutants.

***unh* Leaves and Roots are Less Sensitive to Low Levels of Auxin**

If the *unh* mutant is defective in auxin response, one might expect that elevating the levels of auxin might rescue the defect. I set about to raise the levels of auxin in the leaf in 2 ways: 1) directly through application of exogenous auxin or the introduction of the auxin over-producing mutant *rooty1 (rty1)* (King et al., 1995) and 2) indirectly by

blocking auxin transport in the *unh* leaf either through application of auxin transport inhibitors or the introduction of auxin transport mutants.

RTY1 (*rty1*) is allelic to *SUPERROOT1* (*sur1*) and produces about a 17 and 5 fold increase in IAA in the shoot and root respectively (King et al., 1995). One might expect that the overproduction of auxin would promote excess vascular development and result in a proliferation of veins. Surprisingly, *rty1* leaves showed a drastic simplification in leaf vascular patterning, often resembling that of the cotyledons (Figure 3 s ;Table 3). Moreover, the *unh rty1* double mutant was found to be more extreme than *rty1* in nearly every trait (Figure 3 t ;Table 3). One possible explanation for this is that an optimum level of auxin is required for vein formation, above which vein formation is inhibited.

To test this idea, the level of auxin was manipulated directly by applying 2,4-D. 2,4-D is known to elevate auxin levels in leaf primordia based on *DR5::GUS* expression (Mattson et al., 2003). Consistent with the notion of optimal auxin being essential for vein patterning, wild type seedlings grown on 10^{-8} M 2,4-D also have a significantly reduced leaf vascular pattern (Figure 9 b). The leaves of *unh*, on the other hand, show an increase in leaf vascular complexity when treated with 10^{-8} M 2,4-D (Figure 9 e). At 10^{-7} M 2,4-D both *unh* and wild type are more simplified (Figure 9 c,f) compared with no 2,4-D treatment. It is interesting to note that, while not statistically significant, a similar trend was seen in the roots of *unh* and wild type (Table 8). At 10^{-9} M 2,4-D root growth is slightly inhibited in wild type, but in enhanced in *unh.* At higher concentrations, root growth in both *unh* and wild type is significantly inhibited. Thus it seems that relatively low concentrations of 2,4-D can partially rescue *unh*, supporting the notion that *unh* is defective in auxin response and that increasing auxin ameliorates the phenotype.

However in both wild type and *unh* high auxin inhibits the formation of a complex leaf vascular pattern.

Decreasing Auxin Transport Rescues *unh*

The second method I used to elevate levels of auxin in the leaf was to increase the levels of free auxin indirectly by inhibiting auxin transport. It is believed that around day 4 and 5, the leaf primordium begins producing its own auxin switching from a sink to a source, producing auxin first at the distal tip and margin and later in the lamina (Avsian-Kretchmer et al., 2002). In order to elevate auxin at the source of production, I firstly exposed *unh* seedlings to the auxin transport inhibitor NPA and second I generated double mutants of *unh* in combination with auxin transport mutations. The auxin transport inhibitor NPA has been well characterized and has been shown to increase the levels of auxin predominantly at the leaf margin by blocking the auxin efflux pathway (Mattson et al., 1999; Mattson et al., 2003; Sieburth, 1999). In wild type, this increase leads to increased vascularization at the margin near the distal tip, increased vasculature in the petiole and midvein region and an increase in the number of higher order veins. We predicted that the local marginal increase in auxin might rescue the marginal gaps in the peripheral venation of *unh*. *unh* and wild type grown for 10 days on 10, 30 and 100 μM NPA were compared (Figure 10). The first leaf of all *unh* treated plants showed a completely closed marginal venation pattern at all concentrations of NPA, a phenotype similar to untreated and treated wild type leaves (Figure 10 b, e). *DR5::GUS* expression in both *unh* and wild type was increased at the margin indicative of increased auxin at the margin (Figure 10 c, f). *DR5::GUS* expression was also seen, though to a lesser extent, in

the lamina. This supports the idea that increasing auxin can compensate for the marginal venation gap in *unh*.

To confirm the idea that the margin vein gaps were rescued by NPA because of increased auxin through decreased auxin transport, I generated double mutants with three known auxin transport mutants *pin1-1*, *eir1-1/pin2* and *aux1-7* (Figure 3 ;Table 3). The auxin efflux carrier PIN1 has been shown to be expressed at the earliest stages of leaf primordium development (Reinhardt et al., 2003) and it is thought that it plays an important role in the efflux of auxin in leaves. As we might expect, the loss-of-function *pin1-1* mutant (Figure 3 q; Table 3) has a leaf venation that resembles that of plants grown in low levels of NPA or exposed to NPA late in development (Mattson et al., 1999). Compared to *unh*, the *unh pin1-1* double mutant had a significant increase in the number of areoles, secondary veins and branch points and the marginal vein gaps were consistently closed (Figure 3 r; Table 3). Thus, increasing auxin accumulation by decreasing auxin flow in the *pin1-1* mutant suppressed much of the *unh* phenotype.

To ensure that the effects seen in *pin1-1* were a direct result of decreased auxin transport in the shoot and not associated with the root I generated an *unh eir1/pin2* double mutant. EIR1/PIN2 has been thought to be root specific based on RT-PCR analysis of roots, leaves, stems, flowers and siliques (Luschnig et al., 1998). The leaf vascular phenotype of *eir1* has not been reported previously and surprisingly my analysis indicates that the *eir1* mutant showed a significant decrease in the number of tertiary and quaternary veins, as well as a decrease in branch points and leaf width (Table 3; Figure 3 k). Relative to *unh*, the *unh eir1* double mutant showed a significant decrease in number of tertiary veins, branch points and leaf width and a significant increase in the number of

marginal venation gaps (Figure 3; Table 3). The *eir1* leaf phenotype suggests that either *EIR1* is expressed in the leaf and was not previously detected by RT-PCR or that auxin transport defects in the root inhibit venation complexity in the leaf. If the second possibility is true, the opposite leaf vein phenotypes, despite both mutants showing reduced auxin transport in roots, allows us to assume that the increased venation complexity seen in *pin1-1* is not an indirect result of auxin transport defects in the root.

Auxin influx is also important for the movement of auxin through the leaf and leaf patterning. AUX1 has been localized in the leaf (Reinhardt et al., 2003) and is thought to act redundantly in the shoot with other influx carriers, since the *aux1-7* mutant appears normal (Stieger et al., 2002). Although the *aux1-7* mutant has previously been found to have a normal venation pattern (Steynen & Schultz, 2003) I found that like the *pin1-1* mutant it had a more complex phenotype than that of wild type, displaying a significant increase in areoles, secondary and tertiary veins (Figure 3 c; Table 3). In the *unh aux1-7* double mutant there was a significant increase in the number of areoles, secondary veins and branch points compared to *unh* mutants (Figure 3 d; Table 3). This indicates that some of the extreme characters of *unh* were partially suppressed in the presence of the *aux1* mutation. This suppression of the *unh* phenotype in both the *aux1-7* and *pin1-1* backgrounds provides strong additional evidence that increasing auxin levels by decreasing auxin transport can alleviate the strong phenotype of *unh*.

Discussion

I have identified an *Arabidopsis* mutant, *unhinged* (*unh*) that exhibits reduced leaf vascular complexity and frequently forms an open vascular pattern in the leaves and cotyledons. Based on the abnormal *AtHB-8::GUS* expression seen in young leaves, this defect is present before the procambial patterning process of development. The recessive nature of *unh* indicates that it likely represents a loss-of-function mutation. Double mutant analyses, reduced intensity of *DR5::GUS* expression and response to exogenous auxin and auxin transport inhibitors suggest that *unh* is defective in auxin response.

The *unh* Mutation Reduces Auxin Response

Several lines of evidence indicate that plants mutant for *unh* are defective in auxin response. Firstly, both leaves and roots of *unh* show no change in the pattern of *DR5::GUS* expression, but a reduction in its expression. This phenomenon is similar to that seen in leaves of *van3*, *fkd1* and *cvp2* mutants, all thought to be involved in auxin signaling (Steynen & Schultz, 2003; Carland & Nelson, 2004; Koizumi et al., 2005) as well as in the roots of the auxin response mutants *axr1-3*, *axr1-12* and *axr3* (Sabatini et al., 1999). Moreover, the increase in free ending veins in *unh* is similar to the *cvp2* and *fkd1* phenotypes, while this, combined with a strong reduction of higher order veins, is similar to the leaf vein phenotype of auxin response mutants such as *axr1-3* and *mp*. I reasoned that if the *unh* phenotype was the result of defective auxin signaling, elevating the levels of auxin by applying 2,4-D might compensate for this defect. The *unh* simplified vein pattern was made more complex by treatment with relatively low concentrations of 2,4-D. This suggests that *unh* is defective in auxin signaling, but that

auxin signaling is not completely absent. One explanation for this is that the *unh* mutation is a partial loss of function rather than a null allele. Alternatively, other redundant activities may act with UNH to allow a full auxin response. Redundancy among auxin response mutants is a well known phenomenon, illustrated well by the characterization of the *nph4* mutant in *MP* antisense line *35S::MPAS* (Hardtke et al., 2004). Plants carrying the *35S::MPAS* construct have generally fewer higher order veins in rosette leaves, while *nph4* mutant plants show no vein pattern defects. The combination of the two defects, however, produces a more simplified leaf vascular phenotype than *35S::MPAS* alone.

The increased complexity of the *unh* phenotype when exposed to elevated auxin levels directly was further illustrated by elevating auxin levels indirectly in the *unh* leaf through application of auxin transport inhibitors. Auxin efflux inhibitors have been shown to cause localized increases in auxin at the leaf margin, a condition that allows for distal vein meeting of the normally non-meeting distal veins in *fkd1* mutants (Steynen & Schultz, 2003). Similarly, *unh* plants exposed to the auxin efflux inhibitor NPA, or combined with the auxin efflux mutant *pin1-1*, or the auxin influx mutant *aux1-7* showed a complete restoration of closed marginal vein gaps, consistent with the notion that auxin response can occur in *unh* mutants if auxin is sufficiently high. Although the auxin transport proteins AUX1 and PIN1 have been shown to be important for auxin transport in leaves, *pin1* and *aux1* mutants do not show a drastic increase in marginal venation as seen in NPA treatment; however their ability to eliminate the margin gaps seen in *unh* mutants suggests they do result in somewhat increased levels of auxin at the margin. I propose that the lack of increased marginal venation in *aux1-7* and *pin1-1* plants results from the redundant action of other transport proteins and/or a partial compensatory

mechanism that causes the ectopic expression of other transport proteins. Both scenarios have been observed in the roots of *Arabidopsis*. In wildtype roots cooexpression of PIN1 and PIN7 is seen in vascular bundle and in double and triple mutant *pin* plants, the remaining (non-mutated) PIN proteins are ectopically expressed and partially compensate for the deficiencies in auxin transport (Blilou, et al., 2005). In addition to their restoration of the closed margin gaps and like low levels of 2,4-D, *pin1-1* and *aux1-7* show increased complexity of *unh* venation. This is consistent with the idea that *pin1-1* and *aux1-7* have increased auxin throughout the leaf.

My results show that increasing auxin directly, as well as indirectly through impairing auxin transport, can restore vascular complexity in *unh* leaves. Given that the artificial *DR5* promoter is dependent on ARF binding (Jenik & Barton, 2005) auxin signaling at or upstream of the level of ARF activity must be defective in *unh*. Four models for UNH are consistent with these results (Figure 12):

1.) UNH may be promoting auxin response directly (Figure 12 b). Analysis of leaves in an allelic series of *mp* indicates that higher order veins are more sensitive to reduced auxin signaling (Mattson et al., 2003). Based on our double mutant analysis, *UNH* acts redundantly with *AXR1*, *FKD1* and *CVP2* and would likely be in an auxin response pathway parallel or partially redundant to these genes. For example, *UNH* may inhibit the expression of certain AUX/IAA's, may interact with the SCF/TIR1 to prolong an auxin signal, or may stabilize and prolong binding of specific ARF's to their respective ARE's. The short, gravitropic root phenotype seen in *unh* as well as the sensitivity of *unh* to high levels of 2,4-D is uncharacteristic of known auxin response mutants, suggesting this first model is unlikely.

2) UNH may normally function to enhance auxin response indirectly by acting as a positive regulator of auxin production or a negative regulator of auxin conjugation in the leaf (Figure 12 c). Mutants and transgenic lines that decrease auxin levels either through decreased production (Zhao et al., 2002) or increased conjugation (Romano et al., 1991; Rampey et al., 2004; Tian et al., 2004; Qin et al., 2005) often have smaller leaves and shorter roots, however aberrant leaf vascular patterns have not been reported. Interestingly, one tryptophan-dependent auxin synthesis gene, *NIT3*, shows localized expression in leaf vascular tissue (Rampey et al., 2004) and may function to boost auxin response and thus ensure vascular tissue continuity.

3) UNH may normally function to enhance auxin response indirectly by negatively regulating auxin transport (Figure 12 d). We have seen that when auxin transport is somewhat impaired as in *pin1-1* or *aux1-7*, leaf complexity increases, even in the *unh* background. Thus it may follow that increasing auxin transport will reduce vein complexity, as in *unh* mutants. The scenario that *unh* has increased auxin transport may help explain the partial suppression of *mp* when combined with *unh*. In *mp* the auxin maximum in the embryo is absent (Sabbatini et al., 1999) and the severe *mp* defects are thought to be an indirect effect of *mp* influence on auxin transport (Mattson et al., 1999). In *mp unh* double mutant plants, enhanced auxin transport caused by *unh* could partially compensate for reduced auxin transport caused by *mp*, resulting in a suppression of the *mp* phenotype.

Negative regulation of auxin transport is not without precedent. The mutant *transparent testa4* (*tt4*), which is defective in flavonoid synthesis, shows elevated auxin transport in the inflorescence and hypocotyl (Brown et al., 2001). *UNH* is unlikely to be

involved in flavonoid mediated auxin transport inhibition, since flavonoid biosynthesis mutants lack anthocyanins and produce a yellowish seed coat (testa), characteristics never seen in *unh*. One might expect that increased auxin transport might lead to an increased *DR5::GUS* expression in the root as well as altered gravitropic response. This is not seen in *unh*, suggesting this third model is unlikely.

4.) UNH may normally function to enhance auxin response indirectly through the brassinosteroid (BR) pathway (Figure 12 e). Brassinosteroids have been shown to enhance auxin response and mutants defective in BR production or response also show decreased *DR5::GUS* expression in the leaves (Bao et al., 2004) as well as having shorter, gravitropic roots (Müssig et al., 2003), accumulation of anthocyanins, more leaves at bolting and shorter internodes (Chory et al., 1991), all characteristics seen in *unh*. Examining the leaf vascular phenotypes of BR mutants and exposing *unh* to exogenous BR would be valuable in exploring this possibility.

Auxin Influx and Efflux Activity Inhibit Vascularization

The more complex vein pattern of *aux1-7* relative to wild type indicates that AUX1 is not required for auxin perception in leaves. One might expect that a small decrease in auxin influx would lead to less auxin entering the cell and being perceived and result in a simplified vascular phenotype. Indeed *aux1-7* had long been thought to be involved in auxin perception due to its auxin resistant-like root phenotype, suggesting that it is required for auxin perception in the roots (Timpte et al., 1995). The complex leaf vascular phenotype observed in *aux1-7* suggests that AUX1 is not required for the cell to perceive auxin and that one or more of the putative auxin influx carriers LAX1, LAX2,

LAX3, or AAP1 (Swarup et al., 2004) may be compensating for AUX1 in the *aux1-7* mutant leaves.

The increased complexity of leaf vascular patterns in the *pin1-1* and *aux1-7* single mutants indicate that both auxin influx and auxin efflux are important in inhibiting excess vascularization. According to the auxin canalization model, a positive feedback mechanism causes auxin transporting cells to become more efficient in auxin transport resulting in stable "auxin canals" that drain neighbouring cells of auxin. This model indicates that a decrease in auxin transport would result in an increase in auxin in the non-vascular cells. In support of this idea, the levels of IAA determined by gas chromatography–selected reaction monitoring–mass spectrometry were found to be elevated early in development in the *aux1-7* mutant (Marchant et al., 2002). In both *pin1-1* and *aux1-7*, the decrease in auxin transport may indirectly lead to accumulations of auxin in the leaf, thus producing more vascular tissue. This idea is consistent with the *aux1-7* and *pin1-1* mutants showing a significant increase in areoles, secondary, tertiary veins and branch points and their ability to increase the complexity of both the wild type and the *unh* vein patterns.

Models for Auxin Mediated Vascularization

I have proposed that the increased vein complexity in *aux1-7* and *pin1-1* results from inefficient drainage of auxin into canalization channels, thus increasing auxin levels in non-vascular cells and inducing additional vascularization. This idea implies that auxin concentration normally limits vascular tissue formation in the leaf. That the simplified venation pattern of *unh*, which results from reduced auxin response, is made more

complex by *aux1-7*, *pin1-1*,and low levels of 2,4-D supports this idea. In apparent contradiction to the idea, wild type leaves exposed to low and high levels of 2,4-D, *unh* exposed to high levels of 2,4-D, as well as leaves of the auxin overproducing *rty1* mutant show a simplified vascular pattern. A similar phenomenon is seen in wild type plants grown at higher temperatures (Scarpella et al., 2004; Cormack, unpublished results), a condition thought to increase auxin levels (Gray et al., 1998). These data suggest that while auxin is normally limiting in vein formation in wild type leaves and vein pattern can be made more complex by adding small amounts of auxin, adding large amounts of auxin results in inhibition of vein formation. This suggests that a threshold of auxin is required. I propose two models that may explain this phenomenon. 1.) Increasing auxin initiates other cellular responses (eg. mesophyll differentiation) that indirectly terminate vein formation. Premature mesophyll differentiation has been thought to account for the free-ending nature of high order veins in wild type leaves (Scarpella et al., 2004). According to my model, increases in auxin may cause premature differentiation of mesophyll, which would terminate the formation of veins and the growth of the leaf and lead to a simple vein pattern and smaller leaves. In support of my model, early vascular differentiation near regions of high auxin and delayed vascular differentiation near regions of low auxin has been reported in the NPA treated leaf (Mattson et al., 1999; Sieburth, 1999). Additionally, the apical tip, which has the highest levels of auxin in the young leaf primordia, differentiates mesophyll relatively early (Scarpella et al., 2004) and the auxin over-producing *sur* mutants result in decreased leaf expansion (Ljung et al., 2001). Consistent with these observations, I observed smaller leaves in *rty1* and 2,4-D

exposed plants as well as progressively smaller leaves in plants exposed to higher temperatures at earlier stages (Cormack, unpublished results).

2.) A localized level of auxin above a threshold is required for a pre-canalization patterning event. Recently it was shown that in the root, the first patterning event is the accumulation of auxin, which drives auxin responsive genes *PLT1* and *PLT2* (Xu et al., 2006). Expression of these genes is important in regulating both the expression and localization of PIN proteins. Thus, cells accumulating auxin express *PLT1* and *PLT2*, change cell fate and the changes in fate result in changes to polar auxin flow that reinforce and refine the region of auxin accumulation. In contrast to the auxin canalization model suggested for vein patterning, this model for root patterning suggests that auxin canalization is the result of, rather than the cause of, cell fate. It is tempting to speculate that a similar process may be occurring during leaf development. According to this model, localized accumulations of auxin in the leaf would induce the expression of cell-fate determinants which would trigger a shift the polarity of the auxin influx and efflux proteins so as to focus the auxin accumulation zone to a narrow band of presumptive vascular cells. As the leaf grows and existing veins separate, pockets of auxin accumulation would form and the cycle would repeat. According to this model, when the system is inundated with auxin, more cells would be involved in the focusing of auxin, fewer narrow bands of relative auxin accumulation would occur and fewer veins would form. This is consistent with the observation that 2,4-D treatment and *rty1* leaves have a simplified vein pattern.

Temperature Induction of High Auxin Results in a Simplified Vascular Pattern

It is known that at high temperatures, *Arabidopsis* plants produce more auxin in the leaves and show an increased auxin response, based on gas chromatography–mass spectrometry and increased *pIAA4-GUS* expression respectively (Gray et al., 1998). Thus it is possible that the decreased leaf area and reduced venation seen in plants grown at high temperatures is the result of increased auxin levels throughout the leaf. The fact that high temperature hypocotyl defects seen in wild type were not seen in *axr1-3* or *35s-iaaLys* plants, supports the idea that the effects of high temperature are dependent upon auxin production and auxin response (Gray et al., 1998). This is supported by the similar, simplified, leaf vascular phenotype of the leaves of plants treated with 2,4-D, plants in the *rty1* background and plants grown at temperatures near 29°C. Analyses of the *varicose* mutant grown at 16, 22 and 29°C showed that higher temperatures produced a more simplified vascular pattern while lower temperatures produced a more complex vascular pattern (Deyholos et al., 2003). This would suggest that lower temperatures may correspondingly result in decreased auxin production. One can imagine several implications of having auxin levels dependent upon temperature during seedling development. Since vein patterns are less complex at high and more complex at low temperatures, it is tempting to speculate that decreased venation and leaf size may be one mechanism controlling water loss at higher temperatures. Thus leaves developing at high temperatures form a simpler venation pattern in smaller leaves resulting in less water lost through transpiration, while at low temperatures a more complex venation in larger leaves develops to maximize transport of water and photosynthate.

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Table 1. Primer markers developed and used in mapping *unh*.

Primer Name	Sequence (5' - 3')	SSLP or CAPS	AGI BAC	Col* (bp)	Ler* (bp)
4-13 F	aacgccaaaagacattttattcc	SSLP	T4I9	217	173
4-13 R	tgctgcatacacgtcgtctc				
4-12 F	ttaaaatgcctgacggtgaa	SSLP	T14P8	123	108
4-12 R	caaaagagcaaaagcatatttcaa				
4-11-7 F	gccgtctcccttgtcagata	SSLP	T2H3	148	195
4-11-7 R	cagactactttgcgagagtttca				
4-11-5b F	atcatgtgactgcgttttgc	SSLP	T10M13	120	140
4-11-5b R	tcgagaaactaaagccatgaat				
4-11-4-3 F	tgcattatcttgcctgctatct	CAPS¥	T10M13	342	190 & 152
4-11-4-3 R	aggtagtaaatggcaccaccaa				
4-11-3 F	ataatgggccttactcaaccaa	CAPS¥	T7B11	261 & 141	402
4-11-3 R	acgaactcaatgcaaaacgac				
4-11-2 F	gtggtagcctggacaaaacc	SSLP	T7B11	103	95
4-11-2 R	ttccactgtcctcaaaattgtc				
4-11 F	gacaataaccttgcgtggtaca	SSLP	T7B11	274	297
4-11 R	tggttactgtatgccaaatgaa				
4-10.5 F	tctgggatacaaaatgccaaa	SSLP	T7B11	258	462
4-10.5 R	gattgatcgttaaaccccattt				
4-10 (Ciw5F) §	ggttaaaaattagggttacga	SSLP	T15B16	163	129
4-10 (Ciw5R) §	agatttacgtggaagcaat				
4-9-bF	cttcatcgtcaggtctgcaa	SSLP	F11O4	204	194
4-9-bR	gctgctgagaaacgagcttt				
4-8F	tcggaccacagttgataagaa	SSLP	F2N1	265	235
4-8R	ctgagctgtttaggcccgta				
4-3bF	gagcaaacggtggcaataat	SSLP	T18A10	198	235
4-3bR	ggtggttttgtccaagctgt				
4-3aF	ttcgatttggtttgattcagttt	SSLP	F15P23	154	178
4-3aR	ccatttaccaatgtgagctttct				
4-0.5 F	caaacaaaatggggaagttttc	SSLP	F6N15	331	174
4-0.5 R	aagagtggggttagaatagaagaca				

*Indicates lengths of final polymorphic fragment(s) of each ecotype.

¥The restriction enzyme *Dde*I was used in both CAPS

§CIW5 is a known primer marker (sequence obtained through TAIR)

Table 2. Protein coding genes between primer markers 4-11-3 and 4-11-5b

TAIR Accession	Locus	Description*
Gene:2141429	AT4G01980	hypothetical protein, contains similarity to hypothetical proteins of Arabidopsis thaliana
Gene:1006229042	AT4G01985	expressed protein
Gene:2141359	AT4G01990	pentatricopeptide (PPR) repeat-containing protein, low similarity to DNA-binding protein (Triticum aestivum) GI:6958202; contains Pfam profile PF01535: PPR repeat
Gene:504953079	AT4G01995	expressed protein
Gene:3440096	AT4G02000	expressed protein, low similarity to zinc finger protein (Arabidopsis thaliana) GI:976277
Gene:2132167	AT4G02010	protein kinase family protein, contains protein kinase domain, Pfam:PF00069
Gene:2132177	AT4G02020	zeste-like protein 1 (EZA1), identical to enhancer of zeste-like protein 1(EZA1) (GI:4185507) (Arabidopsis thaliana); similar to polycomb group (Arabidopsis thaliana) GI:1903019 (curly leaf); contains Pfam profile PF00856: SET domain
Gene:2132192	AT4G02030	expressed protein
Gene:2132202	AT4G02040	expressed protein,
Gene:2132212	AT4G02050	sugar transporter, putative, similar to SP|Q10710 Sugar carrier protein A {Ricinus communis}, glucose transporter (Saccharum hybrid cultivar H65-7052) GI:347855; contains Pfam profile PF00083: major facilitator superfamily protein
Gene:2132222	AT4G02060	prolifera protein (PRL) / DNA replication licensing factor Mcm7 (MCM7), identical to DNA replication licensing factor Mcm7 SP|P43299 PROLIFERA protein {Arabidopsis thaliana}; contains Pfam profile PF00493: MCM2/3/5 family
Gene:2132232	AT4G02070	DNA mismatch repair protein MSH6-1 (MSH6-1) (AGAA.3), identical to SP|O04716 DNA mismatch repair protein MSH6-1 (AtMsh6-1) cress) {Arabidopsis thaliana}
Gene:3705781	AT4G02075	zinc finger (C3HC4-type RING finger) family protein, contains InterPro Entry IPR001841 Zn-finger, RING
Gene:2132147	AT4G02080	Arabidospis thaliana GTP binding protein, Sar1 homolog (ASAR1) mRNA, complete
Gene:2132172	AT4G02090	expressed protein,
Gene:2132182	AT4G02100	DNAJ heat shock N-terminal domain-containing protein, contains Pfam profiles PF00226: DnaJ domain, PF00515: TPR Domain

* Description information obtained through TAIR

Table 3. First leaf vascular pattern characters for various genotypes at 21 DAG

	Areoles	Secondary Veins	Tertiary Veins	Quaternary Veins	Branch Points	Leaf Width Maximum (mm)	Free Ending Veins	Marginal Venation Gaps	Vascular Islands
Wild type (36)	14.86 ± 0.9	8.42 ± 0.3	14.67 ± 1.0	1.60 ± 0.2	40.28 ± 2.0	6.59 ± 0.2	10.56 ± 0.6	0.00 ± 0.0	0.03
unh (41)	7.75 ± 0.4 ¥	7.20 ± 0.2 ¥	8.43 ± 0.5 ¥	0.50 ± 0.1 ¥	23.30 ± 1.0¥	5.19 ± 0.2 ¥	7.78 ± 0.6 ¥	0.90 ± 0.2 ¥	0.05
aux1-7 (31)	22.84 ± 1.3 ¥	9.65 ± 0.3 ¥	19.00 ± 1.4 ¥	0.87 ± 0.2 ¥	51.74 ± 2.9¥	4.52 ± 0.2 ¥	6.06 ± 0.6 ¥	0 ± 0.0	0
unh aux1-7 (37)	10.16 ± 0.4 *§	9.30 ± 0.3 *	9.97 ± 0.8 §	0.19 ± 0.1 *§	28.59 ± 1.1*§	4.84 ± 0.1	8.27 ± 0.6 §	0.68 ± 0.2 §	0
axr1-3 (32)	5.91 ± 0.4 ¥	5.75 ± 0.2 ¥	6.41 ± 0.5 ¥	0.75 ± 0.2 ¥	18.84 ± 0.9¥	5.23 ± 0.1 ¥	7.03 ± 0.5 ¥	0.84 ± 0.2 ¥	0.06
unh axr1-3 (33)	1.55 ± 0.3 *§	5.42 ± 0.2 *	4.93 ± 0.4 *§	0.36 ± 0.1	12.06 ± 0.8*§	5.09 ± 0.2	8.97 ± 0.5 §	3.79 ± 0.2 *§	0.42 *§
axr2-1 (40)	14.50 ± 1.1	7.88 ± 0.3	11.00 ± 1.1 ¥	0.63 ± 0.1 ¥	33.75 ± 2.4¥	4.12 ± 0.2 ¥	4.75 ± 0.4 ¥	0.03 ± 0.0	0.03
unh axr2 (58)	7.27 ± 0.3 §	7.43 ± 0.2	6.12 ± 0.6 *§	0.16 ± 0.1 *§	20.92 ± 1.0§	3.34 ± 0.2 *§	6.39 ± 0.5 §	0.55 ± 0.1 §	0
cvp2 (32)	8.84 ± 0.4 ¥	10.63 ± 0.3 ¥	24.03 ± 1.5 ¥	3.16 ± 0.5 ¥	46.78 ± 1.9¥	7.95 ± 0.2 ¥	29.09 ± 1.7 ¥	2.5 ± 0.2 ¥	2.81 ¥
unh cvp2 (31)	3.71 ± 0.5 *§	8.10 ± 0.2 *§	10.19 ± 0.6 *§	0.64 ± 0.2 §	21.45 ± 1.0*§	5.89 ± 0.1 *§	14.03 ± 0.9 *§	4.06 ± 0.4 *§	2.39 *
eir1 (33)	14.31 ± 0.8	8.33 ± 0.3	11.89 ± 0.8 ¥	0.50 ± 0.2 ¥	34.53 ± 1.7¥	5.29 ± 0.1 ¥	5.92 ± 0.5 ¥	0 ± 0.0	0
unh eir1(32)	6.81 ± 0.4 *§	7.13 ± 0.2 §	6.66 ± 0.5 *§	0.38 ± 0.1	20.31 ± 0.9*§	4.27 ± 0.1 *§	6.69 ± 0.6	1.38 ± 0.2 *§	0
fkd1 (29)	5.72 ± 0.2 ¥	9.00 ± 0.2	9.03 ± 0.5 ¥	0.21 ± 0.1 ¥	23.9 ± 0.7 ¥	5.90 ± 0.1 ¥	12.5 ± 0.6 ¥	2.41 ± 0.2 ¥	0.86 ¥
unh fkd1 (36)	2.47 ± 0.2 *§	8.11 ± 0.2 *§	2.47 ± 0.3 *§	0.03 ± 0.0 *	12.7 ± 0.5 *§	5.30 ± 0.2 §	7.77 ± 0.5 §	4.64 ± 0.2 *§	0.72 *
mp (27)	1.81 ± 0.5 ¥	1.81 ± 0.4 ¥	0.15 ± 0.1 ¥	0.00 ± 0.0 ¥	3.81 ± 0.9¥	0.67 ± 0.1 ¥	0.19 ± 0.1 ¥	0.07 ± 0.1	0
unh mp (37)	2.97 ± 0.4 *§	3.24 ± 0.3 *§	1.11 ± 0.2 *§	0.05 ± 0.0 *	7.27 ± 0.8*§	1.12 ± 0.1 *§	1.36 ± 0.3 *§	0.19 ± 0.1 *	0
pin1-1 (19)	23.00 ± 1.4 ¥	9.11 ± 0.4	23.63 ± 1.5 ¥	3.63 ± 0.9 ¥	59.68 ± 3.5 ¥	7.56 ± 0.3 ¥	13.68 ± 1.2 ¥	0 ± 0.0	0.05
unh pin1-1 (23)	18.96 ± 1.8 *	15.83 ± 4.4	21.30 ± 2.0 *	1.65 ± 0.4 *	53.35 ± 4.2 *	7.19 ± 0.3 *	15.43 ± 1.2 *	0 ± 0.0 *	0.09
rty (43)	6.56 ± 0.4 ¥	5.55 ± 0.3 ¥	2.49 ± 0.3 ¥	0 ± 0.0 ¥	14.40 ± 0.9¥	2.26 ± 0.1 ¥	1.27 ± 0.2 ¥	0.05 ± 0.0	0
unh rty (46)	5.35 ± 0.3 *§	4.72 ± 0.2 *§	1.20 ± 0.2 *§	0 ± 0.0	11.41 ± 0.6*§	1.49 ± 0.1 *§	0.73 ± 0.1 *§	0.15 ± 0.1 *	0

Values represent means ± SEM. Number in bracket represents number of plants scored.

*The double mutant is significantly different from *unh* ($p<0.05$).

§The double mutant is significantly different from its corresponding single mutant ($p<0.05$)

¥The single mutant is significantly different from wild type ($p<0.05$).

Table 4. Cotyledon vascular pattern characters for various genotypes

	Areoles		Veins		Free ending veins	
Wild type (34)	3.32 ±	0.1	3.77 ± 0.1		0.45 ± 0.1	
unh (53)	1.94 ±	0.2¥	3.91 ± 0.1	¥	1.96 ± 0.2	¥
mp (144)	0.40 ±	0.1¥	1.16 ± 0.1	¥	0.76 ± 0.1	¥
unh mp (70)	0.41 ±	0.1*	1.21 ± 0.2	*	0.8 ± 0.1	*

Values represent means ± SEM. Number in bracket represents number of plants scored.

*The double mutant is significantly different from *unh* ($p<0.05$).

§The double mutant is significantly different from its corresponding single mutant ($p<0.05$).

¥Significantly different from wild type ($p<0.05$).

Table 5. Appearance of leaf vein characteristics in wild type and *unh*¥

	Wild type		*unh*	
Areoles				
5 DAG	0.07 ± 0.07	(14)	0.07 ± 0.07	(15)
6 DAG	2.56 ± 0.38	(39)	0.77 ± 0.17	(30)*
7 DAG	4.47 ± 0.66	(34)	2.24 ± 0.37	(17)*
Free Ending Secondaries				
5 DAG	0.00 ± 0.00	(14)	0.13 ± 0.09	(15)
6 DAG	0.21 ± 0.08	(39)	0.67 ± 0.15	(30)*
7 DAG	0.12 ± 0.08	(34)	1.59 ± 0.26	(17)*
Differentiated Midvein				
5 DAG	0.54 ± 0.13	(14)	0.47 ± 0.13	(15)
6 DAG	0.92 ± 0.04	(39)	0.97 ± 0.03	(30)
7 DAG	1.00 ± 0.00	(34)	1.00 ± 0.00	(17)
Secondaries				
5 DAG	0.57 ± 0.17	(14)	0.40 ± 0.19	(15)
6 DAG	3.23 ± 0.35	(39)	1.87 ± 0.13	(30)*
7 DAG	4.09 ± 0.39	(34)	3.65 ± 0.28	(17)
Tertiaries				
5 DAG	0.00 ± 0.00	(14)	0.00 ± 0.00	(15)
6 DAG	0.51 ± 0.20	(39)	0.10 ± 0.06	(30)*
7 DAG	1.71 ± 0.59	(34)	0.29 ± 0.19	(17)*
Quaternaries				
5 DAG	0.00 ± 0.00	(14)	0.00 ± 0.00	(15)
6 DAG	0.00 ± 0.00	(39)	0.00 ± 0.00	(30)
7 DAG	0.21 ± 0.09	(34)	0.00 ± 0.00	(17)
Tertiaries and Quaternaries				
7 DAG	1.91 ± 0.70	(34)	0.29 ± 0.19	(17)*
Branch Points				
5 DAG	0.70 ± 0.29	(14)	0.40 ± 0.19	(15)
6 DAG	6.21 ± 0.87	(39)	2.80 ± 0.26	(30)*
7 DAG	10.06 ± 1.49	(34)	5.53 ± 0.66	(17)*

Values represent means ± SEM. Number in bracket represents number of leaves scored.

¥ Scoring based on xylem lignification

*Significantly different from wild type ($p<0.05$).

Table 6. *AtHB-8::GUS* expression patterns in *unh* and wild type

		Wild type		*unh*
Free ending expression among uppermost presumptive secondaries [§]				
	5 DAG	0.32±0.10	(41)	1.05 ± 0.16 (19)*
	7 DAG	0.04±0.02	(57)	0.93 ± 0.10 (46)*
Expression in presumptive tertiaries	5 DAG	0.02±0.02	(41)	0.00 ± 0.00 (19)
	7 DAG	3.77±0.40	(57)	0.80 ± 0.15 (46)*

Values represent means ± SEM. Number in bracket represents number of leaves scored.
[§]Only the first two arches of presumptive secondaries were considered. Leaves lacking these arches were not considered.
*Significantly different from wild type ($p<0.05$).

Table 7. Analysis of several developmental characteristics in *unh*.

	Internode length (mm) at 30 DAG	Days to bolting of 50% of plants	Leaves on primary shoot at bolting	Shoots at bolting
Wild type	14.4	15.0	6.6	1.0
unh	12.9*	18.0	8.00*	1.3

Values represent means. With the exception of the bolting traits, all data was collected on 30 DAG plants.
*Significantly different from wild type ($p<0.05$).

Table 8. Primary root growth of seedlings exposed to 2,4-D

	0 M	10-9 M	10-8 M	10-7 M
Wild type	29.28 ± 1.0 mm (25)	29.00 ± 0.9 mm (27)	28.65 ± 1.2 mm (26)	2.38 ± 0.10 mm *§ (26)
unh	18.72 ± 1.4 mm * (25)	20.65 ± 1.0 mm * (26)	17.33 ± 1.2 mm * (27)	1.73 ± 0.20 mm *§ (22)

Values represent means ± SEM. Number in bracket represents number of leaves scored.
*Significantly different from wild type ($p<0.05$).
§Significantly different from 0 M ($p<0.05$)

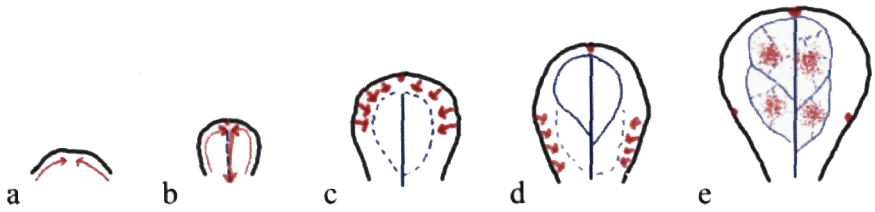

Figure 1. Young leaves switch from an auxin sink to an auxin source. In the early leaf (a), external auxin is transported into the leaf by polar auxin transport (red arrows). As auxin is transported out of the leaf, procambial tissue forms in the midvein region (b). The production of auxin at the margin (c, d) and within the lamina (e) results in the production of secondary and tertiary procambial tissue.

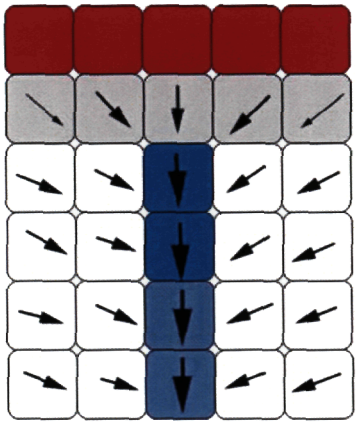

Figure 2. The auxin canalization model. According to the auxin canalization model, transport (arrows) of auxin from auxin producing cells (red) lead to preferential channels of auxin transport (blue). The increased conductivity and high auxin flux in these canalized cells (blue) not only lead to their vascular differentiation, but also deplete neighbouring cells (white) of auxin, preventing them from taking on a vascular cell fate. Taken from Mattson et al. (1999).

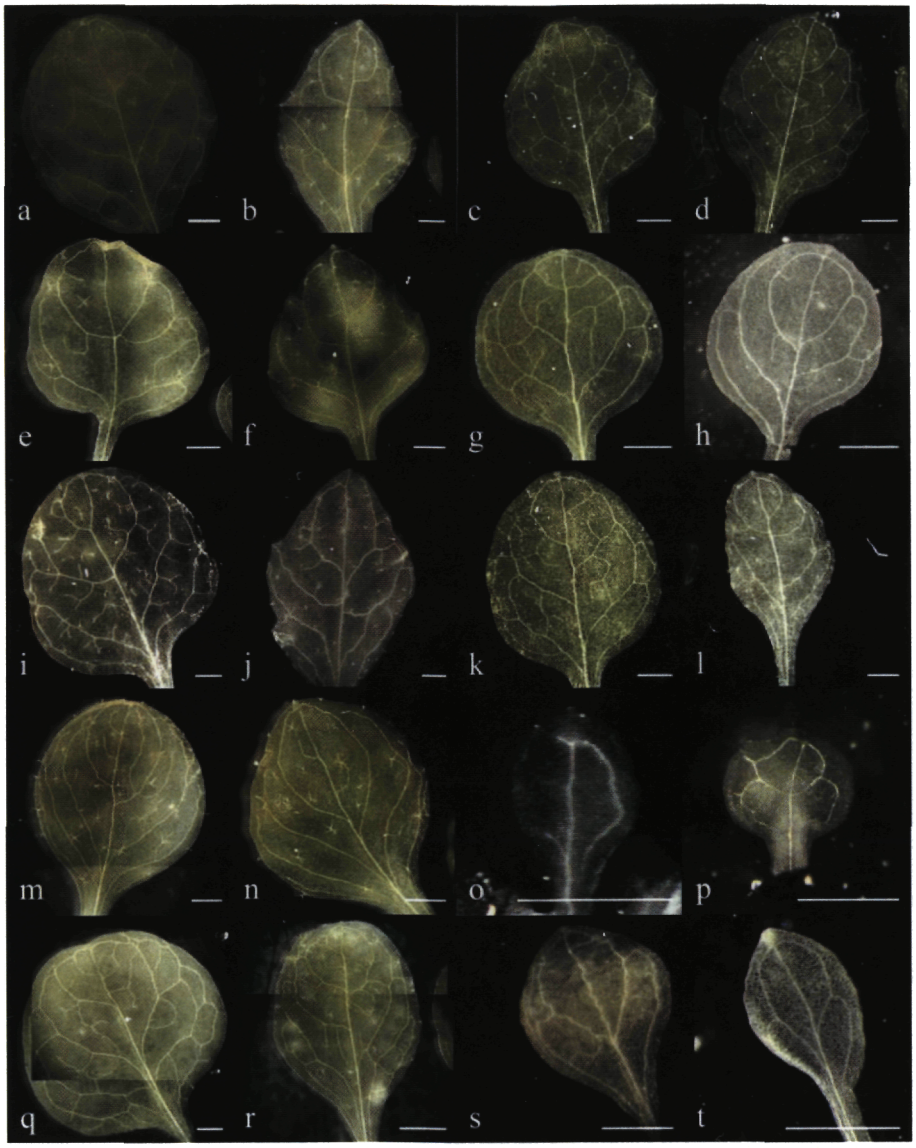

Figure 3. Vascular pattern of cleared 21 DAG first leaves from wild type (a), *unh* (b), *aux1* (c), *unh aux1* (d), *axr1-3* (e), *axr1-3 unh* (f), *axr2* (g), *axr2 unh* (h), *cvp2* (i), *cvp2 unh* (j), *eir1* (k), *eir1 unh* (l), *fkd1-1* (m), *fkd1-1 unh* (n), *mp* (o), *mp unh* (p), *pin1-1* (q), *pin1-1 unh* (r), *rty1* (s), *rty1 unh* (t). Viewed by translumination on a dissecting scope. Scale bar: 1 mm.

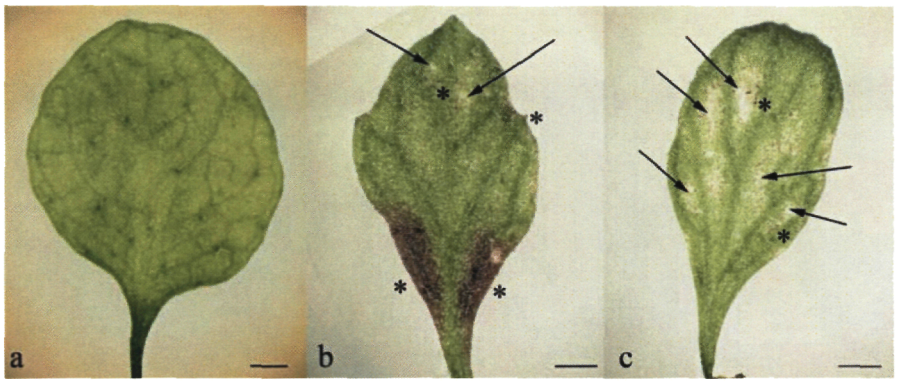

Figure 4. Senescence and anthocyanin accumulation in leaves of *unh*. The first leaf of wild type (a) and *unh* (b, c) at 21 DAG. Unlike the wild type leaf, *unh* often shows early leaf senescence (arrows) in the interveinal regions as well as regions of anthocyanin accumulation (*). Viewed by translumination on a dissecting scope. Scale bar: 1mm.

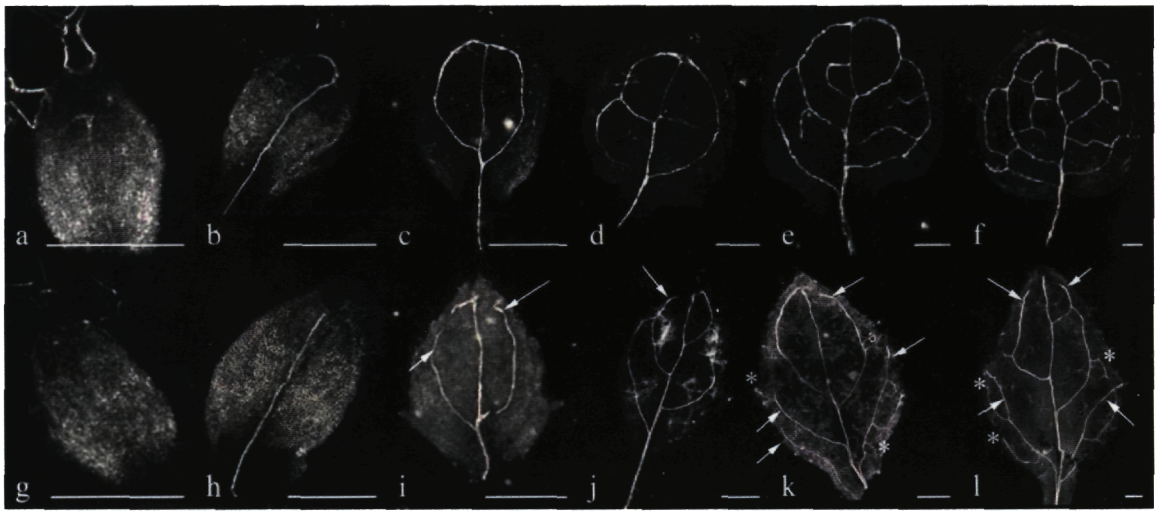

Figure 5. Vascular pattern development in the first leaf of wild type (a-f) and *unh* (g-l). The formation of the midvein in wild type (a, b) is indistinguishable from *unh* (g,h). The formation of the first two pairs of secondary veins initiate from the midvein in wild type, whereas one or both of these veins initiates freely in *unh* (arrow). This pattern continues in subsequent secondary veins. Tertiary veins begin to form around 8 DAG in wild type as well as *unh*, but fewer tertiaries form in *unh* and these are often freely ending (*). Viewed by dark field optics on a compound microscope. Scale bar: 250 μm.

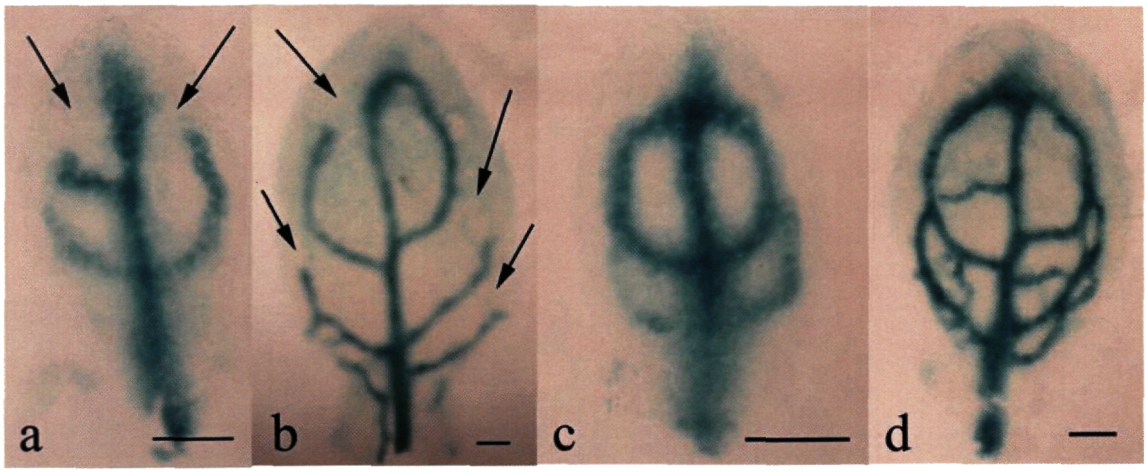

Figure 6. *AtHB-8::GUS* expression in *unh* (a, b) and wild type (c,d) leaves at 5 (a, c) and 7 (b, d) DAG. Unlike wild type, which shows contiguous loops of expression, *unh* shows an open, disconnected pattern with gaps (arrows) in *AtHB-8* expression. Leaves stained for 6 hours. Viewed by phase contrast on a compound microscope. Scale = 50 μm.

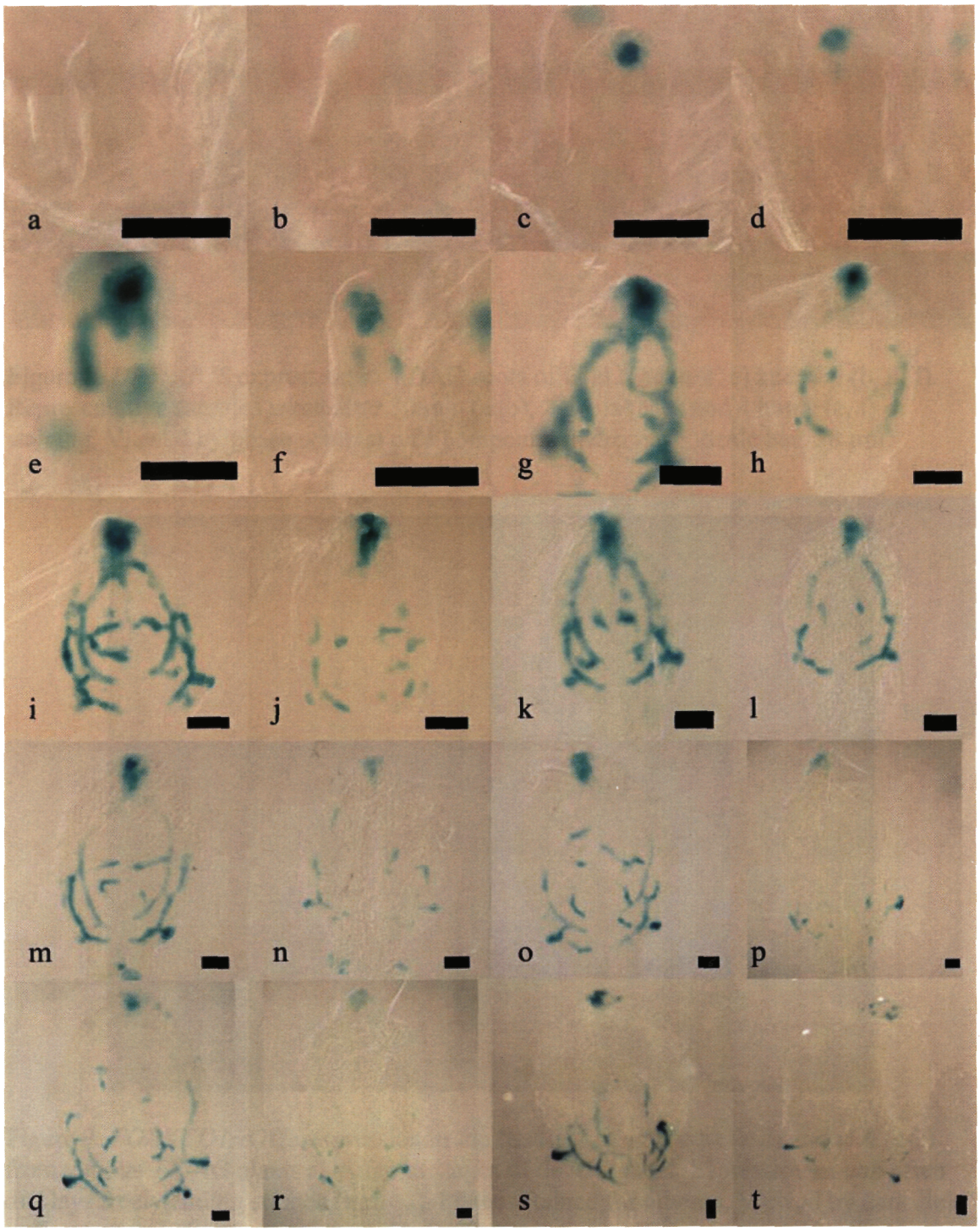

Figure 7. *DR5::GUS* expression in developing leaves of wild type (a, c, e, g, i, k m, o, q, s) and *unh* (b, d, f, h, j, l, n, p, r, t). Representative samples taken from 3 DAG (a-f), 4 DAG (g-j), 5 DAG (k-n), 6 DAG (o-r) and 7 DAG (s, t). Leaves stained for 12 hours. Note the decreased intensity of *DR5::GUS* expression in *unh* leaves. Viewed by phase contrast on a compound microscope (a-r) and translumination on a dissecting scope (s, t). Scale bar: 500 μm.

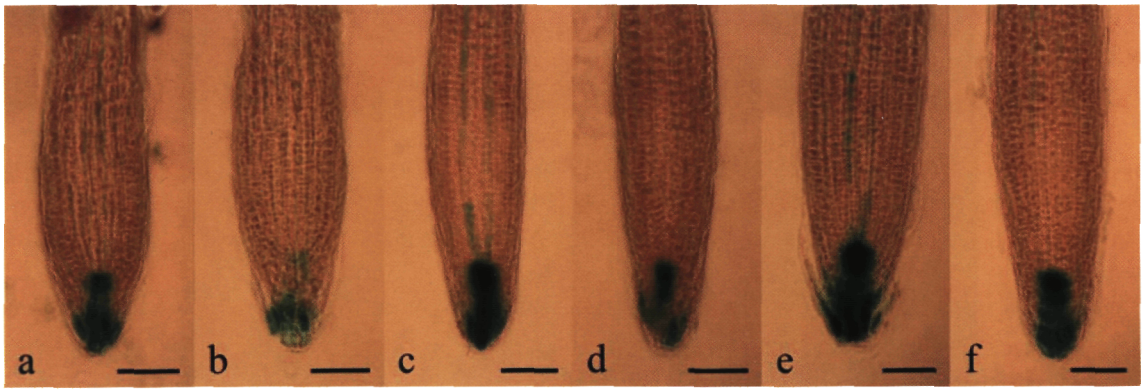

Figure 8. *DR5::GUS* expression in 4 DAG roots of wild type (a, c, e) and *unh* (b, d, f). Representative samples taken after 30 min (a, b), 2 hours (c, d) and 4 hours (e, f) of staining. Viewed by phase contrast on a compound microscope. Scale bar: 50 μm.

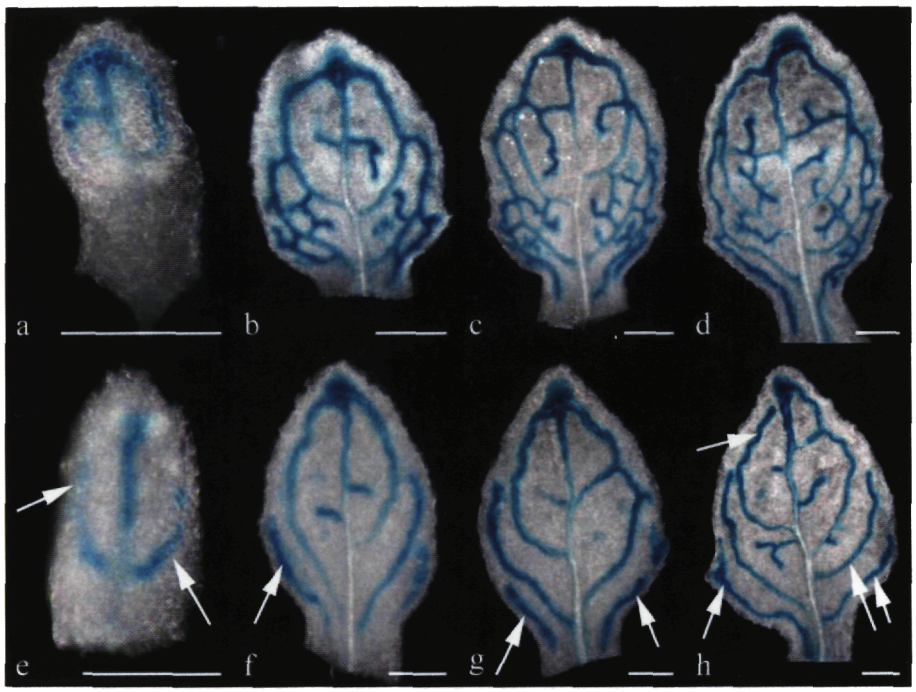

Figure 9. *FORKED1::GUS* expression in the first leaf of wild type (a-d) and *unh* (e-h) from various 7 DAG plants at different stages of development. Expression in *unh* often displays freely ending strands (arrows). Leaves stained for 6 hours. Viewed by dark field optics on a compound microscope. Scale = 100 μm.

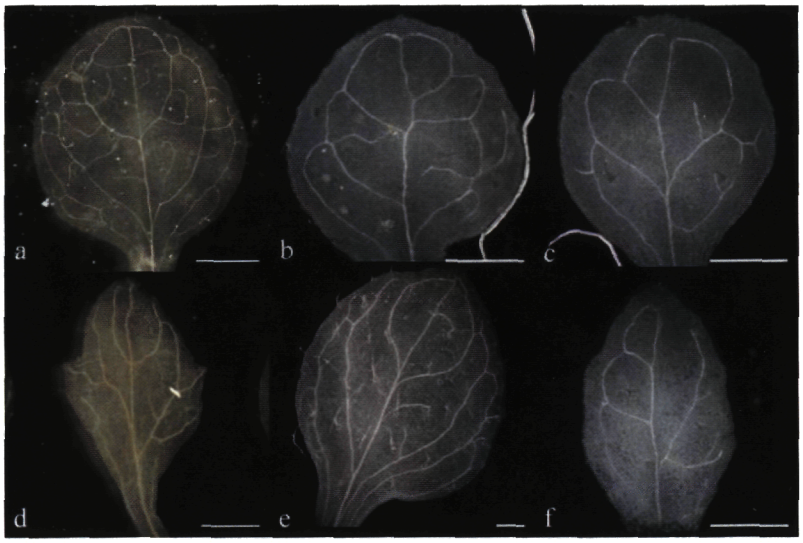

Figure 10. Vascular pattern of 10 DAG wild type (a, b, c) and *unh* (d, e, f) first leaves exposed to 0 M (a, d), $1x10^{-9}$ M (b, e) and $1x10^{-8}$ M (c, f) of 2,4-D. Viewed by translumination on a dissecting scope (a,d) and dark field on a compound microscope (b,c,e,f). Scale bar: 1 mm.

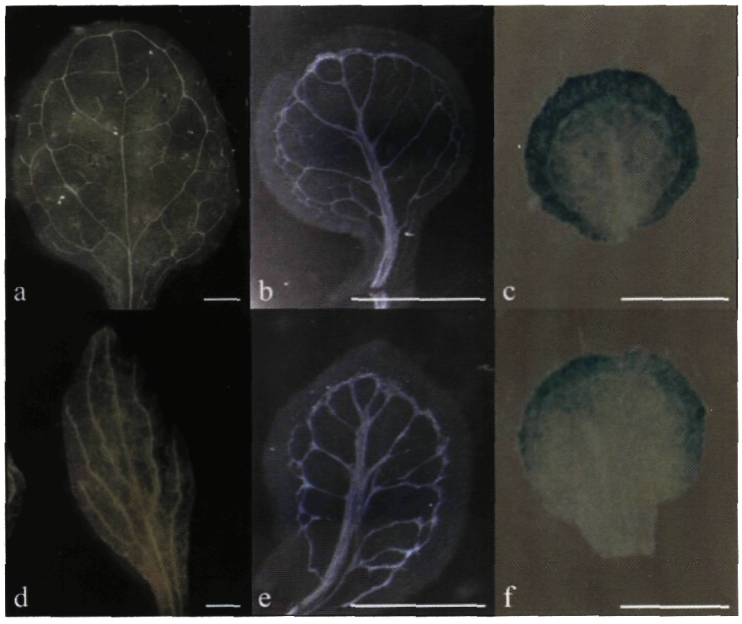

Figure 11. Vascular pattern and *DR5::GUS* expression of 10 DAG wild type (a-c) and *unh* (d-f) first leaves exposed to 0 M (a,d) and 30 µM NPA (b,c,e,f). *DR5::GUS* expression pattern (c, f) demonstrates auxin accumulation predominantly at the margin. Viewed by translumination on a dissecting scope (a, c, d, f) and dark field optics on a compound microscope (b,e). Scale bar = 500 µm.

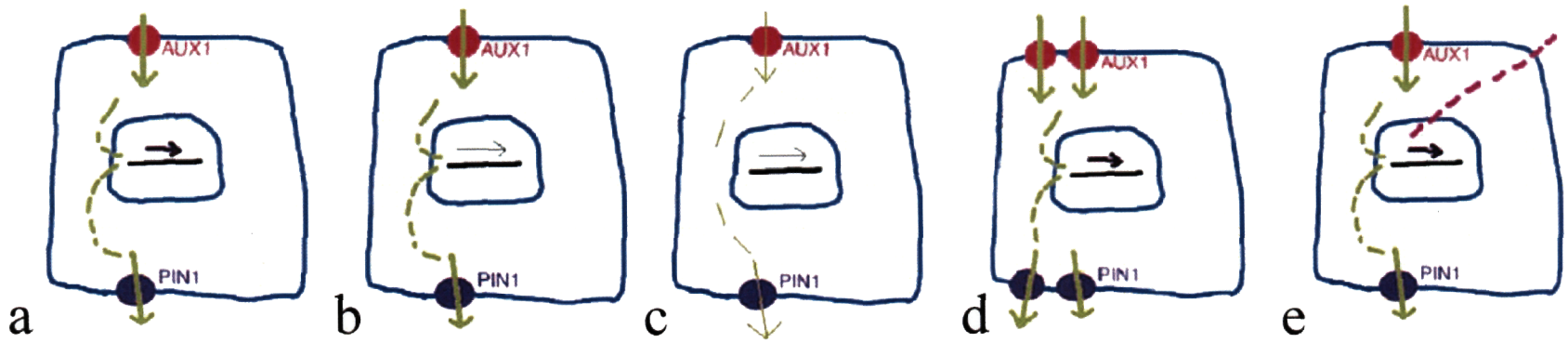

Figure 12. Four models for UNH activity. In wildtype cells (a) auxin (green) enters the cell through an influx carrier (red circle) such as AUX1, a transcription-based auxin response occurs (black arrow) and auxin is then removed from the cell through auxin efflux carriers (blue circle) such as PIN1.

1. UNH is a positive regulator of auxin response and directly associated with the TIR1 auxin response pathway, such that in the *unh* mutant (b), auxin response is directly repressed.
2. UNH is a positive regulator of auxin production, such that in the *unh* mutant (c) less auxin is produced.
3. UNH is a negative regulator of auxin transport, such that in the *unh* mutant (d) auxin is depleted from a larger number of neighbouring cells (not shown).
4. UNH is a positive regulator of auxin response and acts through the brassinosteroid pathway (e), such that in the *unh* mutant, brassinosteroid response is repressed and this leads to a decreased auxin response.