Vacuolar SNAREs Function in the Formation of the Leaf Vascular Network by Regulating Auxin Distribution

Makoto Shirakawa\(^1\,\,^2\), Haruko Ueda\(^1\,\,^2\), Tomoo Shimada\(^1\), Chiaki Nishiyama\(^1\) and Ikuko Hara-Nishimura\(^1\,\,^\ast\)

\(^1\)Department of Botany, Graduate School of Science, Kyoto University, Kyoto, 606-8502 Japan

In normal leaf development, a two-dimensional pattern of leaf veins is known to form by differentiation of vascular cells from ground meristem cells in a manner that is regulated by the polar flow of auxin. However, the mechanisms regulating the distribution of auxin in the leaf primordium are largely unknown. Here we show that vacuolar SNAREs (soluble N-ethylmaleimide-sensitive factor attachment protein receptors), VAM3 and VTI11, are required for the formation of the leaf vascular network in a dosage-dependent manner. This is the first report to show that the pre-vacuolar compartment (PVC)–vacuole traffic pathway is required for the formation of the leaf vascular network. \(vam3-4\), a VAM3-defective mutant, was found to have an immature vascular network. An analysis of the DR5 reporter in \(vam3-4\) indicated that VAM3 is involved in the proper pattern formation of auxin maxima in the leaf primordium. This suggests that the immature vascular network in \(vam3-4\) was mainly determined at the stage of procambium formation in the leaf primordium.

The abnormal distribution of auxin maxima was caused by the non-polarized localization of the auxin efflux carrier PIN1 (PIN-FORMED 1) in leaf primordium cells. VAM3 is the first key protein which is required for the proper localization of PIN1 in leaf cells. Finally, we found that PIN1 proteins were constitutively transported to vacuoles in leaf and roots cells. Our findings demonstrate that the PVC–vacuole pathway is required for the formation of vacuolar SNAREs Function in the Formation of the Leaf Vascular Network by Regulating Auxin Distribution

**Keywords:** *Arabidopsis thaliana* • Auxin • PIN1 • SNARE • VAM3 • Vascular network.

**Abbreviations:** DAG, days after germination; GFP, green fluorescent protein; GUS, β-glucuronidase; mRFP, monomeric red fluorescent protein; PIN1, PIN-FORMED 1; PVC, pre-vacuolar compartment; RT–PCR, reverse transcription–PCR; SNARE, soluble N-ethylmaleimide-sensitive factor attachment protein receptor; TGN, trans-Golgi network; VAM, vacuolar morphology; VPS, vacuolar protein sorting; VTI, VPS10 interacting.

**Introduction**

Spatial and temporal pattern formation requires the generation of complex organizations of cell fate in animals and plants. The two-dimensional vascular network of leaves is an ideal model of pattern formation in plants. The molecular mechanism underlying vascular network formation in leaf primordia is poorly understood. Leaf vein patterns form by differentiation of the procambium, which is the precursor of vascular cells, from the ground meristem in a regulated manner through the polar flow of auxin (Scarpella et al. 2006). Many studies have shown that auxin flow is controlled by the polarized localization of auxin efflux carriers, called PIN (PIN-FORMED) proteins (Petrasek et al. 2006). Little is known about the process in leaves, although much is known about the molecular mechanism underlying the polarized localization of PIN proteins in roots. No mutants with a defect in the localization of PIN proteins in leaves have been reported.

In the root cells of *Arabidopsis thaliana*, the polarized localization of PIN1 and PIN2 to the plasma membrane is mediated by membrane trafficking machinery, including GNM (Geldner et al. 2003), VAM3/SCAREFACE (Koizumi et al. 2005, Sieburth et al. 2006), SNX1 (Jaillais et al. 2006) and VPS29 (Jaillais et al. 2007). These factors might be responsible for the recycling of PIN proteins between the plasma membrane and endosomes. Although gnom and van3/sfc mutants have defects in leaf vein patterning, the localization
of PIN proteins in leaves of these mutants is not known. Precellular compartments (PVCs) and vacuoles have recently been reported to function in the regulation of PIN proteins (Kleine-Vehn et al. 2008, Laxmi et al. 2008, Spitzer et al. 2009).

Previously, we reported that the patterning of myrosin cells is controlled by VAM3, which is a key component of the vacuolar SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor), which is composed of VAM3 (Qa-SNARE), VTI11 (Qb-SNARE), SY5 (Qc-SNARE) and VAMP727 (R-SNARE) (Sanderfoot et al. 2001, Yano et al. 2003, Ohtomo et al. 2005, Jahn and Scheller 2006, Ueda et al. 2006, Ebine et al. 2008). VAM3 is reported to localize to both PVCs and the vacuolar membrane (Sato et al. 1997, Sanderfoot et al. 1999). This raises the question of whether the membrane traffic that occurs via the vacuolar SNARE complex is involved in the formation of the leaf vascular network.

Here we show that the vacuolar SNARE complex is required for the formation of the leaf vascular network in a dosage-dependent manner and that the immature vein network in vam3-4 is mainly determined at the stage of procambium formation by the poor distribution of auxin maxima. In addition, we show that VAM3 is required for the localization of PIN1 in leaf cells and that PIN1 is constitutively transported to vacuoles in leaf cells. Our findings demonstrate that vacuolar SNAREs are required for the polarized localization of PIN1, which may be essential for the formation of vascular network in leaves.

**Results**

**VAM3 is involved in the formation of the vascular network in leaves**

We previously reported that vam3-4 had wavy leaves with a large number of myrosin cells (Ohtomo et al. 2005, Ueda et al. 2006). As myrosin cells develop along the vein in the wild type, we focused on the leaf vein patterning in vam3-4. We found that a deficiency of VAM3 abolished development of the third and fourth veins in juvenile leaves (the first and second true leaves) (Fig. 1A, L). The leaf vein patterning becomes increasingly complex during the leaf developmental stages of leaves. We analyzed the leaf vein network from the first true leaf to the 12th true leaf. All leaf vein networks of vam3-4 at DAG30 (30 d after germination) were less mature than those of the wild type (Fig. 1A–V). The complexity of the vein pattern in a leaf can be expressed by the number of vein junctions. The numbers of vein junctions in vam3-4 remained lower than those in the wild type throughout the developmental stages (Fig. 1W). These results showed that VAM3 is involved in the formation of the vascular network in leaves.

**The vacuolar SNARE complex is required for the formation of the vascular network in leaves**

To answer the question of whether VAM3 actually functions in the development of vascular cells in the vacuolar SNARE complex, we established single and double mutants of VAM3 and VTI11 (Qb-SNARE), a protein that interacts with VAM3 (Sanderfoot et al. 2001, Yano et al. 2003, Ebine et al. 2008), by using vam3-4 and the weaker allele vam3-3. Neither vam3-3 nor vti11 exhibited defects in leaf vein patterning (Fig. 2B, F, upper panels), while vam3-4 and vam3-3 vti11/+ had immature third and fourth order leaf veins (Fig. 2C, D, upper panels). vam3-4 vti11/+ exhibited a more severe vascular immaturity: the first and second order veins were unattached and neither the third nor fourth order veins developed (Fig. 2E, upper panels). The most severe phenotypes among the mutants were found in vam3-3 vti11: the primary veins were disconnected, the secondary veins were not attached caudally to the primary veins and the higher order veins had disappeared (Fig. 2G, upper panels). The homozygous vam3-4 vti11 was not obtained, possibly because the mutation is embryonically lethal (Supplementary Table S1). The degree of immaturity of the veins of the mutants paralleled the degree of dwarfism (Fig. 2A–G, lower panels; 2H–J). Our findings of genetic interactions showed that the vacuolar SNARE complex containing VAM3 and VTI11 regulates the development of vascular cells in a dosage-dependent manner.

**VAM3 deficiency causes abnormal distribution of auxin in leaf primordia**

The formation of auxin maxima in the leaf primordium is important for the formation of the vascular network and the differentiation of the procambium (Scarpella et al. 2006). To see to what degree the plant hormone auxin is involved in the development of vascular cells of vam3-4, we expressed each of two reporter genes driven by the promoter DR5, which is responsive to auxin maxima (Ulmasov et al. 1997, Benkova et al. 2003), in vam3-4 and investigated the distribution of auxin in juvenile leaves at DAG5 when the ground meristem was determined to differentiate the procambium. Expression of the reporter β-glucuronidase (GUS) was weaker in vam3-4 than in the wild type (Fig. 3A, B). Similarly, reporter green fluorescent protein (GFP) expression showed that wild-type leaves, except in the apex and margin regions, exhibited a GFP network pattern that would become leaf veins in the future (Fig. 3C, lower panel), while vam3-4 leaves exhibited a much simpler GFP network than did the wild-type leaves (Fig. 3D, lower panel). These results indicate that the simpler leaf vein pattern in vam3-4 is mainly determined at the stage of procambium formation, and that the VAM3 deficiency caused the poor generation of auxin maxima.
Fig. 1 VAM3 is involved in the formation of the vascular network in leaves. (A–V) True leaves (the first true leaf to the 12th true leaf) were harvested from wild-type and vam3-4 plants at DAG30. Compared with the leaf vein development of the wild type (A–K), vam3-4 had a defect in development of the higher order veins throughout the stages (L–V). (W) The numbers of vein junctions in a pair of juvenile leaves (leaf 1 and leaf 2) and in each rosette leaf (leaf 4 to leaf 12) of wild-type and vam3-4 plants (A–V) were counted. vam3-4 exhibited fewer vein junctions at any leaf stages than the wild type. Bars = 1 mm.
The finding that the auxin levels in the tissues were not affected by the VAM3 deficiency (Fig. 3E) indicates that VAM3 is required for the proper formation of the auxin maxima, which determine where procambium cells are located in the leaf primordium. The abnormal distribution of auxin reflected the reduction of the gene expression of two auxin-responsive family proteins and three IAA-induced proteins (IAA6, IAA5/AUX2-27 and IAA2) in the young leaves of vam3-4 (Fig. 3F). These results indicated that VAM3 regulates auxin-responsive gene expression by controlling the distribution of auxin in the leaf primordium.

VAM3 is required for the proper expression pattern of PIN1

The auxin maximum and the auxin gradients are formed by a positive feedback loop between auxin and the auxin efflux carrier PIN1 during leaf vein pattern formation (Scarpella et al. 2006, Wenzel et al. 2007). We hypothesized that VAM3 controls the distribution of auxin by regulating the localization of PIN1, which determines where procambium cells are located in the leaf primordium. To visualize the distribution of PIN1, we expressed pPIN1:PIN1-GFP in vam3-4. The PIN1–GFP network pattern in the inner plane of the juvenile leaves of the wild type at DAG5 was similar to the expected future vein pattern (Fig. 4A). On the other hand, vam3-4 exhibited a simpler PIN1–GFP network pattern in the inner plane than did the wild type (Fig. 4B). The simpler PIN1–GFP network pattern in vam3-4 is very similar to the simpler pattern of both DR5:GUS and DR5:GFP in vam3-4. The simpler pattern of PIN1–GFP was supported by the finding that the level of endogenous PIN1 expression in vam3-4 was slightly lower than in the wild type (Supplementary Fig. S1). These results indicated that VAM3 is involved in the proper expression pattern of PIN1 in the leaf primordium and that the abnormal expression pattern of PIN1 caused the abnormal auxin distribution.

VAM3 is involved in the polarized localization of PIN1 in leaf cells

The distribution of auxin has been shown to be regulated by the polarized localization of PIN1 on the plasma membrane in root cells (Benkova et al. 2003). However, no mutants with...
a defect in PIN1 distribution have been reported in leaf cells. 
In the wild type we observed abaxial epidermal cells (n = 92) 
in juvenile leaves at DAG3 and procambium cells (future secondary 
veins, n = 31) in juvenile leaves at DAG3.5. A polarized localization of PIN1–GFP fluorescence on the plasma 
membrane was detected in 85.9% of abaxial epidermal cells 
observed (Fig. 5A, B) and in 96.8% of procambium cells 
observed (Fig. 5E, F). We also observed abaxial epidermal 
cells (n = 106) in juvenile leaves at DAG3 and procambium 
cells (future secondary veins, n = 21) in juvenile leaves at 
DAG3.5 of vam3-4. Interestingly, in vam3-4, non-polarized localization of PIN1–GFP fluorescence on the plasma membrane was detected in 84.0% of abaxial epidermal cells 
observed (Fig. 5C, D) and in 90.5% of procambium cells 
observed (Fig. 5G, H). These results indicate that VAM3 is required for the polarized localization of PIN1 to the plasma membrane in leaf primordia.

In contrast, the expression of PIN1–GFP in roots revealed that it was localized on the basal side of the plasma membrane in both vam3-4 and the wild type (Supplementary Fig. S2A–D). PIN2–GFP also exhibited a similar polarized localization on the apical side of the plasma membrane in the root epidermis of vam3-4 and the wild type (Supplementary Fig. S2E–H). GFP–PIP2 also exhibited non-polarized localization at the plasma membrane in the root of vam3-4 and the wild type (Supplementary Fig. S2I–L). These results indicate that VAM3 is not required in roots for the polarized localization of PIN1 and PIN2, and the non-polarized localization of PIP2.

PIN1 is constitutively transported to vacuoles, which is labeled with VAM3

The subcellular localization of PIN1–GFP in young leaves was explored by focusing on endomembranes including vacuoles in a transgenic plant that co-expressed PIN1–GFP and mRFP–VAM3 (monomeric red fluorescent protein, a marker of vacuolar membranes), both of which are reported to be functional in planta (Benkova et al. 2003, Ebine et al. 2008). PIN1–GFP showed a polarized localization on the plasma membrane in primordia of juvenile leaves at DAG4 (Fig. 6A–D). We previously reported that GFP undergoes conformational changes in the presence of blue light and acidic
pH and that these changes increase the sensitivity of GFP to vacuolar proteinases (Tamura et al. 2003). To avoid the light-dependent degradation of GFP, we transferred the plants from the light into the dark at 12 h before inspection with a fluorescent microscope. Unexpectedly, specifically under the dark condition, GFP fluorescence was detected in vacuoles, all of which were surrounded by mRFP–VAM3, in addition to the polarized localization on the plasma membrane as under the light condition (Fig. 6E–I). Similar results were obtained in roots (Supplementary Fig. S3A–G). These results suggest that PIN1 proteins are constitutively transported to vacuoles in the leaf and root cells. Our findings suggest that VAM3 is involved in the polarized localization of PIN1 in leaf cells, apparently by constitutively transporting PIN1 into vacuoles for degradation. One possibility is that PIN1 is transported to vacuoles efficiently from the side opposite to where PIN1 is localized, resulting in the polarized localization of PIN1 in leaf cells.

PVC–vacuole pathway is involved in vascular network formation in leaves

To test our hypothesis that the PVC–vacuole pathway is required for the formation of the leaf vein pattern, we searched for mutants that had defects in membrane traffic and vascular network formation. We found that the vti11 vti12/+ and vps9a-2 mutants had defects in the formation of the leaf vein pattern (Fig. 7). The immaturity of the leaf vein pattern in the vti11 vti12/+ and vps9a-2 mutants was very similar to that in vam3-4 (compare Fig. 7 with Fig. 2D). VPS9A is the homolog of yeast VPS9 which is involved in vacuolar sorting, and it was reported that the vti11 vti12/+ mutant had defects in the sorting of seed storage protein (Goh et al. 2007, Sanmartin et al. 2007). This result showed that the PVC–vacuole pathway is involved in the formation of the leaf vein pattern.

**Discussion**

**Vacuolar SNAREs are required for formation of the leaf vascular network**

Our results showed that the vacuolar SNARE complex is required for the formation of the leaf vascular network in a dosage-dependent manner and the immature vein network in vam3-4 is mainly determined at the stage of procambium formation by the poor distribution of auxin maxima. This is the first report to show that the PVC–vacuole pathway is required for the formation of the leaf vascular network. It is
reported that VAN3/SFC, CVP1 and VAB1, which localize to the trans-Golgi network (TGN), are essential for the vascular tissue continuity and that GNOM, which localizes to recycling endosomes, is required for leaf vascular formation (Geldner et al. 2004, Naramoto et al. 2009). As the leaf vascular phenotypes of these mutants are different from those of both vam3 and vam3 vti11 mutants, the role of the PVC–vacuole trafficking pathway in the formation of the leaf vascular network might be different from the roles of the TGN and recycling endosomes.

Previously, we reported that VAM3 is involved in the patterning of myrosin cells in leaves of *A. thaliana* (Ueda et al. 2006). Myrosin cells accumulate large amounts of myrosinase in their vacuoles and function as a plant defense system called a myrosinase–glucosinolate system. Myrosinase hydrolyzes glucosinolates to produce toxic compounds, such as isothiocyanates, against pathogens and plant-eating insects (Bones and Rossiter 2006). Interestingly, myrosin cells are distributed along the leaf vascular network on the abaxial side of the leaves (Husebye et al. 2002, Ueda et al. 2006). In contrast to the immaturity of the leaf vascular network in *vam3-4*, myrosin cells are increased in *vam3-4* and the increased myrosin cells in *vam3-4* form the network-like structures. It is possible that there is some developmental interaction between the formation of the leaf vascular network and the development of myrosin cells in leaves.

Vacuolar SNAREs are required for the polarized localization of PIN1

Our findings suggest that VAM3 is involved in the polarized localization of the auxin efflux carrier PIN1 in leaf cells. In *Arabidopsis* root, three factors (GNOM, VAN3 and VPS29) participate in the localization of PIN1 (Geldner et al. 2003, Sieburth et al. 2006, Jaillais et al. 2007). In leaves, PIN1 localization is also polarized but the factors involved in this polarization are unknown. This is the first report to show a defect in the localization of PIN1 in leaf cells. However, VAM3 was not required for the proper localization of PIN1 in roots. This might be due to the redundant functions of two VAM3 homologs of *A. thaliana* (AtSYP21 and AtSYP23) that are more highly expressed in roots than in leaves. To understand the different regulation of PIN1 in roots and in leaves, it will be necessary to characterize additional mutants that have defects in the localization of PIN1 in leaves. Additionally, we found that vps9a-2 and vti11 vti12/+ had a defect in the formation of the leaf vascular network. The polarized localization of PIN1 in the embryo and roots of *Arabidopsis* was recently found to require VPS9a (Dhonukshe et al. 2008). These results suggest that the PVC–vacuole pathway is involved in the polarized localization of PIN1.

Why does the polar localization of PIN1 require the vacuolar SNAREs? One possibility is that PIN1 is transported to vacuoles efficiently from a side other than the side where PIN1 is localized in a polarized manner, resulting in the formation of the polarized localization of PIN1 in leaf cells. Because vacuoles in vegetative tissues function in the degradation of proteins, transport of PIN1 into the vacuoles may be required for the degradation of PIN1. To examine this possibility, a quantitative analysis of PIN1–GFP accumulation by using a chase experiment is required. However, there is a significant experimental problem: GFP signals in vacuoles are quickly lost by irradiation during observation, as reported previously (Tamura et al. 2003). Further analysis with stable fluorescent proteins under light and acidic conditions will be necessary to understand these phenomena.
demonstrate a VAM3-dependent transport of PIN1 into the vacuoles.

Materials and Methods

Plant materials

Arabidopsis thaliana ecotype Col-0 was used as the wild-type plant. We used the following Arabidopsis mutants and transgenic lines: vam3-3 and vam3-4 that we isolated previously (Ohtomo et al. 2005, Ueda et al. 2006), and vti11 (Kato et al. 2002), vti11 vt112/+ (Surpin et al. 2003), vps9a-2 (Goh et al. 2007), DR5rev:GFP (Benkova et al. 2003), p35S:GFP-PIP2 (Cutler et al. 2000), pPIN1:PIN1-GFP (Benkova et al. 2003), pPIN2:PIN2-GFP (Xu et al. 2006), pDR5:GUS (Ulmasov et al. 1997) and pVAM3:mRFP-VAM3 (Ebine et al., 2008) that were kind gifts. Seeds were surface-sterilized with 70% ethanol and then sown onto 0.5% (w/v) Gellan Gum (Wako, Tokyo, Japan) that contained 1% (w/v) sucrose and Murashige–Skoog medium (Wako). The seeds were incubated at 4°C for 3–5 d to break seed dormancy, and were grown at 22°C for 20 d under continuous light (100 mE s⁻¹ m⁻²). The plants were transferred onto vermiculite for subsequent growth.

Generation of double mutants

We crossed a homozygote for vti11 with a homozygote for either vam3-3 or vam3-4. Double mutants were isolated by genotyping F₂ progeny. Genotyping for vti11 was performed by PCR using the primers: a5RACEGSP3, ZTAILF and MPO12. P7. The primer pair ZTAILF and MPO12.P7 was used to confirm the deletion of VTI11, and the primer pair ZTAILF and a5RACEGSP3 was used to confirm the VTI11 gene. Primers used are given in Supplementary Table S2. Genotyping for vam3-3 or vam3-4 was done by immunoblotting using an anti-VAM3 antibody (Sato et al. 1997).

Transgenic plants

We introduced the binary vector containing mRFP–VAM3 into the PIN1–GFP transgenic plant, and T₁ seeds were selected in a medium containing 25–50 mg l⁻¹ hygromycin B; three independent T₁ lines were then analyzed. We also generated the following transgenic lines by crossing pPIN1:PIN1-GFP in vam3-4, pDRS:GUS in vam3-4, pDRS_vam3-4:GFP in vam3-4, p35S:GFP-PIP2 in vam3-4 and pPIN2:PIN2-GFP in vam3-4. We analyzed both F₁ and F₂ generations of these lines.

Analysis of vein pattern

Juvenile leaves were harvested from DAG15 plants of single (vam3-3, vam3-4 and vti11) and double (vam3-3 vti11+/+ and vam3-4 vti11+/+) mutants, and from the DAG23 plant of vam3-3 vti11. The leaves were made transparent overnight in a chloral hydrate solution [chloral hydrate:water:glycerol, 8:2:1 (by vol.)], and dehydrated in 60% glycerol overnight. The leaves were mounted on a slide glass and were inspected under dark-field illumination with a microscope (model MVX10; Olympus, Tokyo, Japan).

Confocal laser scanning microscopy

The fluorescent images were inspected with a confocal laser scanning microscope (LSM510 META, Carl Zeiss, Jena, Germany) using a 488 nm line of a 40 mW Ar/Kr laser. We used a water immersion objective (×63, 1.20 NA), and dry objectives (×40, 0.95 NA; ×20, 0.80 NA; ×0, 0.50 NA). The 505–530 band-pass filter was used for GFP, while the 505–550 band-pass filter was used for Venus. The images were analyzed using an LSM image-examiner software (Carl Zeiss). The data was exported as 12-bit TIFF files and processed using Adobe Photoshop Elements 4.0 (Adobe Systems, Tokyo, Japan). The signal intensity was analyzed using the software ImageJ.

RT–PCR analysis

We removed both cotyledons and roots from DAG5 plants of the wild type and vam3-4 to collect differentiating tissues. Total RNA was isolated from the tissues using a kit (RNaseasy plant mini kit, QIAGEN, Valencia, CA, USA). Reverse transcription was performed using SuperScript First-Strand Synthesis System for RT–PCR (Invitrogen, Carlsbad, CA, USA) with the oligo(dT)₁₂₋₁₈ primer (Invitrogen). We performed PCR of 25, 30 and 35 cycles with each primer set. Primer sets used are given in Supplementary Table S2.

Supplementary data

Supplementary data are available at PCP online.

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References


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