

# DRP1A Is Responsible for Vascular Continuity Synergistically Working with VAN3 in Arabidopsis<sup>1</sup>

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In most dicotyledonous plants, vascular tissues in the leaf have a reticulate venation pattern. We have isolated and characterized an Arabidopsis (*Arabidopsis thaliana*) mutant defective in the vascular network defective mutant, *van3*. *van3* mutants show a discontinuous vascular pattern, and VAN3 is known to encode an ADP-ribosylation-factor-GTPase-activating protein that regulates membrane trafficking in the trans-Golgi network. To elucidate the molecular nature controlling the vein patterning process through membrane trafficking, we searched VAN3-interacting proteins using a yeast (*Saccharomyces cerevisiae*) two hybrid system. As a result, we isolated the plant Dynamin-Related Protein 1A (DRP1A) as a VAN3 interacting protein. The spatial and temporal expression patterns of DRP1A::GUS and VAN3::GUS were very similar. The subcellular localization of VAN3 completely overlapped to that of DRP1A. *drp1a* showed a disconnected vascular network, and the *drp1a* mutation enhanced the phenotype of vascular discontinuity of the *van3* mutant in the *drp1a van3* double mutant. Furthermore, the *drp1* mutation enhanced the discontinuous vascular pattern of the *gnom* mutant, which had the same effect as that of the *van3* mutation. These results indicate that DRP1 modulates the VAN3 function in vesicle budding from the trans-Golgi network, which regulates vascular formation in Arabidopsis.

Plant vascular tissue forms a continuous system throughout the plant body and provides transport pathways for water, dissolved materials, and signaling molecules. Within the leaves of vascular plants, the vascular system is constructed in a complex network pattern called venation, which shows a rich variety among different groups of plants. However, it is thought that a common basic mechanism underlies this spatial arrangement. The formation of this vein pattern has been widely studied as a paradigm of tissue pattern formation in plants. Especially in the model plant Arabidopsis (*Arabidopsis thaliana*), several mutants have been characterized with the view of studying vein pattern formation. *vascular network defective1* to 6 (*van1–6*; Koizumi et al., 2000), *scarface* (Deyholos et al., 2000), and *cotyledon vein pattern 1* and 2 (*cvp1, cvp2*; Carland et al., 1999) have discontinuous secondary vascular strands in their cotyledons and leaves. Recently, the causal gene for one of these

mutants, *cvp1*, was identified. *CVP1* encodes STEROL METHYLTRANSFERASE 2 (SMT2), an enzyme in the sterol biosynthetic pathway (Carland et al., 2002). However, the molecular function of this enzyme in the generation of venation is unknown. A mutation of the *SMT1* gene, which encodes another sterol methyltransferase, disturbed the membrane location of PIN1 and PIN3. This implies a role for sterol biosynthesis in polar auxin flow (Willemsen et al., 2003). The *smt1* mutant also has a defect in the formation of continuous vascular networks in leaves. However, unlike the *smt2* mutant, it shows an obvious abnormality in the overall architecture of its vascular pattern. The difference in the venation phenotypes of these *smt* mutants requires further analysis to clarify the relationship between sterol biosynthesis and vein pattern formation.

In this situation, a large number of studies indicate that polar auxin transport plays a crucial role in continuous vascular pattern formation (Nelson and Dengler, 1997; Berleth et al., 2000; Sachs, 2000; Aloni, 2001; Dengler, 2001; Ye, 2002; Turner and Sieburth, 2002). Indeed, the prevention of polar auxin transport by specific inhibitors causes the formation of local aggregates of vascular cells in the margin of newly developed leaves (Mattsson et al., 1999; Sieburth, 1999). Sachs (1991) proposed the auxin signal flow canalization hypothesis, which suggests that polar auxin flow induces continuous vascular formation. The mutation in the *GNOM* gene that encodes a brefeldin A sensitive ADP-ribosylation factor guanine-nucleotide exchange factor (ARF-GEF) responsible for targeted PIN1 recycling (Shevell et al., 1994; Busch et al., 1996; Steinmann

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et al., 1999; Geldner et al., 2003) causes the formation of tracheary elements arranged in clusters or as single cells instead of a formation lined up to form continuous strands (Mayer et al., 1991, 1993; Koizumi et al., 2000). These results support the role of the vesicle trafficking system, which includes PIN1 and GNOM as being responsible for vascular formation. Recently, the *VAN3* gene, whose mutation induces fragmented venation, has been shown to encode a unique type of ARF-GTPase-activating protein (GAP), in which the *VAN3* protein is shown to locate in the trans-Golgi network (TGN; Koizumi et al., 2005). A phenotypic analysis of the *van3* mutant suggests that the *VAN3* ARF-GAP may play an important role in the vesicle transport responsible for the auxin signaling that is required for vascular differentiation (Koizumi et al., 2000, 2005).

To examine the detailed molecular mechanisms of *VAN3* function, especially in vesicle trafficking, we isolated the *VAN3* binding protein using the yeast (*Saccharomyces cerevisiae*) two hybrid system. Here, we showed the association between *VAN3* and a dynamin-like protein, Dynamin-Related Protein 1A (DRP1A). Based on results from genetic, histochemical, and cell biological analyses, we propose that DRP1A is responsible for the vascular formation synergistically working with the *VAN3* protein.

## RESULTS

### DRP1A Associates with *VAN3*

To isolate *VAN3* interacting proteins, we performed a yeast two hybrid screening with *VAN3* as bait. From the screening of an Arabidopsis cDNA library, we isolated DRP1A as a *VAN3* binding protein. DRP1A is a known member of the dynamin family that constitutes a structurally related, but functionally diverse, family of large GTP binding proteins that share a conserved N-terminal GTPase domain and a C-terminal GTPase effector domain (Danino and Hinshaw, 2001). To confirm the association between DRP1A and *VAN3*, a swapping test was conducted. *VAN3* was fused with the DNA activation domain in pACT2, and DRP1A was fused with the DNA binding domain of pAS2 in the yeast two hybrid system. When pACT2-*VAN3* and pAS2-DRP1A were cotransformed in the yeast strain Y190, the yeast could survive on a selection agar plate without His. This indicated an interaction between *VAN3* and DRP1A in yeast (Fig. 1A).

To validate the protein-protein interaction revealed by the yeast two hybrid, a glutathione S-transferase (GST) pull-down assay was performed with a recombinant protein of GST full-length DRP1A as bait and a recombinant *VAN3*-GAL4 DNA binding domain protein as predator. Results showed that a GST-DRP1A recombinant protein pulled down the *VAN3*-GAL4 DNA binding domain recombinant protein band. However, GST itself did not (Fig. 1B).

### DRP1A Has GTPase Activity

DRP1A is known to have a GTPase domain and a GTPase effector domain. To examine the GTPase activity of the DRP1A protein, recombinant GST-tagged DRP1A protein was purified, and the hydrolysis of GTP by the recombinant DRP1A was measured. Radionucleotide-labeled GTP bound to DRP1A formed the substrate DRP1A-GTP. Further incubation under a condition that minimized interference by nucleotide exchange or contaminating nucleotidases resulted in GTP hydrolysis on the DRP1A in a time dependent manner (Fig. 1C). This result suggested that the DRP1A protein functions as a GTPase.

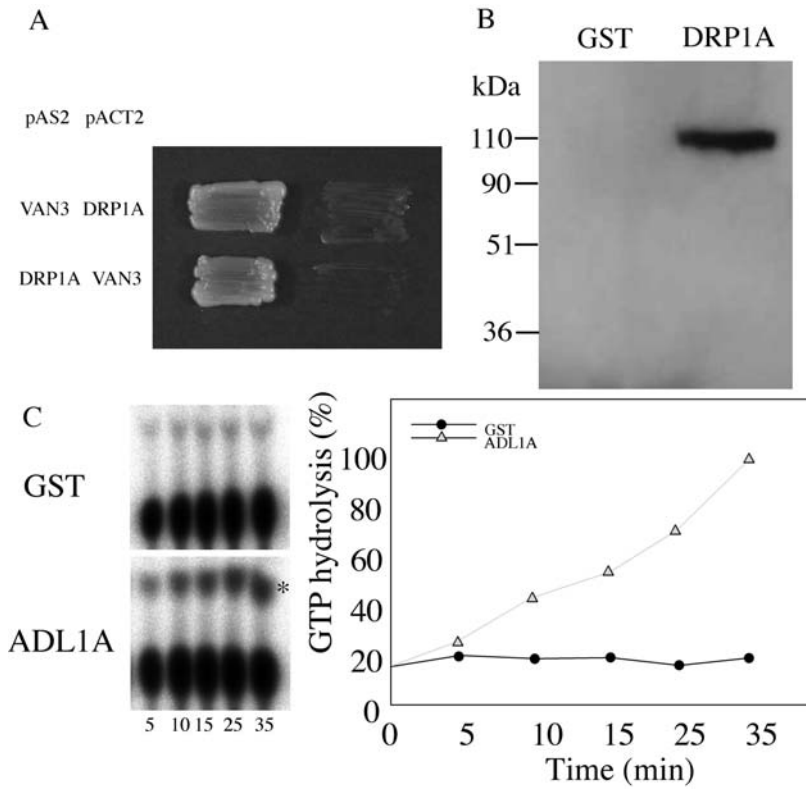
### Vascular Discontinuity of *drp1a*

Kang et al. (2003) showed that the *drp1a/adl1a* mutant had defects in the anisotropic expansion of stigmatic papillae and in the development of leaf trichomes. These results suggested that DRP1A might be responsible for expansion during single cell differentiation. They also discussed the possibility of DRP1A function in vascular formation (Kang et al., 2003).

To examine the DRP1A function in vascular formation, we cleared cotyledons of the *drp1a* mutants and observed vascular tissues. The cotyledon of Arabidopsis has a very simple continuous vein pattern: one midvein and three or four lateral veins (Fig. 2A). Approximately 10% of *drp1a* mutants showed disconnected vascular network (19 abnormal plants in 200 mutants; Fig. 2B), and the leaf size and shape is not so affected. This disconnected venation pattern of the *drp1a* mutant was similar to that of the *van3* mutant, although the discontinuity in *drp1a* was weaker than that of the *van3* mutant (Fig. 2C). In contrast, we could not observe disconnection in the wild type ( $n = 200$ ).

### Genetic Interaction of DRP1A and *VAN3*

To observe the genetic interaction between the *van3* and *drp1a* mutants, we produced *van3 drp1a* double mutants. The *van3 drp1a* double mutant had a defect in germination, although its embryo development was anatomically indistinguishable to that of the wild type (data not shown). In the  $F_2$  progeny of a cross between *van3* and *drp1a*, mutants from each combination segregated at ratios of about 9:3:3:1 (wild type:*van3*:*drp1a*:*van3 drp1a* = 176:64:56:18;  $\chi^2$  0.002 <  $P$  < 0.45). Genotypes of the 18 *van3 drp1a* double mutants were confirmed by PCR methods (Sawa et al., 1997). Seventeen of the 18 mutants failed to germinate, and only one mutant line showed seedling lethality without producing rosette leaves. This *van3 drp1a* double mutant had severely fragmented and poorly matured vasculature (Fig. 2D). In repeated experiments, about 5% of *van3 drp1a* double mutants could germinate (9/200), and all double mutants showed a similar phenotype. Thus, the *drp1a* mutation enhanced the



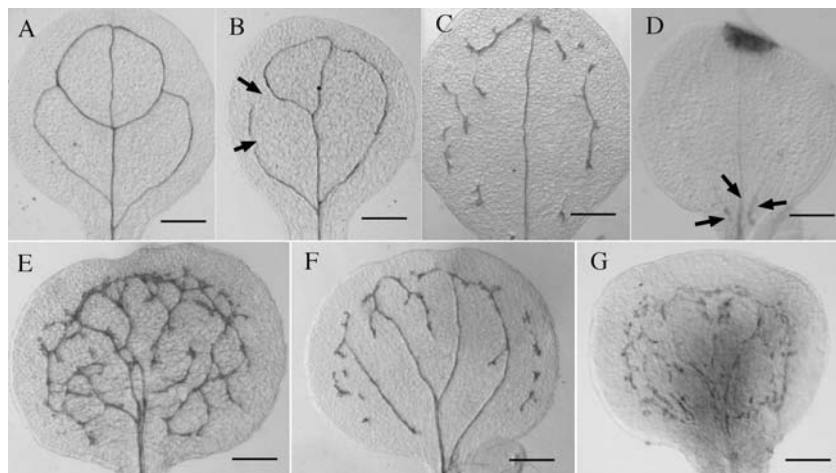
**Figure 1.** VAN3 interaction with DRP1A. A, Yeast two hybrid analysis. The Y190 yeast strain was transformed with pAS2-VAN3 and pACT2-DRP1A. The yeast harboring pAS2-DRP1A and pACT2-VAN3 were grown on agar plates without His. The Y190 yeast strain was transformed with pAS2-VAN3 and the pACT2 vector or pAS2-DRP1A and the pACT2 vector are shown as negative controls. B, Protein-protein interaction assay with GST pull down. VAN3-GAL4 DNA binding domain pull down with purified GST and GST-DRP1A used as bait. The VAN3 protein was detected with a western-blot analysis. C, Time course of DRP1A GTPase activity. Recombinant DRP1A protein was incubated with 5  $\mu\text{M}$  [ $\alpha$ - $^{32}\text{P}$ ] GTP. GTP hydrolysis was observed (\*) in the left section. GTP hydrolysis was quantitatively analyzed with a BIO-imaging analyzer in the right section.

*van3* phenotype, suggesting that the *DRP1A* gene may be responsible for synergistic vascular formation with the *VAN3* gene.

**Genetic Interaction of DRP1A and GNOM**

The *gnom/van7* mutant is known to show an abnormal venation pattern (Koizumi et al., 2000). About one-half of the *gnom* seedlings (10/23) produced fused cotyledons, and the *gnom* cotyledon produced excess vascular tissues (Fig. 2E). Although the *gnom* mutant sometimes produces partially fragmented vascular

tissues, the fragmentation is not as obvious, and the fragmentation was never observed in the primary veins. *GNOM* is known to encode an ARF-GEF, which is needed in PIN1 recycling between the plasma membrane and endosomes. On the other hand, *VAN3* is shown to encode an ARF-GAP (Koizumi et al., 2005). In seedlings of the *van3 gnom* double mutants, about one-half produced fused cotyledons (21/43). The cotyledon of the *van3 gnom* double mutants contained one or two midveins and highly fragmented lateral veins (Fig. 2F). Because we revealed that DRP1A could bind with VAN3, genetic interactions between the *drp1a* mutant



**Figure 2.** Genetic interaction of the *drp1a* mutant. A, Wild type. B, About 10% of *drp1a* mutants show a disconnected vasculature. Arrows show the vascular disconnection. C, *van3* mutants show a fragmented vasculature. D, *drp1a van3* double mutants showed a severe defect in vascular development. Arrows show the restricted maturation of the tracheary element. E, *gnom* mutants show a concentrated vasculature. F, *gnom van3* double mutants show a highly fragmented vasculature. G, *gnom drp1a* double mutants show a fragmented vasculature similar to the *gnom van3* double mutant. Bars = 500  $\mu\text{m}$ .

and the *gnom* mutant were also examined. In seedlings of the *gnom drp1a* double mutant, about one-half produced fused cotyledons and a highly fragmented vasculature (Fig. 2G). Interestingly, the fragmentation was also observed even in the primary veins (Fig. 2G). This was similar to that of the *van3 gnom* double mutant. Thus, the *drp1a* mutation appears to have a similar effect to that of the *van3* mutation, which enhances the fragmentation of vasculature in the *gnom* mutants. These results suggested that the *DRP1A* gene might be responsible for vascular formation.

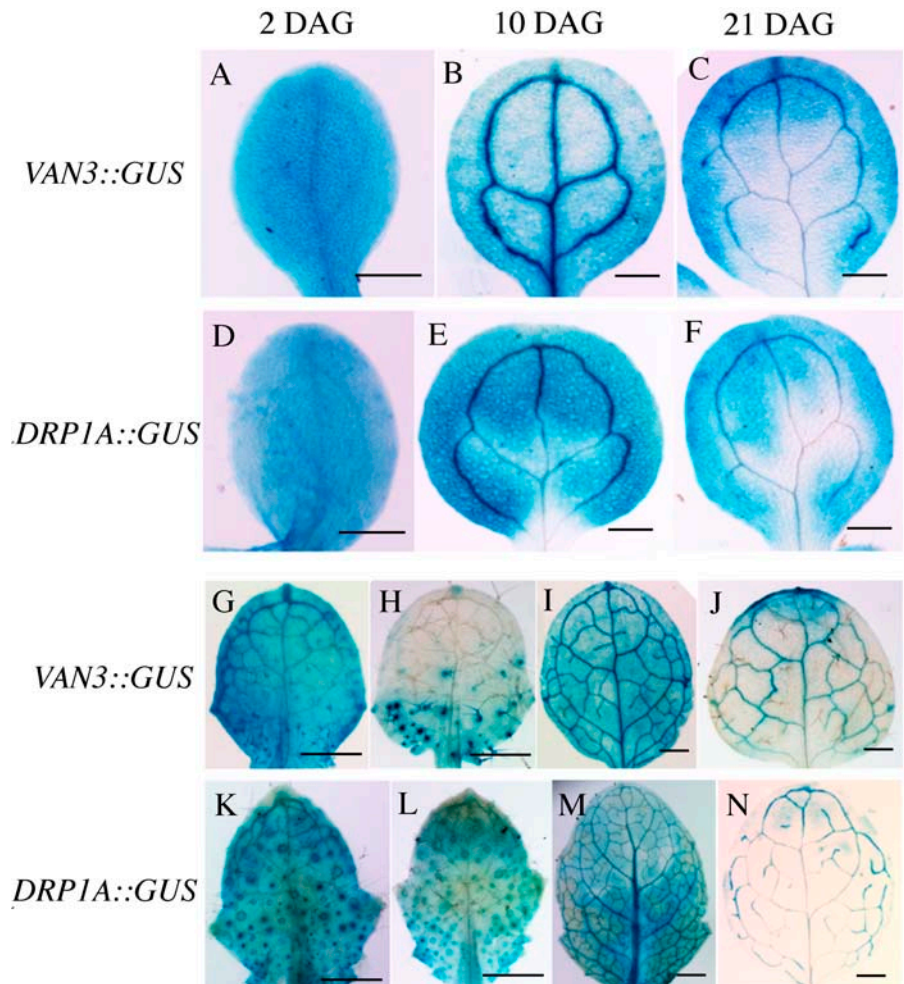
### Overlapping Expression of the *DRP1A* and *VAN3* Genes

To examine the temporal and spatial interrelationship between *DRP1A* and *VAN3* during leaf development, we analyzed the expression of *DRP1A::GUS* (Kang et al., 2003) and *VAN3::GUS* transgenic plants. The *VAN3* promoter sequence used in this  $\beta$ -glucuronidase (*GUS*) fusion constructs includes 1.1 kb upstream from the putative transcription start site, and it is sufficient to complement the *van3* mu-

tant phenotype in the complementation test (Koizumi et al., 2005). The progeny from three independent *VAN3::GUS* transgenic plant lines were used for a histochemical analysis of *GUS* activity.

*DRP1A::GUS* and *VAN3::GUS* transgenic plants showed overlapping reporter gene expression profiles during leaf development. *GUS* staining was ubiquitously observed in the cotyledons of both the *DRP1A::GUS* and *VAN3::GUS* transgenic seedlings 2 d after germination (DAG; Fig. 3, A and D). Strong *GUS* staining was characteristically observed in vascular tissues at 10 DAG (Fig. 3, B and E). At 21 DAG, *GUS* staining was observed at the margin of the cotyledons (Fig. 3, C and F). A similar expression pattern was observed in the rosette leaves, while a ubiquitous expression of the two genes was observed at early stages of rosette leaf development (Fig. 3, G and K). *GUS* staining was also observed in developing trichomes (Fig. 3, G, H, K, and L). As leaves developed, the ubiquitous expression was weakened (Fig. 3, H–J, L–N) and restricted to vascular tissues. At this stage, *GUS* staining was not obvious in the trichomes. This overlapping spatial expression pattern of *DRP1A* and

**Figure 3.** Temporal and spatial expression pattern of *DRP1A* and *VAN3* during leaf development. Histochemical localization of *VAN3::GUS* (A–C, G–J) and the *DRP1A::GUS* (D–F, K–N) reporter gene expression in the cotyledon (A–F) and in rosette leaf (G–N). Samples were fixed and incubated in a reaction buffer including 2 mg/mL of X-Gluc for 10 min. Bars = 500  $\mu$ m (A–F), 1 mm (G–N).



*VAN3* strongly suggests that *DRP1A* and *VAN3* work in the same tissues and cells.

#### Overlapped Localization of the *DRP1A* and *VAN3* Proteins in Subcellular Compartment

To understand the functional interaction between *DRP1A* and *VAN3*, we examined the localization of *DRP1A* and *VAN3*. Red fluorescent protein (RFP)-tagged *DRP1A* was introduced into *Arabidopsis* suspension-cultured cells, and their subcellular locations were observed with a confocal laser scanning microscope (Fig. 4A). *DRP1A* localized to a fiber like structure. In addition to the fiber like structure, a *DRP1A* positive signal was obvious in some punctate structures (Fig. 4A). It has been suggested that these fiber like structures are microtubules (Kang et al., 2001; Hong et al., 2003b). To clarify that this is the case, we used green fluorescent protein (GFP)-tagged MAP4 as a microtubule marker (Fig. 4B). When RFP-tagged *DRP1A* and the GFP-tagged MAP4 were cointroduced, the *DRP1A* positive signal overlapped to that of MAP4. This indicated that *DRP1A* was localized to the microtubules under our conditions (Fig. 4, D–F).

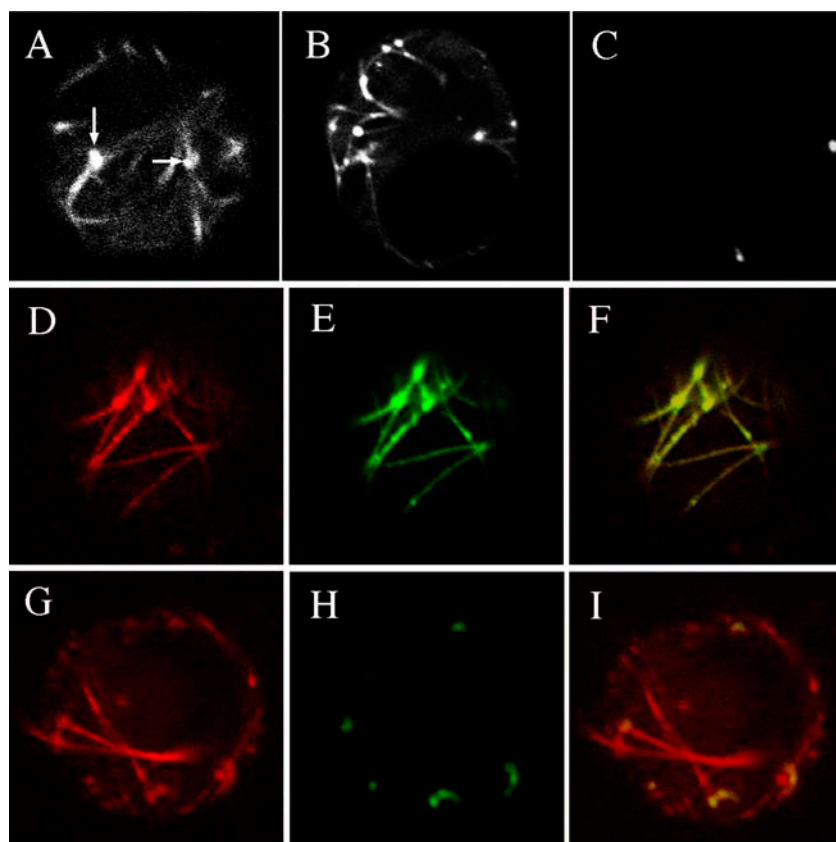
The *VAN3* protein is known to be located at the TGN (Koizumi et al., 2005). We confirmed this by VENUS (Nagai et al., 2002)-tagged *VAN3* (Fig. 4C). When *VAN3*-VENUS and RFP-tagged *DRP1A* were cointro-

duced, *VAN3*-VENUS-positive compartments overlapped the punctate structures of the RFP-*DRP1A* positive signal (Fig. 4, G–I). Similar overlapping figures were obtained in with tobacco (*Nicotiana tabacum*) BY2 suspension-cultured cells (data not shown). These results suggest that the *VAN3* protein can colocalize with *DRP1A* at the TGN, which might be associated with microtubules.

## DISCUSSION

### *DRP1A* and *VAN3* Interaction in Vascular Formation

Here, we isolated a plant dynamin, *DRP1A*, as a *VAN3* interaction protein using yeast two hybrid screening, and their interaction was confirmed by swapping test and a biochemical pull-down assay. We analyzed the interaction between *DRP1A* and *VAN3* in situ with different types of experiments. A histochemical analysis of the *drp1* mutant revealed disconnected vascular formation, which is weaker, but significantly resembled that of the *van3* phenotype. The phenotype of the *drp1a van3* double mutants indicated a genetic interaction between the two mutations with regard to vascular formation. Furthermore, the *drp1a* mutation enhanced the vascular fragmentation of the *gnom* mutants in the same manner as the *van3* mutation. A promoter analysis of *VAN3* and



**Figure 4.** Subcellular localization of *DRP1A* and *VAN3*. 35S-promoter-driven RFP-tagged *DRP1A*. Arrows show punctate structures. A, GFP-tagged MAP4 as a microtubule marker (B) and VENUS-tagged *VAN3* (C) were introduced into protoplasts of *Arabidopsis* suspension-cultured cells. RFP-*DRP1A* and GFP-MAP4 (D–F), and RFP-*DRP1A* and *VAN3*-Venus (G–I) were cointroduced. Localization of RFP-*DRP1A* (D), MAP4-GFP (E), and merged image (F) are shown. The location of RFP-*DRP1A* (G), *VAN3*-VENUS (H), and merged image of RFP-*DRP1A* and *VAN3*-VENUS (I) are shown. Fluorescence was observed by confocal laser microscopy (LSM510 META, Carl Zeiss).

*DRP1A* indicated spatially and temporally overlapped expression patterns of these two genes. Taken together with the finding of subcellular colocalization, these results strongly support the *in vivo* interaction of *DRP1A* and *VAN3* in controlling vascular formation.

#### Vesicle Trafficking Mechanisms Regulated by *DRP1A* and *VAN3* in Vascular Formation

Formation of transport vesicles involves the assembly of distinct coat complexes that induces membrane budding and the selection of cargo proteins. This process is regulated by small GTPases such as ARFs, which are required for the formation of TGN-derived clathrin coated vesicles (Bednarek and Falbel, 2002). In mammalian cells, the release of clathrin-coated TGN-derived vesicles involves the action of another GTPase dynamin (Jones et al., 1998). Animal dynamin was originally isolated from bovine brain extracts as a microtubule-binding protein (Shpetner and Vallee, 1989), and its GTPase activity was shown to be activated by microtubules (Shpetner and Vallee, 1992). It has been suggested that dynamin GTPase may be involved in the transport of Golgi-derived vesicles along the microtubules, from the minus end to the plus end (Fullerton et al., 1998). Using an antibody against Arabidopsis *DRP1A*, Kang et al. (2001) observed significant immunolabeling of punctate structures with the antibody in Arabidopsis suspension cultured cells, although they did not determine the nature of the structures. Furthermore, Murphy et al. (2002) indicated that  $\beta$ -adaptin, a subunit of the clathrin adaptor protein complex, and the AtSEC14 protein were co-fractionated with *DRP1A* by affinity chromatography on immobilized-naphthylphthalamic acid. These results suggest that dynamins may play a role in vesicle transport from the TGN (Murphy et al., 2002). We have indicated that *VAN3* is located on the TGN (Koizumi et al., 2005). In this paper, we revealed that *DRP1A* is colocalized with *VAN3* and also with microtubule marker, MAP4. Hong et al. (2003b) also revealed the colocalization of *DRP1A* with microtubules in tobacco cultured cells. All of these findings strongly suggest that the *DRP1A* is responsible for the vesicle trafficking from the TGN, which is associated with microtubules, synergistically working with *VAN3*.

#### Diverse *DRP1A* Function in Vesicle Trafficking and in Plant Development

*drp1a* showed pleiotropic abnormalities in plant development including normal embryogenesis, seedling development, and reproduction (Kang et al., 2003). We also observed the fragmented vascular tissue formation. *DRP1A* appears to be localized at different subcellular compartments such as punctate organelles and microtubules, probably depending on cell stages and cell types, and therefore, *DRP1A* may have pleiotropic functions in plant development. *DRP1A* is categorized to be a member of five phrag-

moplastin-like DRPs in Arabidopsis (Hong et al., 2003a). *DRP1A* and *DRP1E* are suggested to have overlapped functions in embryo development because of the embryo lethality in the *drp1a drp1e* double mutant (Kang et al., 2003). Although it is still unclear about the physiological function of other three members of *DRP1* homologs, these genes may have diverse physiological functions in plants.

*VAN3* is an ACAP-type ARF-GAP, which have BIN/amphiphysin/RVS, pleckstrin homology, ARF-GAP, and ankyrin repeats domains, and have been shown to localize at subpopulation of TGN (Koizumi et al., 2005). In Arabidopsis, there are three other *VAN3* homologs, which possess all the four domains. The domain structures strongly suggest that the *VAN3* homologs may also be responsible for vesicle trafficking. The preliminary examination with their promoters fused to the GUS gene suggested spatial expression patterns of the *VAN3* and three *VAN3* homologs are different from each other; i.e. some show distinct tissue specificity and others show ubiquitous expression (data not shown). This result suggests that *VAN3* and the *VAN3* homologs function in vesicle trafficking, responsible for diverse physiological events even in plants. Here, we indicated the interaction of *DRP1A* with *VAN3*, which complexes locate on TGN (Koizumi et al., 2005). Therefore, a unique set of a dynamin and a *VAN3* homolog might play a specific role in vesicle trafficking at distinctive TGN during various developmental processes in plants. Detailed analysis of subcellular localization and functions of the dynamin and *VAN3* family genes in various cell types and cell stages may cast a new light on the molecular mechanisms of vesicle trafficking underlying various plant developmental events.

## MATERIALS AND METHODS

### Plant Materials

The *van3* mutant was isolated from Arabidopsis (*Arabidopsis thaliana*) Landsberg *erecta* by ethylmethane sulfonate mutagenesis (Koizumi et al., 2000). Seeds were sown on the surface of vermiculite in small pots and incubated at 4°C for 3 d. Plants were grown in a laboratory under continuous illumination of 50 to 100  $\mu\text{E}/\text{m}^2$  per second at 22°C. For histochemical analysis, GUS staining was performed as described by Koizumi et al. (2000), except that samples were incubated in the GUS substrate solution for 10 min. All of the *drp1a* plants were grown in the Murashige and Skoog medium containing 100 mM Suc to suppress the developmental defects (Kang et al., 2001).

### Yeast Two Hybrid Screening

Full-length cDNA of the *VAN3* was ligated to the plasmid pAS-2, and the resulting pAS-VAN3 was used as bait to screen an Arabidopsis cDNA library constructed in the pACT2 vector. The cDNA library constructed in pACT2 was transformed to yeast strain Y190 containing pAS-VAN3. Positive colonies were selected on SC-Trp-Leu-His medium. After the confirmation by the retransformation test and X-gal test, the inserts were sequenced.

### Purification of the *DRP1A* Protein

To express the GST-tagged *DRP1A* protein in a bacteria, an expression plasmid was constructed by use of pGEX-5T. The *DRP1A* gene was amplified

by PCR, and the obtained fragment was ligated into pGEX-5T. The sequence of the DRP1A in the expression plasmid was confirmed by cycle sequencing. Expression of the DRP1A protein was induced in *Escherichia coli* BL21 cells harboring the expression plasmid by addition of isopropylthio- $\beta$ -galactoside. Cultivation of the *E. coli* was carried out at 14°C. When the absorbance of the culture at 600 nm reached 0.8, 1 mM isopropylthio- $\beta$ -galactoside was added to the culture media, and the cultivation was then continued for an additional 2 d. Purification procedure was carried out at 4°C. A 1-liter culture of *E. coli* cells was suspended in 50 mL of 10 mM Tris-HCl at pH 7.5, 0.1 mM EDTA, 5 mM  $\beta$ -mercaptoethanol, and sonicated on ice for 2 min, and centrifuged at 15,000 rpm (27,000g) for 30 min. The supernatant was applied to a glutathione column (Pharmacia Biotech, Uppsala). After the column had been washed with a phosphate-buffered saline, DRP1A was eluted with a solution containing glutathione.

### GTPase Assay

DRP1A GTPase activity was determined using an in vitro assay that measures a single round of GTP hydrolysis on recombinant DRP1A protein (Makler et al., 1995; Damke et al., 2001). A total of 5  $\mu$ M DRP1A was first loaded with 5  $\mu$ M [ $\alpha$ - $^{32}$ P] GTP in a buffer (25 mM HEPES, pH 7.5, 1 mM dithiothreitol, 2 mM EDTA, 1 mM MgCl<sub>2</sub>, 10 mM ATP, and 0.1% sodium cholate). DRP1A-GTP binding efficiency was calculated by filter assay (Koizumi et al., 2005). GTPase assay was performed at 37°C in 50 mM HEPES, pH 7.5, 4 mM MgCl<sub>2</sub>, and 10 mM ATP. The reaction was initiated by the addition of 1  $\mu$ M of [ $\alpha$ - $^{32}$ P] GTP-loaded DRP1A, and stopped by the addition of 250 mM EDTA, followed by placing on ice. The nucleotides were separated by thin layer chromatography on PEI-cellulose sheets (Merck, Rahway, NJ) developed with 1 M LiCl and 1 M HCOOH. The sheets were dried by air dryer, autoradiographed on an imaging plate, and quantitatively analyzed with a BIO-imaging Analyzer (BAS 2000, Fuji Photo Film, Tokyo). By using the DRP1A-GTP binding efficiency value, we calculated the efficiency of the GTP hydrolysis. In the control experiment, GST protein was incubated in the reaction buffer, and no GTP hydrolysis was observed.

### Pull-Down Assay

Yeast strain Y190 carrying pAS-VAN3 was sonicated on ice for 2 min and centrifuged at 15,000 rpm (27,000g) for 30 min. Proteins included in the supernatant were precipitated by ammonium sulfate. Precipitant was dissolved with Tris-HCl, pH 7.5, and it was dialyzed by Tris-HCl, pH 7.5, to remove ammonium sulfate, resulting in partially purified VAN3-GAL4 DNA binding domain recombinant protein. Recombinant GST-tagged DRP1A protein was also dialyzed against 25 mM Tris-HCl, pH 7.5. A total of 15  $\mu$ g of partially purified VAN3-GAL4 DNA binding domain recombinant protein was mixed with 5  $\mu$ g of purified GST-DRP1A recombinant protein or purified GST recombinant protein in 100  $\mu$ L reaction buffer (25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Triton X-100). The reaction mixture was shaken at 4°C for 45 min, and 15  $\mu$ L of glutathione Sepharose 4B bed was then added to the mixture that was shaken for an additional 30 min at 4°C. The glutathione Sepharose 4B bead was then washed five times with 1 mL reaction buffer and boiled in SDS-PAGE sample buffer. After SDS-PAGE, we detected the VAN3 protein by the western-blot experiment, using anti-VAN3 antibody as a probe. Polyclonal anti-VAN3 antibodies were produced against the synthetic peptide EKMQEYKRQVDRESR by a rabbit. Western-blot experiment was performed as described (Ito and Fukuda, 2002).

### Subcellular Localization

Full-length VAN3 cDNA was isolated by reverse transcription-PCR from Columbia ecotype, and an *Xho*I/*Nco*I restriction site was introduced at both ends. The fragment was translationally fused to the N terminus of VENUS yellow fluorescent protein. The chimeric gene was subcloned under the control of the cauliflower mosaic virus 35S promoter and the Nos terminator. 35S::RFP-DRP1A was also produced in the same way to the VAN3-VENUS, except for the DRP1A fusion to the C terminus of RFP protein. 35S::MAP4-GFP and 35S::Talin-GFP were used as intracellular markers of tubulin and actin, respectively. Double transient expression of 35::VAN3-Venus and of intracellular markers in the protoplasts of cultured Arabidopsis cells were analyzed as described by Ueda et al. (2001, 2004). Fluorescence was observed by confocal laser microscopy (LSM510 META, Carl Zeiss, Jena, Germany).

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