CONTINUOUS VASCULAR RING (COV1) is a trans-Golgi Network-Localized Membrane Protein Required for Golgi Morphology and Vacuolar Protein Sorting

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The trans-Golgi network (TGN) is a tubular–vesicular organelle that matures from the trans cisternae of the Golgi apparatus. In plants, the TGN functions as a central hub for three trafficking pathways: the secretory pathway, the vacuolar trafficking pathway and the endocytic pathway. Here, we describe a novel TGN-localized membrane protein, CONTINUOUS VASCULAR RING (COV1), that is crucial for TGN function in Arabidopsis. The COV1 gene was originally identified from the stem vascular patterning mutant of Arabidopsis thaliana. However, the molecular function of COV1 was not identified. Fluorescently tagged COV1 proteins co-localized with the TGN marker proteins, SYNTAXIN OF PLANTS 4 (SYP4) andvacuolar-type H\(^+\)-ATPase subunit a1 (VHA-a1). Consistently, COV1-localized compartments were sensitive to concanamycin A, a specific inhibitor of VHA. Intriguingly, cov1 mutants exhibited abnormal Golgi morphologies, including a reduction in the number of Golgi cisternae and a reduced association between the TGN and the Golgi apparatus. A deficiency in COV1 also resulted in a defect in vacuolar protein sorting, which was characterized by the abnormal accumulation of storage protein precursors in seeds. Moreover, we found that the development of an idioblast, the myrosin cell, was abnormally increased in cov1 leaves. Our results demonstrate that the novel TGN-localized protein COV1 is required for Golgi morphology, vacuolar trafficking and myrosin cell development.

Keywords: Arabidopsis thaliana • COV1 • Myrosin cell • TGN • Vacuolar protein sorting • Vacuolar patterning.

Abbreviations: BFA, brefeldin A; CBB, Coomassie Brilliant Blue; ConCA, concanamycin A; COV1, CONTINUOUS VASCULAR RING; DMSO, dimethylsulfoxide; GFP, green fluorescent protein; GUS, β-glucuronidase; mRFP, monomeric red fluorescent protein; PBS, phosphate-buffered saline; PEP, carboxypeptidase Y-deficient; PSV, protein storage vacuoles; PVC, prevacuolar compartment; 2SC, C-terminal vacuolar targeting signal of pumpkin 2S albumin; SNAP, soluble N-ethylmaleimide-sensitive factor attachment protein receptor; SYP, syntaxin of plants; TGG, β-thioglucoside glucohydrolase; TGN, trans-Golgi network; UTR, untranslated region; VHA, vacuolar-type H\(^+\)-ATPase; VHA-a1, vacuolar-type H\(^+\)-ATPase subunit a1; VSR, vacuolar sorting receptor.

Introduction

In eukaryotic cells, the membrane trafficking machinery regulates transport of newly synthesized and pre-existing proteins, the organization of endomembrane organelles and cellular homeostasis. Endomembrane systems similar to those of modern eukaryotes might have been generated in the last common eukaryotic ancestor (Dacks and Field 2007, Dacks et al. 2008). Many genes encoding regulatory proteins for membrane trafficking pathways are well conserved in eukaryotes, while some have duplicated and diverged, and others were lost during evolution (Dacks and Field 2007, Dacks et al. 2008). Multicellular eukaryotes did not arise from a single origin, but are considered to have evolved independently in animals and plants (Meyerowitz 2002). Therefore, it is thought that plants have both universal and plant-specific components of membrane trafficking systems [e.g. the trans-Golgi network (TGN)]. Plants are one of the ideal models for studies of the endomembrane system.

The TGN is a tubular–vesicular organelle that develops from the trans cisternae of the Golgi apparatus (Staehelin and Kang 2008, Kang et al. 2011). Live-cell imaging indicates that the TGN is a highly dynamic organelle, and a subset of the TGN is not associated with the Golgi apparatus (Viotti et al. 2010). The function of the TGN is to transport newly synthesized proteins en route to vacuoles, the plasma membrane and the extracellular space (Richter et al. 2009). In addition to these anterograde trafficking pathways, the plant TGN also functions as an early endosome in the endocytic pathway, although the animal TGN is a different organelle from the early endosome (Dettmer et al. 2006, Richter et al. 2009, Viotti et al. 2010). Therefore, it is important to understand the unique mechanisms regulating...
TGN morphology and protein trafficking through the TGN in plants.

The primary markers for the plant TGN are SYNTAXIN OF PLANTS 4 (SYP4) and SYNTAXIN OF PLANTS 6 (SYP6), which belong to the Qa and Qc subfamilies of the SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) family, respectively (Uemura et al. 2004). Another convincing marker for the TGN is VHA-a1, a subunit of the vacuolar-type H\(^{+}\)-ATPase (V-ATPase) (Dettmer et al. 2006). VHA-a1 rapidly co-localizes with the endocytic tracer FM4-64 within 6 min (Dettmer et al. 2006), indicating the role of the plant TGN as an early endosome. Pharmacological inhibition of V-ATPase activity by concanamycin A (ConcA) or knockdown of VHA-a1 disrupts the Golgi morphology and function in endocytic and secretory trafficking (Dettmer et al. 2006, Brux et al. 2008, Viotti et al. 2010).

In the last 10 years, >10 factors have been identified and localized at the TGN in Arabidopsis. Characterization of these factors revealed that the plant TGN was involved in events such as cell plate formation (Chow et al. 2008, Toyooka et al. 2009, Park et al. 2013, Teh et al. 2013), cell elongation (Brux et al. 2008, Gendre et al. 2011, Gendre et al. 2013), secretion of cell wall materials (Gendre et al. 2013), plant growth (Park et al. 2013, Teh et al. 2013, Wang et al. 2013), leaf vascular patterning (Naramoto et al. 2009, Wang et al. 2013), organ separation (Liljegren et al. 2009, Sauer et al. 2013), abiotic stress response (Zhu et al. 2002, Kim and Bassham 2011, Asaoka et al. 2013) and immune response (Gu and Innes 2011, Uemura et al. 2012, Choi et al. 2013). A recent TGN–proteome analysis identified additional proteins that potentially localize to the TGN (Drakakaki et al. 2012), including CONTINUOUS VASCULAR RING (COV1). COV1 was originally reported to be required for vascular patterning in the Arabidopsis stem (Parker et al. 2003). The mutant cov1 exhibited ectopic differentiation of vascular cells in the stem, but not in the leaves (Parker et al. 2003). Ectopic vascular cells were already differentiated in the shoot apex of cov1, suggesting that COV1 is involved in the early developmental stage of vascular tissue, but not in the secondary growth stage (Parker et al. 2003). The molecular function of COV1 remained unknown for a decade.

Here, we show that COV1 is a TGN-localized membrane protein that is required for Golgi morphology and vacuolar protein trafficking. We also found that COV1 is required for the development of vascular and myrosin cells in leaves. Our results highlight the linkage between COV1-mediated TGN function and plant higher order function, particularly vascular patterning and myrosin cell development in Arabidopsis.

**Results**

**COV1 is a TGN-localized membrane protein**

To determine the subcellular localization of COV1, we constructed translational fusions of either sGFP (green fluorescent protein) or TagRFP (red fluorescent protein) with the genomic COV1 sequence (Fig. 1A), and introduced each of them into cov1-1 mutants. Stereomicroscopic analysis showed that COV1-sGFP was expressed throughout the whole plant examined (Supplementary Fig. S1). Both COV1-sGFP and COV1-TagRFP complemented the defects in root growth and stem elongation in the cov1-1 mutant (Fig. 1B). This indicates that COV1–sGFP and COV1–TagRFP are functional. Confocal microscopic analysis of the transgenic plants revealed that the fluorescently tagged COV1 proteins were localized on mobile punctate structures (Fig. 1C).

To identify the COV1-localized structures, we stained cells expressing COV1–sGFP with the endocytic tracer FM4-64 (Ueda et al. 2001). COV1–sGFP and FM4-64 rapidly co-localized at the punctate structures after 6 min of staining (Fig. 2A–C). One of the earliest organelles labeled by FM4-64 is the TGN (Dettmer et al. 2006); therefore, this observation suggested that COV1–sGFP localized at the TGN. To verify this, we treated seedlings expressing COV1–sGFP with brefeldin A (BFA), which is a fungal toxin that causes an agglomeration of the TGN and endosomes to form BFA compartments in Arabidopsis (Grebe et al. 2003). Co-localization of COV1–sGFP with the BFA compartments would support the notion that COV1–sGFP localized at the TGN. The results showed that BFA treatment caused the relocation of COV1–sGFP to the core of BFA compartments that are labeled by FM4-64 (Supplementary Fig. S2). This was consistent with a rapid co-localization of COV1–sGFP and FM4-64, and strongly suggested that COV1–sGFP localized to the TGN.

We further confirmed the subcellular localization of COV1 by performing co-localization experiments using well-established fluorescent organelle markers. Transgenic Arabidopsis lines stably expressing each of the TGN markers [monomeric RFP (mRFP)–SYP43 (Uemura et al. 2012), VHA-a1–mRFP and VHA-a1–sGFP (Dettmer et al. 2006)] and the prevacuolar compartment (PVC) and tonoplast marker [GFP–PEP12 (Uemura et al. 2010)] were used in combination with either COV1–sGFP or COV1–TagRFP. Confocal microscopy visualization showed that COV1–sGFP co-localized with mRFP–SYP43 (Fig. 2D–F) and VHA-a1–mRFP (Fig. 2G–I). COV1–TagRFP co-localized with VHA-a1–sGFP (Fig. 2J–L). In contrast, no co-localization was detected between COV1–sGFP and GFP–PEP12 (Uemura et al. 2010) were used in combination with either COV1–sGFP or COV1–TagRFP. Confocal microscopy visualization showed that COV1–sGFP co-localized with mRFP–SYP43 (Fig. 2D–F) and VHA-a1–mRFP (Fig. 2G–I). COV1–TagRFP co-localized with VHA-a1–sGFP (Fig. 2J–L). In contrast, no co-localization was detected between COV1–TagRFP and GFP–PEP12 (Fig. 2M–O). In addition, a transient expression analysis in tobacco leaf cells revealed that COV1–TagRFP did not co-localize with ST–GFP, a trans-Golgi marker (Boevink et al. 1998) (Fig. 2P–R). Furthermore, COV1–sGFP punctate structures in Arabidopsis were sensitive to ConcA, a V-ATPase inhibitor (Fig. 3), which caused aggregation of the TGN (Dettmer et al. 2006). Taken together, these results indicate that COV1 is localized to the TGN.

**COV1 is involved in the organization of the Golgi and the TGN**

Previous studies reported that TGN-localized proteins were required for the maintenance of normal morphologies of the
Golgi and TGN (Brux et al. 2008, Liljegren et al. 2009, Gendre et al. 2011, Uemura et al. 2012, Sauer et al. 2013). Therefore, the TGN localization of COV1 prompted us to investigate the ultrastructure of the Golgi apparatus and the TGN in the cov1 mutant. We found that the TGN did not associate with Golgi stacks in cov1-2 (Fig. 4A), suggesting that COV1 was required for the association of the TGN with the trans side of the Golgi apparatus. In addition, the number of cisternae per Golgi stack

**Fig. 1** Complementation of the cov1 phenotypes by expression of fluorescently tagged COV1. (A) Structural organization of the COV1-sGFP and COV1-TagRFP genes that are introduced into cov1-1 mutants. A 3,149 bp genomic fragment of COV1 is used. (B) The cov1-1 mutants exhibit short roots (upper panel) and short stems (lower panel) compared with those of wild-type Ler. The growth defects are complemented by COV1-sGFP or COV1-TagRFP driven by its native promoter. (C) Both COV1-sGFP and COV1-TagRFP label mobile punctate structures in epidermal cells in Arabidopsis roots.

**Fig. 2** COV1 localizes at the TGN. (A–C) Rapid co-localization of COV1-sGFP with the endocytic tracer FM4-64 after incubation for 6 min. (A) Confocal images of COV1-sGFP; (B) FM4-64 staining; (C) overlay. (D–I) COV1-sGFP co-localizes exclusively with TGN markers. (D–F) mRFP-SYP43; (G–I) VHA-a1–mRFP. (J–R) COV1-TagRFP co-localizes exclusively with the TGN marker VHA-a1–sGFP (J–L), but not with the prevacuolar compartment and tonoplast marker GFP–PEP12 (M–O) and the Golgi marker ST–GFP (P–R). The fluorescent images of epidermal cells in Arabidopsis root (A–O) and tobacco leaf (P–R) were inspected with a confocal laser scanning microscope.
COV1 is required for the efficient vacuolar protein sorting

The TGN localization of COV1 suggested that COV1 could be involved in protein sorting at the TGN. To examine the involvement of COV1 in vacuolar protein transport, we analyzed the transport of major storage proteins (12S globulin and 2S albumin) into protein storage vacuoles (PSVs) in *cov1* seeds. A defect in the transport of storage proteins should result in an abnormal accumulation of precursor forms of 12S globulin and 2S albumin in seeds and their missorting out of cells (Shimada et al. 2003). Immunoblot analysis showed that *cov1-1, cov1-2* and their F1 seeds (*cov1 F1*) abnormally accumulated the precursor forms in their seeds (Fig. 5A), suggesting a defect in transport into the PSVs. Immunofluorescent analysis with anti-12S globulin antibody revealed a positive signal surrounding cells in *cov1-1* and *cov1-2*, in addition to the strong signal in the PSVs within cells (Fig. 5B). These results indicate that 12S globulin is abnormally secreted out of the mutant cells in *cov1-1* and *cov1-2* seeds, demonstrating that COV1 functions in the efficient transport of vacuolar proteins. *cov1-2* exhibited more severe defects in vacuolar protein sorting than *cov1-1*. This is consistent with other phenotypes; *cov1-2* showed more severe defects in stem vascular patterning (Parker et al. 2003), stem elongation (Parker et al. 2003) and root growth (Supplementary Fig. S3).

**COV1 is involved in vein pattern formation in leaves**

COV1 was previously shown to be involved in the vascular patterning in stems, but not in leaves and cotyledons (Parker et al. 2003). Consistent with this report, our quantitative analysis of the number of vein junctions in a leaf revealed that the complexity of vascular development in leaves of *cov1-1* and *cov1-2* is indistinguishable from that in the wild type (Fig. 6A). Apparent vein patterning in a whole leaf in each mutant is similar to that of the wild type (Supplementary Fig. S4). However, in a more detailed observation, significant portions of both *cov1-1* (86%, *n* = 14) and *cov1-2* (50%, *n* = 12) leaves showed the V-shaped vein splice at the leaf tips (Fig. 6B, arrows), which was not observed in Ler leaves (0%, *n* = 10). In addition, *cov1* mutants exhibited abnormal tangled branches of leaf veins (Fig. 6B, arrowheads). These results suggest that COV1 functions in vein patterning in leaves.

**COV1 is required for myrosin cell differentiation in leaves**

Next, we examined the development of the myrosin cell in *cov1* mutants, because we previously found an abnormally increased development of myrosin cells in the defective mutants of SIP2 family proteins (Ueda et al. 2006, Shirakawa et al. 2010). To evaluate the development of myrosin cells quantitatively, we measured the accumulation levels of the myrosin cell protein β-thioglucoside glucohydrolase 1 (TGG1), which is one of the two major myrosinases in Arabidopsis (Andreasson et al. 2001,
Both cov1-1 and cov1-2 leaves accumulated higher levels of TGG1 than wild-type leaves (Fig. 7A). These results suggest that COV1 is required for myrosin cell differentiation in leaves.

To analyze the distribution pattern of myrosin cells in a whole leaf, we developed a transgenic line (MYR001:GUS) that specifically expresses the β-glucuronidase (GUS) reporter in myrosin cells. The promoter of the MYR001:GUS construct was derived from the 2 kb upstream region of At3g52850 (VSR1). GUS-stained cells were distributed along the leaf vein in the wild-type background (Fig. 7B; Supplementary Fig. S5, MYR001:GUS). These cells were easily stained by Coomassie Brilliant Blue (CBB) (Supplementary Fig. S6A), which was previously used to stain the myrosin cells (Ueda et al. 2006). To confirm further the specificity of the MYR001 promoter, we compared the expression pattern of MYR001:TagRFP (TagRFP was distributed in the nucleus and cytosol) with that of pTGG2:VENUS-2sc (VENUS-2sc was localized to the endoplasmic reticulum and vacuole). The TGG2 promoter was used previously as a specific promoter for myrosin cells (Barth and Jander 2006). Cells expressing MYR001:TagRFP also expressed pTGG2:VENUS-2sc at the inner tissue layer of leaves (Supplementary Fig. S6B). These results confirmed that the MYR001 promoter was specific for myrosin cells.

We introduced the MYR001:GUS reporter into cov1-2. The cov1-2 leaves had an increased number of myrosin cells that formed denser network patterns (Fig. 7B; Supplementary Fig. S5, cov1-2 MYR001:GUS). Collectively, our results suggest that COV1 is required for the proper development of myrosin cells.

Discussion

Previously, COV1 was predicted to be localized at the plasma membrane (Parker et al. 2003), although there was no direct evidence to prove this prediction. Our confocal microscopy analyses of COV1–sGFP and COV1–TagRFP showed that COV1 was localized to the TGN (Fig. 2). Intriguingly, VHA-a1 was not completely merged with COV1 (Figs. 2, 3), while SYP43 displayed strong co-localization with COV1 (Fig. 2). The complementation of the cov1 phenotype by fluorescently tagged COV1 proteins convincingly validates the TGN localization of COV1 (Fig. 1). Consistent with our results, a recent proteomic analysis of the TGN revealed the presence of endogenous COV1 in the TGN fraction, together with well-known TGN-localized proteins (Drakakaki et al. 2012). Therefore, we conclude that COV1 is localized to the TGN.

Ueda et al. 2006). Both cov1-1 and cov1-2 leaves accumulated higher levels of TGG1 than wild-type leaves (Fig. 7A). These results suggest that COV1 is required for myrosin cell differentiation in leaves.
The function of COV1 in cells was not identified for more than a decade (Parker et al. 2003). In this study, we found that COV1 is required for Golgi morphology (Fig. 4). Abnormal Golgi morphologies such as circled cisternae and smaller cisternae were observed in cells carrying mutations of TGN-localized proteins, including VHA-a1 (Brux et al. 2008), NEVERSHED/AGD5/MTV4 (Liljegren et al. 2009, Sauer et al. 2013), ECHIDNA (Gendre et al. 2011), SYP4 family proteins (Uemura et al. 2012) and MTV1 (Sauer et al. 2013). However, to our knowledge, the cov1 mutant is the first example in plants that shows a reduced number of Golgi cisternae. A similar phenotype was observed in human RNAi (RNA interference) cell lines of GRASP65 (Golgi reassembly stacking protein) (Sutterlin et al. 2005, Tang et al. 2010). GRASP65 is a peripheral Golgi protein that is associated with membranes via N-terminal myristoylation, and its homolog has not been found in plants (Brandizzi and Barlowe 2013). COV1 might maintain the Golgi morphology in plants in a different manner from GRASP65 in humans.

Another cov1 phenotype was the reduced association between the TGN and the Golgi apparatus (Fig. 4A). Currently, it is unknown how the connection between the TGN and Golgi cisternae is formed. A similar phenotype was observed in the Arabidopsis mutant echidna (Gendre et al. 2011). ECHIDNA is a TGN-localized protein that is predicted to contain 3–4 transmembrane domains (Gendre et al. 2011). However, the molecular function(s) of ECHIDNA is largely unknown. It is possible that COV1 function partially overlaps with that of ECHIDNA. Although ECHIDNA homologs are found in most eukaryotes (Gendre et al. 2011), which suggests their conserved cellular function, no obvious homolog of plant COV1 is found in other eukaryotes.

We found that TGN-localized COV1 was required for vacuolar protein trafficking (Fig. 5). In general, vacuolar proteins are sorted at the TGN to the vacuole via the PVC. How does COV1 regulate vacuolar sorting? COV1 deficiency may affect the function of trafficking regulators such as vacuolar sorting receptor 1 (VSR1). VSR1 plays a critical role in the transport of vacuolar proteins by recycling between the TGN and the PVC (Shimada et al. 2003). Another possible candidate that is affected in cov1 is VHA-a1 (Dettmer et al. 2006). Mislocalization of VHA-a1 was found in echidna mutant cells (Gendre et al. 2011). We cannot exclude the possibility that COV1 is also involved in the secretory pathway. To understand the molecular function of COV1 better, it is necessary to isolate factors that interact with COV1.

Originally, cov1 was identified as a stem vascular patterning mutant of Arabidopsis (Parker et al. 2003). In this study, we found that COV1 is required for the development of vascular cells in leaves (Fig. 6) and myrosin cells (Fig. 7). Previously, we reported that the SYP2 family proteins redundantly function in the proper development of myrosin cells (Ueda et al. 2006, Shirakawa et al. 2010). Although cov1 and syp2 mutants exhibited very similar defects in myrosin cell development, COV1 and SYP2 family proteins localized at different subcellular compartments. The SYP2 family proteins mediate vacuolar morphogenesis (Ebine et al. 2008), vacuolar trafficking (Ebine et al. 2008, Shirakawa et al. 2010) and endocytic trafficking (Ebine et al. 2011). However, it is unknown how these molecular functions affect the coordination of myrosin cell development. COV1 does not appear to be involved in vacuolar morphogenesis because cov1 did not exhibit the vacuolar fragmentation phenotype (Fig. 5B). It is possible that the trafficking pathways common to COV1 and SYP2 family proteins (the vacuolar trafficking pathway and/or the endocytic pathway) affect the development of myrosin cells.

We also found that COV1 was involved in vein pattern formation in leaves (Fig. 6B). A similar abnormal vascular pattern was observed in transgenic plants expressing a constitutively active form of RHO OF PLANTS 6 (ROP6) (Chen et al. 2012). ROP6 was shown previously to be involved in the endocytic pathway (Chen et al. 2012). It is possible that COV1 also functions in the endocytic pathway. Further studies will be required to identify the molecular mechanisms of COV1 in regulating the development of myrosin cells and vascular cells in leaves.

**Materials and Methods**

**Plant materials and growth conditions**

*Arabidopsis thaliana* ecotype Landsberg erecta (Ler) was used as the wild type. Two mutant alleles of COV1, cov1-1 and cov1-2,
were previously reported (Parker et al. 2003). These are probably not null mutants but each of them possesses a single amino acid substitution. We crossed a plant that was homozygous for cov1-1 with homozygotes of cov1-2 to obtain cov1 F1 seeds. We used the following transgenic Arabidopsis lines: mRFP–SYR43 (Uemura et al. 2012), VHA-a1–mRFP (Dettmer et al. 2006), VHA-a1–sGFP (Dettmer et al. 2006) and GFP–PEP12 (Uemura et al. 2010). Seeds were surface sterilized with 70% ethanol and then sown onto 0.5% (w/v) Gellan Gum (Wako) that contained 1% (w/v) sucrose and Murashige and 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 μE s⁻¹ m⁻²). The plants were transferred onto vermiculite for subsequent growth.

Transgenic plants

Translational fusions between the cDNA for fluorescent proteins and COV1 (At2g20120) were generated by the technique of fluorescent tagging of full-length proteins [FTFLP (Tian et al. 2004)] using the following primer sets: COV1GF-F, COV1-sGFP/TagRFP-1, COV1-sGFP/TagRFP-2, COV1-sGFP/TagRFP-3, COV1-sGFP/TagRFP-4 and COV1GF-R. The cDNAs encoding GFP and TagRFP were inserted downstream of COV1. The amino acid linker between the fluorescent proteins and COV1 was GGGGGA. The amplified DNA COV1-sGFP and COV1-TagRFP fragments were cloned into the pENTR D-TOPO plasmid (Invitrogen) and pENTR D-TOPO, respectively. We introduced these plasmids into binary vectors, pBGW and pDONR P4-P1R (Invitrogen) and pENTR D-TOPO, respectively. Subsequently, COV1-sGFP and COV1-TagRFP were cloned into the pTGG2:VENUS construct, the 2 kb promoter region of At3g52850 (Shimada et al. 2003) were cloned into pDONR P4-P1R (Invitrogen) and pENTR D-TOPO, respectively. To generate the pTGG2:VENUS construct, the 2 kb promoter region of At3g25980 (TGG2) and the vacuole-targeting VENUS sequence (Nagai et al. 2002) were cloned into pDONR P4-P1R (Invitrogen) and pENTR D-TOPO, respectively. These two plasmids were introduced into the binary vector R4pGWBS13 (Nakagawa et al. 2008) by the LR reaction to generate pTGG2:VENUS. To generate the MYR001::GUS construct, the 2 kb promoter region of At3g25850 (VR1) was amplified, and the 35S promoter region of the binary vector pBI121 was replaced with the amplified fragment using restriction enzymes. To generate the MYR001::TagRFP construct, the same region of At3g25850 (VR1) was cloned into pENDR D-TOPO. Next, we introduced this plasmid into the binary vector pGWB459 (Nakagawa et al. 2007) using an LR reaction. Agrobacterium tumefaciens (strain GV3101) was transformed with these constructs. Plants were infected with the bacteria by the floral dip method (Clough and Bent 1998). T1 seeds were selected in the 2 kb promoter region of At3g52850 (Shimada et al. 2008) by the LR reaction to generate the MYR001::TagRFP construct and the same region of At3g25850 (VR1) was cloned into pENDR D-TOPO. True leaves were harvested from wild-type and mutant plants at 30 d after germination. Analysis of the vein pattern was performed as described previously (Shirakawa et al. 2009).

Chemical treatment

To visualize BFA compartments, the COV1-sGFP-expressing seedlings were stained with 5 μM FM4-64 for 6 min, followed by 1 h incubation in 50 μM BFA at room temperature before proceeding to confocal laser scanning microscopy (CLSM). For ConA treatment, seedlings were incubated in 2 μM ConA for 30 min.

Confocal laser scanning microscopy

The fluorescence of transgenic plants was inspected with a confocal laser scanning microscope (LSM780; Carl Zeiss). The data were processed using Adobe Photoshop (Adobe Systems).

Ultrastructure analysis

Cotyledons were harvested from wild-type and mutant plants at 5 d after germination. Ultrastructural analysis was conducted by the Tokai Electron Microscopy service using the freeze-substitution fixation method. Procedures for microscopic analysis were essentially as described previously (Baba 2008).

SDS–PAGE and immunoblot analysis

SDS–PAGE and immunoblot analysis were performed as described previously (Ueda et al. 2006). Antibodies used were anti-TGG1 (diluted 5,000-fold) (Ueda et al. 2006), anti-12S globulin (diluted 10,000-fold) (Shimada et al. 2003) and anti-253P (diluted 5,000-fold) (Li et al. 2006).

Immunohistochemistry

The methods of preparation of thin sections and the immunofluorescence analysis were slightly different from those described previously (Ueda et al. 2006). Dry seeds were fixed with 4% (w/v) paraformaldehyde and 0.75% glutaraldehyde in 0.05 M cacodylate buffer (pH 7.4) containing 10% dimethylsulfoxide (DMSO) at room temperature for 2 h. After washing with 0.05 M cacodylate buffer (pH 7.4), these tissues were dehydrated with dimethylformamide and embedded in LR white resin at −20°C. Sections were cut on an ultramicrotome (Leica, Reichert Division) for both light microscopic and electron microscopic analyses. The thin sections were fixed on MAS (Matsunami adhesive silane)-coated glass slides (Matsunami Glass Ind.) to perform further analyses. For the immunofluorescence analysis, after incubation in phosphate-buffered saline (PBS; 10 mM phosphate buffer, pH 7.4 and 8.5% NaCl) containing 1% bovine serum albumin (Sigma) for 30 min, the slides were incubated with anti-12S globulin antibody (diluted 200-fold in PBS) at 4°C overnight. After washing three times with PBS for 5 min, the slides were incubated with secondary antibodies, Alexa Fluor 488 goat anti-rabbit IgG (H+L) (Molecular Probes), for 2 h at room temperature. The secondary antibodies were diluted 250-fold. After washing three times with PBS for 5 min, the slides were examined with a fluorescence microscope.

Analysis of vein pattern

True leaves were harvested from wild-type and mutant plants at 30 d after germination. Analysis of the vein pattern was performed as described previously (Shirakawa et al. 2009).
GUS staining

True leaves were harvested from wild-type and mutant plants at 15 d after germination. Samples were first placed into ice-cold acetone for 15 min and then into GUS staining solution containing 0.5 mg ml\(^{-1}\) X-Gluc, 0.1 M sodium phosphate buffer, pH 7.0, 10 mM EDTA, 0.5–5 mM potassium ferricyanide, 0.5–5 mM potassium ferrocyanide and 0.1% Triton X-100. Samples in the GUS staining solution were placed under a vacuum and incubated at room temperature for 12–24 h.

Histological analysis

After GUS staining of true leaves at 19 d after germination, samples were cut into slices with a razor blade and then fixed with 4% (w/v) paraformaldehyde, 1% (v/v) glutaraldehyde, 0.05 M cacodylate buffer, pH 7.4, and 10% DMSO. Procedures for microscopic analysis were essentially as described previously (Hara-Nishimura et al. 1993). Ultrathin sections of 1 μm were stained with CBB.

Supplementary data

Supplementary data are available at PCP online.

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Disclosures

The authors have no conflicts of interest to declare.

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