

# Qa-SNAREs localized to the *trans*-Golgi network regulate multiple transport pathways and extracellular disease resistance in plants

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In all eukaryotic cells, a membrane-trafficking system connects the post-Golgi organelles, such as the *trans*-Golgi network (TGN), endosomes, vacuoles, and the plasma membrane. This complex network plays critical roles in several higher-order functions in multicellular organisms. The TGN, one of the important organelles for protein transport in the post-Golgi network, functions as a sorting station, where cargo proteins are directed to the appropriate post-Golgi compartments. Unlike its roles in animal and yeast cells, the TGN has also been reported to function like early endosomal compartments in plant cells. However, the physiological roles of the TGN functions in plants are not understood. Here, we report a study of the SYP4 group (SYP41, SYP42, and SYP43), which represents the plant orthologs of the Tlg2/syntaxin16 Qa-SNARE (soluble *N*-ethylmaleimide sensitive factor attachment protein receptor) that localizes on the TGN in yeast and animal cells. The SYP4 group regulates the secretory and vacuolar transport pathways in the post-Golgi network and maintains the morphology of the Golgi apparatus and TGN. Consistent with a secretory role, SYP4 proteins are required for extracellular resistance responses to a fungal pathogen. We also reveal a plant cell-specific higher-order role of the SYP4 group in the protection of chloroplasts from salicylic acid-dependent biotic stress.

*Arabidopsis* | membrane traffic | membrane fusion

In eukaryotic cells, membrane fusion is an essential process in protein secretion and endocytosis (1, 2). Selective membrane fusion occurs with the concerted functions of several molecules, including SNAREs (soluble *N*-ethylmaleimide sensitive factor attachment protein receptors), Rab GTPases, tethering factors, and Sec1p/Munc18 (SM) proteins. SNAREs are membrane-anchored proteins that contain  $\alpha$ -helical heptad repeats and a characteristic central amino acid within the SNARE motif. The SNARE complex comprises four SNAREs, including three with a central glutamine residue in SNARE motif (Q-SNAREs: Qa, Qb, and Qc) and one with a central arginine (R-SNARE) in SNARE motif. The Q-SNAREs reside on the target membrane, and the R-SNARE resides on the transport vesicle. This complex first forms a bridge between the target organelle membrane and the vesicle, and then compresses to bring the two membranes close enough to mediate specific membrane fusion. After fusion is complete, the SNARE complex is dissociated by a NSF (*N*-ethylmaleimide-sensitive factor), and the SNAREs are recycled. To execute the correct membrane fusion, SNAREs must be localized on the membrane of specific organelles or transport vesicles. Qa-SNAREs also serve as organelle markers (3–5), by virtue of their specific localization on the membrane of target organelles.

The TGN was first defined as a special organelle on the *trans*-side of the Golgi stack that is responsible for protein sorting to the plasma membrane or lysosomes (6). The TGN contains multiple sorting domains and functions as the compartment of cargo sorting. In addition, the TGN is the interface between the Golgi apparatus

and endocytic pathways (7). Syntaxin 16, a TGN-localized Qa-SNARE in animals, functions in the retrograde transport from early endosomes or recycling endosomes to the TGN (8–10). Tlg2, the yeast ortholog of syntaxin 16, plays a role in transport in the endocytic pathway, but not the secretory pathway. Mutants with a disrupted *Tlg2* gene grow slowly, but remain viable (11–13). In plants, the TGN was also reported to function as the sorting station for secretory and endocytosed cargos (14). In addition, recent evidence has indicated that the plant TGN also functions as the early endosome, the first compartment in the endocytic pathway (15). However, the physiological function of the plant orthologs of the TGN-localized Qa-SNAREs (the SYP4 group) is not fully understood. In this study, we aimed to elucidate the physiological role of the SYP4 group (SYP41, SYP42, and SYP43) in *Arabidopsis*.

## Results and Discussion

***Arabidopsis* SYP4 Group Possesses Redundant Functions.** In *Arabidopsis*, the SYP4 group represents the plant orthologs of Tlg2/syntaxin16 and comprises three proteins, SYP41, SYP42, and SYP43 (16, 17). Previous studies reported that SYP41 and SYP42 localized to different subdomains of the TGN and had non-redundant functions because individual *syp41* and *syp42* knockout mutants were gametophytic lethal (18–20). However, in the present study, we found that individual, single homozygous mutants (*syp41*, *syp42*, and *syp43*) were fully viable in the Col-0 accession. We found that the root length of the *syp42* mutant was slightly shorter than that of the wild-type plant, and the *syp41* and *syp43* mutants exhibited no visible abnormalities. The double mutants, *syp41*<sup>-/-</sup>*syp42*<sup>-/-</sup> and *syp41*<sup>-/-</sup>*syp43*<sup>-/-</sup>, also showed shorter and normal roots like their parents, respectively. However, in the case of the *syp42*<sup>-/-</sup>*syp43*<sup>-/-</sup> double mutant (*syp42syp43*), we observed severe pleiotropic defects, including short roots, a large number of lateral roots, semidwarfism, and early senescence (Fig. 1 *A* and *B* and Fig. S1*a*). Further genetic analyses among the *syp4* mutants revealed that *syp41*<sup>+/-</sup>*syp42*<sup>-/-</sup>*syp43*<sup>-/-</sup> mutants (heterozygous for *syp41* and homozygous for *syp42* and *syp43*) were seedling lethal (Fig. 1*A*, inset). In contrast, *syp41*<sup>-/-</sup>*syp42*<sup>+/-</sup>*syp43*<sup>-/-</sup> mutants grew almost normally except for short roots like the *syp42* single mutant. *syp41*<sup>-/-</sup>*syp42*<sup>-/-</sup>*syp43*<sup>+/-</sup> mutants were not lethal but showed phenotypes of short roots, small and white cotyledons, and

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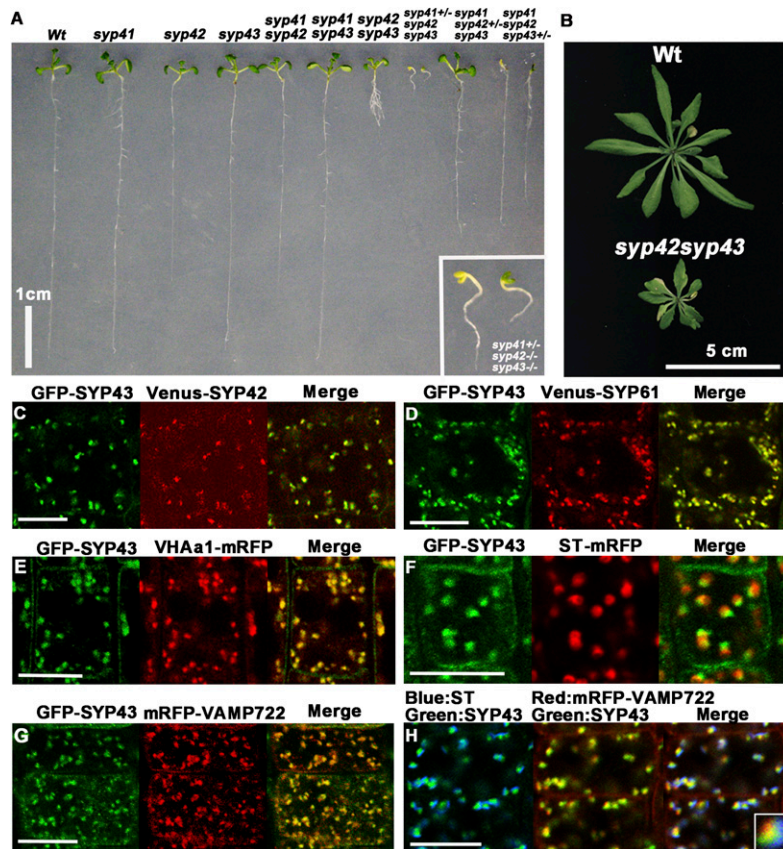
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**Fig. 1.** Members of the SYP4 group have redundant functions. (A) Phenotypes of each *syp4* single mutant and all combinations of SYP4 multiple mutants at the seedling stage. Wild-type (Wt) and mutant plants are 12 d old. (B) Phenotypes of the aerial parts of Wt and *syp42syp43* plants at 35 d. (C–G) GFP-SYP43 colocalized with Venus-SYP42, Venus-SYP61, and VHAa1-mRFP and partially overlapped with ST-mRFP and mRFP-VAMP722. (H) Fluorescent images of root epidermal cells from transgenic plants that expressed GFP-SYP43, ST-Venus, and mRFP-VAMP722. The inset represents the magnified image of a dot-like structure with triple labeling. [Scale bars in C–H: 10  $\mu$ m.]

normal rosette leaves (Fig. 1A and Fig. S1a). We obtained no triple homozygous mutants (*syp41*<sup>-/-</sup>*syp42*<sup>-/-</sup>*syp43*<sup>-/-</sup>) in progeny derived from self-pollinated *syp41*<sup>-/-</sup>*syp42*<sup>+/-</sup>*syp43*<sup>-/-</sup> or *syp41*<sup>-/-</sup>*syp42*<sup>-/-</sup>*syp43*<sup>+/-</sup> mutants. The observed segregation ratio suggested that the triple mutant was impaired in fertilization competence (Tables S1 and S2). These results indicated that SYP41, SYP42, and SYP43 possessed partly overlapping functions and that SYP42 and SYP43 played major roles in the Col-0 accession.

#### Members of the SYP4 Group Localize on the Same TGN Compartment.

To determine the subcellular localizations of the SYP4 group members, we generated transgenic plants expressing SYP4 proteins with fluorescent [green fluorescent protein (GFP), Venus, and monomeric red fluorescent protein (mRFP)] tags, under the control of their respective, native, 5' regulatory sequences. The expression of GFP-tagged SYP42 and SYP43 proteins complemented the abnormalities of the *syp42syp43* mutant, indicating that these GFP-tagged proteins are functional (Fig. S1b). Interestingly, overexpression of GFP-SYP41 (wild-type copies plus externally added GFP-SYP41) also rescued the defects in the *syp42syp43* mutant. This result further supported the notion that the proteins of the SYP4 group had partly overlapping functions. Coexpression of Venus-SYP42 and GFP-SYP43 revealed that they were completely colocalized in root cells (Fig. 1C). In addition, expression of individual, GFP-tagged SYP4 proteins (GFP-SYP41, GFP-SYP42, and GFP-SYP43) demonstrated that they were colocalized with the TGN marker Venus-SYP61 (Fig. 1D and Fig. S2). Further observation with other organelle markers indicated that GFP-SYP43 colocalized with VHAa1-

mRFP (vacuolar ATPase  $\alpha$ 1 subunit; a TGN marker) (Fig. 1E) but not with ST-mRFP (cytoplasmic and transmembrane domains of sialyl transferase; a *trans*-Golgi marker) (Fig. 1F). VAMP722, an *Arabidopsis* R-SNARE, was reported to be localized on mobile, punctate structures; moreover, VAMP722 was shown to function in the secretory pathways that regulate the plant immune response (21). We found that mRFP-VAMP722 partially colocalized with SYP43 but not with ST-Venus or ARA6-GFP (an endosomal marker) (Fig. 1G and Fig. S2b and c). Furthermore, mRFP-VAMP722 generated aggregations after treatment with Brefeldin A (BFA) (Fig. S2c). BFA blocks the function of BFA-sensitive ARF-GEFs, and this leads to the aggregation of the TGN and endosomes in *Arabidopsis* root and cotyledon cells, whereas BFA blocks the retrograde membrane traffic between the Golgi and the endoplasmic reticulum (ER), leading to a block of the early secretory pathway in mature leaves (22). Plants that coexpressed GFP-SYP43, ST-Venus, and mRFP-VAMP722 showed that GFP-SYP43 appeared to localize between the *trans*-Golgi cisternae labeled with ST-Venus and the compartments labeled with mRFP-VAMP722 (Fig. 1H). This observation suggested that mRFP-VAMP722 might localize to the more *trans* side of the SYP43-labeled compartment for secretion. Taken together, these results indicated that SYP41, SYP42, and SYP43 localized on the same TGN compartment and had partly overlapping functions.

**SYP4 Group Regulates Secretory and Vacuolar Transport.** To determine the physiological significance of the SYP4 group in plants, we further investigated the nonlethal *syp42syp43* mutant



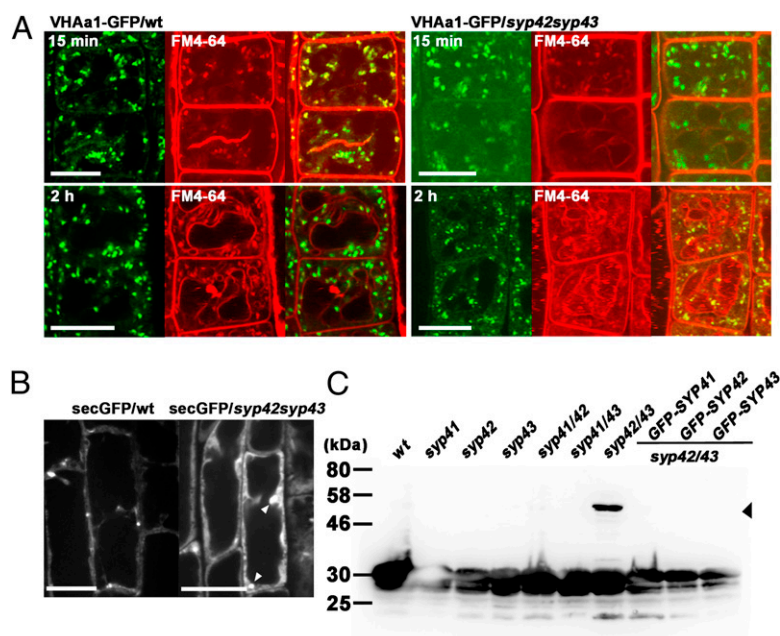
that exhibited several abnormalities. First, we evaluated SYP4-mediated transport in wild type and *syp42syp43* mutant with a tracer of the endocytic pathway, FM4-64. In wild type, 15 min after adding FM4-64, the dye appears on the part of the TGN labeled by VHAa1-GFP, then, after 2 h, it appears on the vacuolar membrane. Notably, in the *syp42syp43* mutant, after 15 min, we observed weak FM4-64 staining on the vacuolar membrane (Fig. 2A). This early arrival of FM4-64 to the vacuolar membrane may have been attributable to impaired recycling from the TGN to the plasma membrane or bypass transport from the plasma membrane to late endosomes. These results indicated that internalization from the plasma membrane to the endocytic pathway was not impaired in the *syp42syp43* mutant.

Next, we used secGFP, a signal peptide added to a variant of GFP, to analyze the secretory pathway. In wild type, secGFP is synthesized in the ER, traverses the secretory pathway and is finally secreted to the apoplast. In the *syp42syp43* mutant cells, we clearly observed secGFP accumulation (Fig. 2B, compare with the wild type), which suggested that secGFP was not secreted. Furthermore, secGFP fluorescence did not accumulate inside the vacuoles after dark treatment (Fig. S3), which suggested that secGFP was not misdirected to the vacuole. These results indicate that the secretory pathway was defective in the *syp42syp43* mutant.

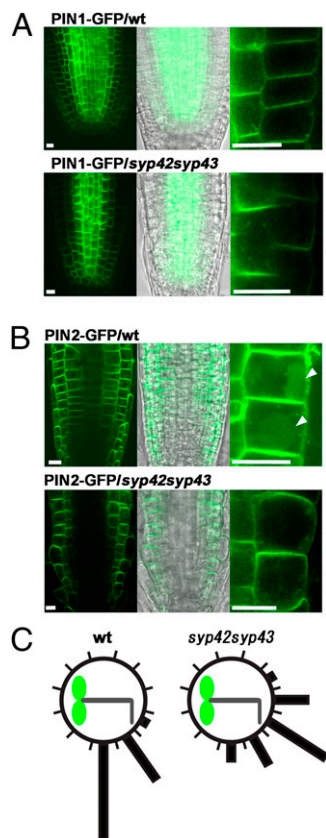
We also investigated the vacuolar transport pathway by monitoring the processing of a seed storage protein, 12S globulin, in dry seeds (23). The precursors of 12S globulin did not accumulate in any of the single mutants or in the double mutants, *syp41syp42* or *syp41syp43*. In contrast, 12S globulin precursor accumulation was substantial in the *syp42syp43* mutant (Fig. 2C). This accumulation of precursors disappeared in the mutant complemented with GFP-tagged SYP4s (Fig. 2C). One possible explanation for this outcome might be that the *syp42syp43* mutant was impaired in the recycling of vacuolar sorting receptors from the late endosomes/prevacuolar compartment (LE/PVC) to the TGN. These findings clearly indicated that the plant SYP4 group members were involved in multiple transport pathways, including the

secretory pathway, the vacuolar transport pathway, and perhaps the retrieval pathway from the LE/PVC to the TGN.

In addition, we investigated the subcellular localization of the PIN proteins (auxin efflux carriers), which are localized on the plasma membrane with a characteristic polarity. As shown in Fig. 3A and B, the expression of PIN1-GFP (24) and PIN2-GFP (24) in *syp42syp43* mutant was a little restricted compared with the wild type, but the polar localization of these proteins was not disturbed. These results could imply that, although the SYP4 group played a substantial role in the secretory pathway (as shown by the secGFP results), it is not necessarily required for all plasma membrane-localized cargos. The PIN2 protein is constitutively recycled and transported to the vacuole for degradation (25). Indeed, we observed that the fluorescent signal from PIN2-GFP was observed in the vacuole under dark conditions in wild type (Fig. 3B, arrowheads). However, in the *syp42syp43* mutant, PIN2-GFP did not accumulate in the vacuole (Fig. 3B). These results implied that the vacuolar transport of PIN2-GFP was impaired. Consequently, we reasoned that this might result in disturbed auxin distribution in the *syp42syp43* mutant. Thus, we monitored auxin distribution by expressing a reporter gene that carried the synthetic auxin response element DR5 fused to the GFP gene (26). Activation by auxin induces proportional expression of the GFP reporter, and thus, reflects the auxin distribution. Until 5 days after germination (DAG), the pattern of fluorescence signals from DR5::GFP in the *syp42syp43* mutant was not altered compared with the wild type. However, from 7 DAG, the fluorescent pattern of the *syp42syp43* mutant began to show clear difference from the wild type (Fig. S4a). The GFP fluorescence became more restricted in the quiescent center of the *syp42syp43* mutant (Fig. S4b). Consistent with these results, the *syp42syp43* mutant showed a weak defect in root gravitropism (Fig. 3C). In addition, this disturbed auxin distribution might be responsible for developmental abnormalities, like the short roots and the large number of lateral roots observed in the *syp42syp43* mutant.



**Fig. 2.** SYP4 group regulates secretory and vacuolar transport and maintains Golgi/TGN morphology. (A) Uptake of FM4-64 by endocytosis is normal in wild-type (wt) (Left) and *syp42syp43* mutant plants (Right). FM4-64 is transported to the part of the TGN labeled with VHAa1-GFP (15 min) and to the vacuoles (2 h). (Scale bars: 10  $\mu$ m.) (B) secGFP accumulated inside *syp42syp43* cells. Arrowheads show the strong signal from secGFP accumulation within the cell. (Scale bars: 10  $\mu$ m.) (C) Immunoblot of seed proteins probed with the anti-12S globulin antibody. Arrowhead indicates unprocessed precursors.



**Fig. 3.** *syp42syp43* mutants exhibit defective PIN2-GFP transport to the vacuole. (A) Expression and subcellular localization of PIN1-GFP in wild type (wt) and the *syp42syp43* mutant. Mislocalization of PIN1-GFP was not observed in the *syp42syp43* mutant. Plants were grown in continuous light for 7 d. (B) Expression and subcellular localization of PIN2-GFP in wt and the *syp42syp43* mutant. Accumulation of PIN2-GFP in the vacuole (arrowheads) was not detected in the *syp42syp43* mutant. Plants were grown in continuous light for 5 d and transferred to dark conditions. (Scale bars: 10  $\mu$ m.) (C) Phenotype of root gravitropism in the *syp42syp43* mutant. Plants were grown in continuous light for 5 d and transferred to dark conditions. Plants were grown in dark conditions for 1 d vertically and turned counter clockwise through 90°. After 24 h incubation, the angles of the roots were measured.

**SYP4 Proteins Are Required to Maintain the Morphology of the Golgi/TGN.** To address whether the *syp42syp43* mutant had altered TGN properties, we treated the plants with BFA and then analyzed the TGN. In wild-type cells, BFA treatment (25  $\mu$ M; 30 min) caused aggregation of the TGN compartment labeled with VHAa1-GFP. In the *syp42syp43* mutant, the aggregation was markedly delayed in cells from roots and leaves (cotyledons) (Fig. S5 *a* and *b*). In contrast, there was no difference between wild type and mutants by treatment with wortmannin (Wm) (a PI3-kinase inhibitor) (Fig. S5*a*).

We also observed the morphology of the TGN at the ultrastructural level in the *syp42syp43* mutant by transmission electron microscopy. The morphology of the *trans* side of the Golgi apparatus, including the *trans*-Golgi cisternae and the TGN, was altered in the *syp42syp43* mutant. The curved *trans* cisternae of the Golgi and TGN were clearly observed in the *syp42syp43* mutant compared with the wild type (Fig. 4*A*). Quantitative analysis measuring the angles of the last cisternae of the *trans* side of the Golgi apparatus, we found that the Golgi or TGN of the *syp42syp43* mutants had a larger number of curved *trans* cisternae than observed in the wild type. In contrast, the total number and lengths of cisternae within individual stacks were not altered compared with the wild type (Fig. 4 *B–D*). In the *syp41<sup>+/-</sup>syp42<sup>-/-</sup>syp43<sup>-/-</sup>*

triple mutant, which exhibited a severe phenotype (Fig. 4*A*, inset), we observed several unusual structures in the Golgi/TGN morphology, including a horseshoe-like shape and a small number of cisternae (Fig. 4*E*). These results implied that the SYP4 group members were required to maintain the morphology of the Golgi/TGN, probably by regulating post-Golgi trafficking.

**SYP4 Group Plays Plant Cell-Specific, Higher-Order Roles.** To reveal the physiological roles of the SYP4 group, we focused on stress responses and investigated the response of the *syp42syp43* mutant to biotic or abiotic stress. Through these experiments, we found prominent phenotypes after inoculation with pathogenic powdery mildew fungi. We noted a striking leaf chlorosis of *syp42syp43* plants 7 d after pathogen challenge with conidiospores of the host-adapted virulent powdery fungus *Golovinomyces orontii* (27) (Fig. 5*A*). This chlorosis is dependent on an intact pathogen-inducible salicylic acid (SA) biosynthesis pathway because leaves of *syp42syp43sid2* triple mutants, lacking additionally the chloroplast-targeted SID2 isochorismate synthase (28, 29), showed a wild-type-like green leaf coloration (Fig. 5*A*). These findings point to a potential chloroplast dysfunction in *syp42syp43* plants during SA-dependent biotic stress. Despite the leaf chlorosis and semidwarfism, *G. orontii* pathogenesis on *syp42syp43* plants was indistinguishable compared with the wild type, as evidenced by the development of a profuse fungal mycelium on the leaf surface in time-series experiments and microscopic inspection of leaf specimen (Fig. S6). The *syp42syp43* mutants also retain the ability to accumulate high SA levels in response to powdery mildew challenge albeit SA levels in non-inoculated *syp42syp43* plants are moderately elevated in the absence of pathogen (Fig. 5*B*). Consistent with an intact SA-dependent defense signaling pathway in *syp42syp43* plants, we observed a fungus-induced activation of the defense response marker gene *PR-1* but not in *sid2* or *syp42syp43sid2* genotypes (Fig. 5*C*). Thus, the biotic stress-induced and SA-dependent chloroplast dysfunction of *syp42syp43* plants occurs in the presence of an otherwise intact pathogen-induced and SA-dependent defense signaling. Together, this reveals an unexpected link between the TGN and chloroplasts and points to a plant-specific higher-order role of the TGN-resident SYP4 group in protecting chloroplasts from SA-dependent biotic stress.

Next, we inoculated *syp42syp43* plants with the nonadapted powdery mildew fungus *Erysiphe pisi*, the pathogenesis of which on the wild type is terminated at an early stage because of pre- and postinvasive resistance responses (27, 30). In comparison with wild type, *syp42syp43* plants fail to effectively restrict hyphal branching of individual *E. pisi* microcolonies (Fig. 5*D* and Fig. S7). Because this step in fungal pathogenesis requires entry of fungal germlings into host cells (30, 31), this assigns to SYP42 and SYP43 a function in secretion-dependent disease resistance responses.

**Possible Mechanism for the Higher-Order Role of the SYP4 Group.** In this study, we demonstrated that the SYP4 group maintained the morphology of the Golgi apparatus and TGN. Although the SYP4 group was localized on the TGN, the morphology of the Golgi apparatus in the *syp42syp43* mutant was abnormal. The mechanism of transport between the *trans*-Golgi cisternae and the TGN remains unknown, even in animal and yeast cells. Thus, it was not clear why the Golgi apparatus morphology was affected in the *syp42syp43* mutant. One possibility is that the SYP4 group might mediate membrane fusion between the transport vesicles budding from the *trans*-Golgi cisterna and the TGN. In that case, in the *syp42syp43* mutant, the abnormal morphology of Golgi apparatus might result from the accumulation of transport vesicles that left the *trans*-Golgi cisternae but could not fuse with the TGN. Another possibility is that the *trans*-Golgi cisternae might not mature sufficiently to form the TGN in the *syp42syp43* mutant because of the defective TGN function, given that the SYP4 group mediated





extracellular defense responses: a defense cargo in the TGN might be sorted by the SYP4 group and delivered via VAMP721/722-containing vesicles to the plasma membrane for subsequent PEN1 (penetration1)-dependent exocytosis in a secretory defense pathway (21, 32). The current findings assign to the TGN compartment a role as an entry portal to post-Golgi transport pathways for several higher-order plant functions. Interestingly, despite the leaf chlorosis of infected *syp42syp43* plants, pathogen-inducible SA accumulation, *PR-1* expression, and pathogenesis of host-adapted *G. orontii* remain largely unaffected in the double mutant. Thus, the SYP4 group is not essential, per se, for the responsiveness of the plants to biotic stress. Our data, instead, show that SYP4 regulates a pathogen-inducible and SA-dependent pathway required for chloroplast function during biotic stress. This points to an unexpected functional link between the TGN and chloroplasts, although it remains to be seen whether this link is direct. For example, SYP4 proteins in the TGN might be essential for sorting nucleus-encoded proteins targeted to chloroplasts during SA-dependent defense responses. Another possibility is that the TGN directly contacts chloroplasts to exchange materials. Taken together, our study reveals the engagement of the SYP4 group in multiple intracellular transport pathways, thereby highlighting functional links between the post-Golgi network and higher-order processes in plants.

## Materials and Methods

Electron microscopy, immunoblot analysis, root gravitropism assay, pathogen assays, and SA measurements were performed as described in *SI Materials and Methods*.

**Plant Materials and Plasmids.** The *syp41* (Salk\_060613), *syp42* (Salk\_116966), and *syp43* (Salk\_144268) *Arabidopsis thaliana* mutants were obtained from the *Arabidopsis* Biological Resource Center. Details regarding plant materials and plasmids are described in *SI Materials and Methods*.

**Confocal Laser-Scanning Microscopy.** For single-color imaging, GFP and mRFP were excited by diode-pumped, solid-state lasers (Cobolt Blues and Cobolt Jive; Cobolt) at 473 nm and observed under a microscope (BX51; Olympus) equipped with a confocal scanner unit (CSU10; Yokogawa Electric) and a cooled CCD camera (ORCA-AG; Hamamatsu Photonics). Multicolor observations were carried out with a LSM710 confocal microscope (Carl Zeiss). Details regarding drug treatment analysis are available in *SI Materials and Methods*.

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