Running title: Role of TNO1 in vesicle trafficking

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TNO1 is involved in salt tolerance and vacuolar trafficking in *Arabidopsis thaliana*

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Abstract

The Arabidopsis t-SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) SYP41 is involved in vesicle fusion at the trans-Golgi network (TGN) and interacts with AtVPS45, SYP61 and VTI12. These proteins are involved in diverse cellular processes, including vacuole biogenesis and stress tolerance. A previously uncharacterized protein, named TNO1, was identified by co-immunoprecipitation as a SYP41-interacting protein. TNO1 was found to localize to the TGN by immunofluorescence microscopy. A tno1 mutant showed increased sensitivity to high concentrations of NaCl, KCl and LiCl, and also to mannitol-induced osmotic stress. Localization of SYP61, which is involved in the salt stress response, was disrupted in the tno1 mutant. Vacuolar proteins were partially secreted to the apoplast in the tno1 mutant, suggesting that TNO1 is required for efficient protein trafficking to the vacuole. The tno1 mutant had delayed formation of the Brefeldin A (BFA) compartment in cotyledons upon application of BFA, suggesting less efficient membrane fusion processes in the mutant. Unlike most TGN proteins, TNO1 does not relocate to the BFA compartment upon BFA treatment. These data demonstrate that TNO1 is involved in vacuolar trafficking and salt tolerance, potentially via a role in vesicle fusion and in maintaining TGN structure or identity.
Introduction

The plant vacuole is a versatile organelle that has important functions including maintaining turgor, ion homeostasis, compartmentalizing toxic material, accumulating defense compounds and storing and degrading proteins (Marty, 1999). To maintain these functions, correct transport of vacuolar proteins is required. Vacuolar proteins are synthesized at the ER, and are transported co-translationally into the ER lumen. From the ER, they are transported to the trans-Golgi network (TGN) via the Golgi (Tormakangas et al., 2001). In the TGN, vacuolar proteins with sequence-specific vacuolar sorting determinants are thought to be recognized by vacuolar sorting receptors (VSRs) (Kirsch et al., 1994; Ahmed et al., 2000; daSilva et al., 2005), although a recent report has suggested that recognition of cargo by VSRs may occur as early as the ER (Niemes et al., 2010). Storage proteins with C-terminal sorting sequences are also bound by VSRs or by a second putative sorting receptor, RMR1 (Jiang et al., 2000; Shimada et al., 2003a; Park et al., 2005; Hinz et al., 2007; Park et al., 2007). After the receptors recognize their cargo, vacuolar proteins are transported to the pre-vacuolar compartment (PVC) (Sanderfoot et al., 1998; Happel et al., 2004; Song et al., 2006) before transport on to the vacuole. In mammalian cells, cargo receptors are recycled from their destination to the site of cargo binding (Seaman, 2005). VSR1 was found to localize to the TGN and the PVC (Paris et al., 1997; Sanderfoot et al., 1998); thus, VSR1 was also suggested to cycle between these organelles.
SNAREs are integral membrane proteins required for the fusion of vesicles with their target membrane. SNAREs can be classified based on their site of function: v (vesicle) -SNAREs are localized to the vesicle membrane and t (target) -SNAREs are localized to the target membrane (Rothman, 1994; Sogaard et al., 1994). Generally, one v-SNARE on the vesicle and three t-SNAREs on the target membrane form a trans-SNARE complex, bringing the two membranes in contact and driving membrane fusion (McNew et al., 2000). Clathrin-coated vesicles containing VSR1 contain the v-SNARE VTI11, which forms a SNARE complex with SYP5, SYP2 and VAMP727 at the PVC during anterograde trafficking from the TGN (Kirsch et al., 1994; Sanderfoot et al., 2001a; Ebine et al., 2008). However, the mechanism of recycling of VSR1 from the PVC to the TGN is not yet fully understood. A complex containing the SNAREs SYP41, SYP61 and VTI12 and the Sec1/ Munc18-like protein AtVPS45, a potential regulator of vesicle fusion (Dulubova et al., 2002; Bryant and James, 2003), is found at the TGN in Arabidopsis (Bassham and Raikhel, 1998; Bassham et al., 2000). SNAREs in the SYP41 complex, along with another SNARE YKT61/62, were found to be sufficient for vesicle fusion in vitro (Chen et al., 2005). Recently, AtVPS45 was suggested to be involved in recycling of VSR1, as RNAi knockdown of AtVPS45 expression causes mis-localization of VSR1 (Zouhar et al., 2009).

SYP41 and SYP42 are highly homologous t-SNAREs with 62% amino acid sequence identity. However, they form separate complexes with AtVPS45 in different subdomains of the TGN (Bassham et al., 2000) and are not functionally redundant, as individual syp41 and syp42 knockout mutants are gametophytic lethal (Sanderfoot et al., 2001b). A mutation in SYP61, another t-SNARE in the SYP41 complex (Zhu et al., 2002), caused defects in
osmotic stress tolerance and ABA regulation of stomatal responses. The v-SNARE VTI12 is involved in trafficking of storage proteins (Sanmartin et al., 2007) and a vti12 mutant showed an early senescence phenotype under starvation conditions, suggesting that VTI12 is also involved in the autophagy pathway for the degradation of cellular contents (Surpin et al., 2003). In addition to their function together in vesicle fusion, components of the SYP41 complex therefore also appear to be involved in distinct processes, suggesting they may function in additional SNARE complexes.

In this study, we identified a novel SYP41-interacting protein that we named TNO1 (TGN-localized SYP41 interacting protein). TNO1 is a membrane protein at the TGN and is required for efficient vacuolar trafficking, consistent with the hypothesis that it functions together with the SYP41 SNARE complex. A tno1 knockout mutant has a salt and osmotic stress-sensitive phenotype, possibly due to the partial mislocalization of SYP61. A Brefeldin A (BFA)-treated tno1 mutant showed a delay in formation of the BFA compartment indicating that TNO1 could be important in TGN/endosome fusion events. We propose that TNO1 is involved in vacuolar trafficking and salt stress resistance by facilitating the vesicle fusion process.
Results

Identification of TNO1

SYP41 is a t-SNARE at the TGN that is required for vesicle fusion in *Arabidopsis thaliana* and forms a complex with VTI12, SYP61 and AtVPS45 (Bassham and Raikhel, 2000; Chen et al., 2005). To identify additional SYP41-interacting proteins, detergent-solubilized membrane extracts of Arabidopsis suspension cells were immunoprecipitated using SYP41 antibody. Proteins that co-immunoprecipitated with SYP41, or with pre-immune antibody as a control, were separated by SDS-PAGE and visualized by silver staining. Four bands corresponding to proteins migrating at approximately 200, 67, 35 and 15 kD were found only in the SYP41 precipitate and were not present in the SYP41 pre-immune precipitate, suggesting that they specifically interacted with SYP41 (Fig. 1A).

The 200, 67, 35 and 15 kD bands were analyzed by tandem mass spectrometry, comparing the fragmentation patterns with the predicted Arabidopsis proteome. The 67 and 15 kD bands were identified as AtVPS45 and a degradation product of SYP61, respectively (Sanderfoot et al., 2001a). The 35 kD band contained SYP41, SYP42 and also SYP43, an additional protein in the SYP4 family that has not yet been characterized. Nine peptides from the 200 kD band (Fig S1B, blue bars) could be matched to the predicted protein encoded by At1g24460, corresponding to 5.2% amino acid coverage, and the protein was named TNO1 (*TGN*-localized SYP41 interacting protein). A cDNA corresponding to the open reading frame of *TNO1* was obtained by RT-PCR. A comparison of the sequence of this cDNA with the sequence in The Arabidopsis Information Resource (TAIR) revealed
the presence of an additional 63 bp (from 4190 to 4252, Fig. S1A), suggesting the presence of an extra exon in our TNO1 sequence (Fig. S1B, red bar) and that either the predicted sequence in TAIR has incorrectly predicted splice sites, or multiple splice variants exist.

TNO1, a previously uncharacterized protein of predicted MW 209 kD, was predicted to have 6 coiled-coil domains in the arabi-coil database (Rose et al., 2004) based on the MultiCoil algorithm (Wolf et al., 1997) and one transmembrane domain at the extreme C-terminus by TopPred-transmembrane prediction (Fig. S2; von Heijne, 1992; Claros and von Heijne, 1994). To confirm that the approximately 200 kD band in the SYP41 precipitate corresponds to TNO1, SYP41 and pre-immune immunoprecipitates were analyzed by immunoblotting using antibodies against SYP41 or TNO1 (see below). TNO1 was detected in the SYP41 precipitate, but not in the pre-immune precipitate, confirming the correct identification of the 200 kD band (Fig. 1B).

To investigate the expression pattern of TNO1, RT-PCR was performed using RNA extracted from different Arabidopsis plant organs. TNO1 was ubiquitously expressed in roots, rosette leaves, cauline leaves and flowers, with lower expression in stems. SYP41 also showed highest expression in flowers and lowest expression in stems (Fig. 1C).

Expression of TNO1 in Arabidopsis plant organs and throughout development was also analyzed using GENEVESTIGATOR, a database and Web-browser data mining interface for Affymetrix GeneChip data (Hruz et al., 2008). The broad expression pattern observed was consistent with the RT-PCR results, with TNO1 found to be highly expressed during germination, flower development and silique maturation (Fig. S3). This expression pattern
suggests that \textit{TNO1} may have an important role throughout the plant, and particularly during reproductive stages.

\textbf{TNO1 is a TGN-localized membrane protein}

To study the function of TNO1, an antibody was raised against a 66 kD fragment from the C-terminus of TNO1. The specificity of affinity-purified TNO1 antibody was tested by immunoblotting against total cell extract, total membrane and total soluble proteins from 2 week old Arabidopsis seedlings. Purified TNO1 antibody specifically recognized a band of approximately 200 kD in Arabidopsis extracts (Fig. 2A), which was not detected by the pre-immune serum (Fig. 2B).

To investigate the potential membrane association of TNO1, the presence of TNO1 in membrane fractions was analyzed. An Arabidopsis suspension cell extract was centrifuged sequentially at 20,000 x \textit{g} and 100,000 x \textit{g}, producing three different fractions, P20 (pellet at 20,000 x \textit{g}), P100 (pellet at 100,000 x \textit{g}) and S (supernatant from 100,000 x \textit{g}). These fractions were analyzed by immunoblotting using TNO1 and SYP61 antibodies. The integral membrane protein SYP61 was detected strongly in all membrane fractions and was not present in the soluble fraction. TNO1 was also present in all the membrane fractions, but not in the soluble fraction, suggesting that TNO1 is associated with membranes (Fig. 2C). The band present above the TNO1 band in the P20 fraction corresponds to the top of the gel where occasionally some aggregated protein is seen.
To determine the mode of association of TNO1 with membranes, total membrane fractions were isolated from 2 week old seedlings and treated with 1% (v/v) Triton X-100, 2 M urea, 1 M NaCl or 0.1 M Na$_2$CO$_3$, followed by re-pelleting of non-solubilized proteins. Immunoblotting using TNO1 and SYP41 antibodies indicated that SYP41, an integral membrane protein control, was solubilized by 1% Triton X-100 and TNO1 was solubilized by 1% Triton X-100 and partially by 2 M urea (Fig. 2D), but not by 1 M NaCl or 0.1 M Na$_2$CO$_3$. This suggests that TNO1 is a membrane-integrated protein consistent with the transmembrane domain prediction of a C-terminal membrane anchor.

To analyze TNO1 subcellular localization, immunofluorescence microscopy was performed and TNO1 labeling compared with that of known markers. Cotyledons of WT and tno1 mutant seedlings (see below) were immunolabeled using purified TNO1 antibody to confirm the specificity of TNO1 labeling. In WT seedlings, TNO1 antibody labeled endogenous TNO1 in punctate structures, while no specific signal was observed in the cotyledons of tno1 mutant seedlings (Fig. 3A), confirming the specificity of the antibody. In addition, omission of the first antibody, second antibody and both first and second antibody were tested as controls for non-specific staining or cross-talk between different fluorescence channels. No specific fluorescence was observed in any of the controls (data not shown).

Purified TNO1 antibody was then used to label endogenous TNO1 in VHAa1-GFP (Dettmer et al., 2006), truncated ST-GFP (Boevink et al., 1998) or RHA1-YFP (Preuss et al., 2004) transgenic Arabidopsis seedlings, which label TGN, Golgi and PVC,
respectively. VHAa1-GFP, RHA1-YFP and ST-GFP all exhibited a dot-like pattern in cotyledons and TNO1-labeled structures completely overlapped with VHAa1-labeled structures, but were distinct from those labeled with ST-GFP or RHA1-YFP (Fig. 3B-D). This result indicates that TNO1 localizes to the TGN.

**Identification of a tno1 knockout mutant**

To analyze the function of TNO1, we obtained a knockout mutant containing a T-DNA insertion in the *TNO1* gene. A homozygous mutant was identified by PCR of genomic DNA using *TNO1* gene-specific primers (LP and RP) and a T-DNA left border primer (LBa1) in three combinations (Fig. 4A). A 900 bp fragment corresponding to the intact *TNO1* gene could be amplified from genomic DNA of WT plants using LP and RP primers. This band was absent from the *tno1* mutant due to gene disruption by the T-DNA. PCR using LBa1 and LP or RP primers generated 450 bp bands, showing the existence of a T-DNA insertion in the *tno1* mutant. This indicates that the *tno1* mutant has a complex insertion in its third exon, as T-DNA left border sequences are present at both ends of the insertion (Fig. 4A, B). Lack of TNO1 protein expression was confirmed by immunoblotting of total protein extracts from WT and *tno1* mutant plants using affinity-purified TNO1 antibodies (Fig. 4C). No difference in overall morphology or development was seen between *tno1* mutant and WT plants grown in MS medium or soil throughout their lifecycle.

*ttno1* mutant plants are sensitive to salt and osmotic stress
SYP61/ OSM1, a t-SNARE in the SYP41 complex, has been reported to be involved in general salt/ osmotic stress responses (Zhu et al., 2002). As TNO1 also interacts with SYP41, TNO1 was hypothesized to be involved in similar stress responses. Sensitivity of homozygous tno1 mutant plants to salt, ionic and osmotic stress was therefore tested. WT and tno1 mutant seedlings were grown on MS solid medium for 5 days and transferred to MS solid medium containing 130 mM NaCl (Fig. 5C, G), 140 mM KCl (Fig. 5D, H), 17 mM LiCl (Fig. 5E, I) or 300 mM mannitol (Fig. 5F, J), and root length was measured after 4 and 8 days. Under normal growth conditions, tno1 and WT seedlings were indistinguishable (Fig. 5A, B). In contrast, tno1 mutant seedlings exhibited shorter roots and earlier leaf chlorosis than WT seedlings under Na+, K+, Li+ and non-ionic osmotic stresses. This result shows that lack of TNO1 causes sensitivity to salt and osmotic stresses.

To confirm that the observed phenotype of the tno1 mutant is due to disruption of the TNO1 gene, a genomic fragment of TNO1 including the native promoter was introduced into the tno1 mutant. TNO1 expression was restored to approximately WT levels in the tno1/TNO1 promoter::TNO1 lines as determined by immunoblotting using TNO1 antibody (Fig. 4D). This TNO1 genomic fragment complemented the salt, ionic and osmotic sensitive phenotype of the tno1 mutant (Fig. 5).

To test whether expression of salt or osmotic stress-responsive genes was altered in the tno1 mutant, expression of TNO1, SYP61 and known salt (SOS1) or osmotic (RD29 and DREB2A) stress responsive genes were analyzed by RT-PCR. TNO1 was expressed at a basal level in the non-treated condition, and its mRNA level increased slightly during NaCl
and KCl treatment, while no expression of TNO1 was detected in the tno1 mutant as expected (Fig. S4). An increase in the transcript level of SYP61 was observed in both WT and tno1 mutant in all stress conditions. SOS1, DREB2A and RD29 increased as expected (Yamaguchi-Shinozaki and Shinozaki, 1993; Liu et al., 1998; Qiu et al., 2004), and no difference between WT and tno1 mutant plants was observed (Fig. S4). This result suggests that the phenotype of the tno1 mutant is not caused by disruption of expression of genes involved in salt or osmotic stress responses.

**SYP61 is partially mislocalized in a tno1 mutant**

TNO1 interacts with the SYP41 complex, and a tno1 mutant has a phenotype similar to that of a syp61 mutant. Thus, we hypothesized that a mutation in TNO1 may affect components of the SYP41 complex. The localization of SYP61 and SYP41 was investigated in WT and tno1 mutant protoplasts. Previously, SYP61 has been shown to co-localize with VHAa1-GFP (Dettmer et al., 2006). To investigate localization of SYP61 in the tno1 mutant, a VHAa1-GFP transgenic tno1 mutant was generated. Immunofluorescence analysis was performed using protoplasts generated from VHAa1-GFP transgenic WT and tno1 mutant plants, and the percentage of co-localization (SYP61-labeled structures overlapping with VHAa1/ total SYP61-labeled structures) was analyzed in ImageJ (Abramoff et al., 2004) using the colocalization finder plugin. In WT protoplasts, 70% of SYP61 co-localized with VHAa1-GFP, while 47% of SYP61 was found to co-localize with VHAa1-GFP in the tno1 mutant protoplasts (Fig. 6). This difference was statistically significant (t-test, p=0.00011; n=17). When co-localization of SYP41 with VHAa1-GFP was compared between WT and
tno1 mutant, no difference was observed (Fig. 6), suggesting that loss of TNO1 specifically affects localization of SYP61 and not SYP41.

**Lack of TNO1 causes secretion of vacuolar proteins**

Disruption of vacuolar transport typically leads to secretion of vacuolar proteins by a default pathway (Shimada et al., 1997; Shimada et al., 2003a). Previously, disruption of the SYP41-interacting proteins AtVPS45 or VTI12 was shown to cause secretion of aleurain and of a vacuolar marker consisting of CLAVATA 3 (CLV3) fused to the vacuolar sorting signal from barley lectin (Sanmartin et al., 2007; Zouhar et al., 2009). Therefore, we hypothesized that lack of TNO1 may also cause secretion of vacuolar proteins.

To test the potential involvement of TNO1 in vacuolar trafficking, we compared the amount of the vacuolar proteins Arabidopsis aleurain-like protein (AALP), RD21 and invertase in WT and tno1 mutant vacuoles. Vacuoles were isolated from WT plants and tno1 mutants and analyzed by immunoblotting using AALP, RD21 and invertase antibodies. No significant differences were seen between WT and tno1 mutant plants, suggesting that overall vacuolar trafficking is not inhibited in the tno1 mutant (Fig. 7A).

Although the tno1 mutant does not appear to have a general block in vacuolar trafficking, this does not preclude a mild or partial disruption in this pathway as seen in a vti12 mutant (Sanmartin et al., 2007). Thus, intercellular wash fluid (ICF) was collected from leaves of WT and tno1 mutant plants to determine whether any vacuolar proteins were partially secreted (Neuhaus et al., 1991). Proteins in the ICF were analyzed by immunoblotting using
antibodies against AALP, RD21 and invertase, with the cytoplasmic proteins ChlI (stromal) and PEX11d (peroxisomal) used as controls to rule out differences in cell breakage between mutant and wild-type plants. AALP, RD21 and invertase bands in ICF-depleted leaf extract showed no difference between WT and tno1 mutant, as expected from the above results with isolated vacuoles. However, the tno1 mutant had increased levels of AALP and RD21 in the ICF compared with wild-type plants, suggesting that lack of TNO1 caused some secretion of AALP and RD21 (Fig. 7B). By contrast, no secretion of vacuolar invertase was observed, although vacuolar invertase was suggested previously to be transported to the vacuole by a similar pathway to RD21 (Rojo et al., 2003). RD21 may therefore have an alternative or additional transport pathway to the vacuole. These data suggest that TNO1 is required for efficient trafficking of some vacuolar proteins.

**Involvement of TNO1 in vesicle fusion**

In Arabidopsis, the trafficking inhibitor brefeldin A (BFA) targets the Arf-GEF GNOM and induces the formation of BFA compartments (Geldner et al., 2001), most likely by homotypic fusion of the TGN (which is also an early endosome) and heterotypic fusion between the TGN and other endosomes (Dettmer et al., 2006; Lam et al., 2009; Viotti et al., 2010). Therefore, SNAREs at the TGN may function in these fusion events upon BFA treatment. As TNO1 interacts with SYP41 and loss of TNO1 disrupted localization of SYP61, TNO1 could be involved in membrane fusion at the TGN together with the SYP41 complex.
Formation of the BFA compartment in VHAa1-GFP transgenic WT and *tno1* mutant seedlings was therefore investigated. Without BFA treatment, there was no difference in the appearance of VHAa1-GFP positive structures in roots and cotyledons between WT and *tno1* mutant seedlings (Fig. 8A-D). After 1 hour BFA treatment, VHAa1-GFP accumulated in the BFA compartments in roots of WT seedlings as reported previously and in *tno1* mutant seedlings (Fig. 8E, F; Dettmer et al., 2006). In WT cotyledons, the BFA compartments were seen in the peripheral region. By contrast, VHAa1-GFP in *tno1* mutant cotyledons did not accumulate in BFA compartments after 1 h BFA treatment (Fig. 8G, H). VHAa1-GFP labeled BFA compartments in cotyledons of the *tno1* mutant could be seen only after 5 h of BFA treatment (Fig. 8K). No difference was seen in recovery from BFA treatment between WT and *tno1* mutant (Fig. 8I, J).

The response of TNO1 to BFA treatment was also investigated. VHAa1-GFP transgenic seedlings were treated in MS liquid medium supplemented with 50 μM BFA for 2 h, followed by fixing and immunolabeling using TNO1 antibody. TNO1 was not found in the VHAa1-GFP-labeled BFA compartment, but instead remained in small punctate structures similar to those in the absence of BFA. After BFA treatment, TNO1 co-localized with the remaining small VHAa1-GFP-labeled structures (Fig. 8L-O), suggesting that TNO1 is present in TGN remnants that do not undergo fusion.

Together, these results suggest that lack of TNO1 delays homotypic fusion between TGN or fusion between TGN and other endosomes in cotyledons. The BFA compartment therefore forms more slowly in the *tno1* mutant, presumably due to delayed fusion. TNO1
may function in maintaining TGN structure or identity, marking TGN remnants that do not participate in BFA compartment formation.
Discussion

Identity of TNO1

TNO1 was identified as a novel protein that co-immunoprecipitates with SYP41. As SYP41 is a t-SNARE residing at the TGN, we hypothesize that TNO1 may also be involved in vesicle fusion at this organelle. We have demonstrated that TNO1 is involved in salt and osmotic stress responses and is required for efficient vacuolar trafficking. Based on the effects of BFA treatment, TNO1 is proposed to be involved in the formation of the BFA compartment and may function in maintaining TGN structure or identity.

When we analyzed its potential domain structure using the Conserved Domain Architecture Retrieval Tool, two previously characterized domains (SMC_prok_B and SMC_N) containing long coiled-coil regions for protein interaction were identified in TNO1 (Akhmedov et al., 1998; Geer et al., 2002). Although SMC domains are commonly found in structural maintenance of chromosomes proteins, they are also found in other proteins with multiple coiled-coil domains such as vesicular tethering factors and cytoskeleton binding proteins. A search for proteins with sequence similarity to TNO1 using the BLAST algorithm (Altschul et al., 1998; Altschul et al., 2005) identified several hypothetical proteins from other plant species as most closely related to TNO1. In addition, myosin-related proteins, a putative ATP-binding protein and tethering factors including USO1 in yeast, tglgin-1 in mouse and gigantin in human were identified with high significance, primarily due to the presence of long coiled-coil regions in these proteins.
USO1, which has 20% amino acid sequence identity and 39% amino acid similarity to TNO1, is a tethering factor involved in ER to Golgi trafficking that tethers COPII vesicles at the cis-Golgi (Cao et al., 1998). Tethering factors have been reported to function in vesicle fusion by interacting with their tethering partner to pull vesicles close to their target membrane (Cao et al., 1998). Some are also involved in maintaining organelle structure and assisting in the formation of SNARE complexes via direct interaction with a SNARE (Shorter et al., 2002; Vasile et al., 2003). The presence of multiple predicted coiled-coil domains and the similarity to USO1 suggest that TNO1 could be a tethering factor at the TGN.

**Phenotype of tno1 KO mutant**

To determine the function of TNO1, a tno1 mutant was isolated and four major phenotypes were identified – sensitivity to high salt and osmotic stress, partial secretion of some vacuolar proteins, mis-localization of SYP61, and delayed BFA compartment formation in cotyledon cells. Differing effects on salt tolerance have been seen for mutations in different trafficking proteins. Knockdown of AtVAMP7C, a v-SNARE involved in vesicle fusion with the vacuole, was found to confer salt tolerance by preventing H$_2$O$_2$ containing vesicles from fusing with the tonoplast (Leshem et al., 2006). Knockout of SYP22, a t-SNARE in the PVC and tonoplast, also increased salt tolerance in shoots, and a decreased level of Na$^+$ in shoots was observed (Hamaji et al., 2009). Thus, vesicle fusion appears to directly or indirectly affect salt tolerance at the PVC and vacuoles. By contrast, the tno1 mutant and a mutation in SYP61 (Zhu et al., 2002) showed a salt sensitive phenotype. TNO1 and SYP61
co-localize to the TGN and are likely to exist in the same protein complex. The salt-sensitive phenotype of the tno1 mutant may be due to its effect on SYP61 (Fig. 6). In a tno1 mutant, less SYP61 co-localized with VHAa1-GFP-labeled TGN compared with WT plants, leading to possible reduced SYP61 function in salt tolerance. It was hypothesized that a cation transporter in the osm1/syp61 mutant may not function correctly, affecting guard cell turgor and salt sensitivity (Zhu et al., 2002). Extensive investigations of cation transporters in yeast, plants and mammalian cells have shown that cation/\(H^+\) antiporters in the Golgi, TGN, endosome and the PVC regulate the pH and cation concentration in these organelle, affecting vesicle trafficking, and a knock-down of VHAa1 in Arabidopsis also leads to increased salt sensitivity (Brett et al., 2002; Brett et al., 2005; Nakamura et al., 2005; Pardo et al., 2006; Fuji et al., 2007; Krebs et al., 2010). Thus, one possibility is that the salt sensitive phenotype of the tno1 and osm1/syp61 mutants could be caused by defects in the transport of cation/\(H^+\) antiporters or in the function of cation/\(H^+\) antiporters at the TGN.

Another noticeable phenotype is the secretion of normally vacuolar proteins in the tno1 mutant. Analysis of ICF from the tno1 mutant demonstrated partial secretion of AALP, in common with mutants in the additional SYP41 complex components VPS45 and VTI12 (Sanmartin et al., 2007; Zouhar et al., 2009). AALP has dual vacuolar targeting signals and is a cargo for VSR1 (Watanabe et al., 2004; Hinz et al., 2007). TNO1, AtVPS45 and VTI12 may therefore cooperate in VSR1-mediated vacuolar trafficking, via recycling of VSR1 from PVC to TGN. RD21 is another protein that is partially secreted in the tno1 mutant. RD21 is a cys-protease like AALP, but is probably transported via a VSR1-independent
pathway. RD21 and invertase are transported to the vacuole by ER bodies rather than by the VSR1-mediated transport pathway (Hayashi et al., 2001; Rojo et al., 2003); however, invertase was not secreted in the tno1 mutant. This result may indicate that RD21 can follow an alternative transport pathway to the vacuole. Protein profiles from isolated vacuoles of wild-type and tno1 mutant leaf protoplasts were compared by 2D gel electrophoresis but no significant differences were observed (data not shown). This is most likely due to only a small fraction of proteins being secreted, with most protein still being correctly transported to the vacuole. The partial secretion of vacuolar proteins may therefore be an indirect effect of the loss of TNO1.

Although salt sensitivity and secretion of some vacuolar proteins in the tno1 mutant may appear to be independent phenotypes, these two phenotypes are closely related. A connection between salt sensitivity and vacuolar trafficking has been well established. In yeast and mammalian cells, post-Golgi organelles involved in vacuolar trafficking were shown to be targets for salt stress, resulting in inefficient vacuolar trafficking (Bachert et al., 2001; Pardo et al., 2006; Hernandez et al., 2009). In Arabidopsis, the endocytic pathway to the vacuole has also been shown to be important for salt tolerance (Leshem et al., 2006; Leshem et al., 2007). Thus, our results provide additional evidence that vacuolar trafficking is important for salt tolerance.

**BFA treatment**

In this study, we used BFA to investigate the involvement of TNO1 in fusion events, as TNO1 is a member of a protein complex predicted to be required for membrane fusion at
the TGN, and BFA treatment causes the formation of BFA compartments by fusion between multiple types of endosomes. As expected, VHAa1-GFP transgenic plants formed BFA compartments after 1 hour BFA treatment, but VHAa1-GFP transgenic \textit{tno1} mutant plants showed formation of BFA compartments only after 5 h of BFA treatment in cotyledon cells, while mutant root cells behaved identically to wild-type in this assay. This delay in BFA compartment formation implies that TNO1 could be involved in homotypic fusion of the TGN/early endosomes or fusion between TGN and other types of endosomes upon BFA treatment.

VHAa1-GFP and TNO1 co-localize extensively under normal conditions; however, loss of co-localization was observed upon BFA treatment, indicating that TNO1 does not enter BFA compartments (Fig. 8). A similar effect was observed for the mammalian golgin GM130, which, unlike most Golgi proteins, does not relocate to the ER in the presence of BFA (Seemann et al., 2000). GM130 is a tethering factor that also interacts with SNAREs during vesicle fusion and a Golgi matrix protein required for the integrity of the Golgi structure (Diao et al., 2008). After BFA treatment, GM130 remains in a Golgi remnant, while Golgi luminal proteins are transported back to the ER, indicating that GM130 is involved in maintaining Golgi structure (Seemann et al., 2000). Although there is no sequence homology between TNO1 and GM130, based on the properties of TNO1 it is possible that TNO1 also functions as a tethering factor and is involved in maintaining TGN structure or identity. \textit{In vitro} fusion assays using recombinant TNO1 and TGN SNAREs may provide insight into the role of TNO1 in membrane fusion events.
Materials and Methods

Identification of TNO1

Protein extracts were made essentially as in Bassham et al. (2000) and Sanderfoot et al. (2001). Arabidopsis suspension cells were ground in extraction buffer (phosphate buffered saline (PBS), 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride (PMSF) and 1% (v/v) Triton X-100), and solubilized for 2 h at 4°C. Solubilized proteins were added to a column containing immobilized SYP41 antibodies or SYP41 pre-immune serum, and incubated for 2 h at 4°C. The columns were washed 5 times with extraction buffer, and bound proteins were eluted using 0.2 M glycine pH 2.5. Immunoprecipitates from SYP41 and pre-immune antibody were separated by SDS-PAGE and visualized using silver staining.

Protein bands migrating at an approximate molecular mass of 200, 67, 35 and 15 kD were excised and analyzed at the Protein Microsequencing and Proteomic Mass Spectroscopy Lab at the University of Massachusetts Medical School (Worcester, MA). Proteins were digested in gel using trypsin, the resulting peptides were applied to a Finnigan Electrospray LCQ Deca ion trap mass spectrometer and the peptide masses were used to identify potential matches within the Arabidopsis proteome.

cDNA cloning and sequencing

RNA was extracted from Arabidopsis siliques and treated with DNase I, followed by reverse transcription using an oligo dT primer. cDNAs were amplified using Herculase long and accurate polymerase mix (Agilent Technologies, Wilmington, DE) and TNO1
cDNA forward and reverse primers (Table 1) according to the manufacturer’s protocol. The amplification product was inserted into pGEM T-Easy vector (Invitrogen, Carlsbad, CA) and DNA sequencing was performed at the W.M. Keck Foundation (Yale University, New Haven, CT). The sequence was deposited in GenBank (Accession number HM776995).

**Plant materials and growth conditions**

*Arabidopsis thaliana* (ecotype Col-0) seeds were surface sterilized and grown on soil or MS (Murashige–Skoog Vitamin and Salt Mixture (Caisson Lab, Inc., North Logan, UT)) solid medium containing 1% (w/v) sucrose. A *tno1* knockout mutant line (Salk_112503) which has a T-DNA insertion in the third exon was obtained from the Arabidopsis Biological Resource Center. A homozygous *tno1* mutant was identified by PCR using *TNO1* internal forward and reverse primers and T-DNA left border-a1 primer (Table 1).

**Generation of transgenic plants**

GFP-fused vacuolar H⁺ATPase (GFP-VHAa1) plasmid (Dettmer et al., 2006) was introduced into *Agrobacterium tumefaciens* strain GV2260, which was used to transform *tno1* mutant plants by the floral dipping method (Clough and Bent, 1998). The resulting VHAa1-GFP transformed *tno1* plants were selected on MS solid medium containing 50 μg mL⁻¹ kanamycin.

For *tno1* mutant complementation, the full length *TNO1* gene and its promoter were separately amplified by PCR with *TNO1* promoter primers and genomic *TNO1* primers with corresponding restriction sites (Table 1). The *TNO1* promoter fragment was digested
using \textit{SalI} and \textit{NotI}, and the \textit{TNO1} genomic fragment was digested using \textit{NotI} and \textit{KpnI}. The digested \textit{TNO1} promoter and genomic DNA were ligated into the binary vector pCAMBIA1300MCS1 digested using \textit{SalI} and \textit{KpnI} (Sanderfoot et al., 2001a). The resulting plasmid was introduced into \textit{Agrobacterium tumefaciens} strain GV2260, which was used to transform \textit{tno1} mutant plants. The transgenic \textit{tno1} mutant plants were identified by resistance to 50 μg mL\(^{-1}\) hygromycin and immunoblotting with TNO1 antibody.

**RT-PCR analysis of \textit{TNO1}, \textit{SYP41} and salt/ osmotic stress responsive genes**

Total RNA was extracted from Arabidopsis organs using the TRIzol RNA isolation method (http://www.Arabidopsis.org/portals/masc/AFGC/RevisedAFGC/site2RnaL.htm#isolation). Roots were harvested from 4-week old Arabidopsis plants grown in liquid culture (MS plus 1% (w/v) sucrose) and stems, rosette leaves, cauline leaves, flowers and siliques were harvested from 7-week old Arabidopsis plants grown in soil. Extracted RNA was treated with DNase I, followed by reverse transcription using an oligo dT primer. cDNAs were amplified for 25 cycles using \textit{TNO1} internal forward and reverse primers or \textit{SYP41} forward and reverse primers (Table 1).

One week old wild-type and \textit{tno1} mutant seedlings were treated with 300 mM NaCl, 40 mM LiCl, 300 mM KCl or 600 mM mannitol for 5 hours. Total RNA was extracted from the seedlings and expression of salt-overly sensitive 1 (\textit{SOS1}), DRE binding protein (\textit{DREB2A}), \textit{RD29}, \textit{TNO1} and \textit{SYP61} was analyzed by RT-PCR using gene specific primers...
(Table 1; Yamaguchi-Shinozaki and Shinozaki, 1993; Liu et al., 1998; Shi et al., 2000; Zhu et al., 2002).

**Antibody production and purification**

A 1.7kb C-terminal fragment of the *TNO1* cDNA was cloned into pET28b(+) expression vector (Novagen, Madison, WI) to produce a His-partial TNO1 fusion construct. The fusion protein construct was introduced into *Escherichia coli* BL21 (DE3) and expression and purification of partial TNO1 was as described in Bassham et al. (2000). The partial TNO1 was purified using Ni-NTA agarose and used to immunize rabbits at Cocalico Biologicals (Reamstown, PA) after gel purification.

For purification of TNO1-specific antibodies, purified His-tagged TNO1 protein was separated by SDS-PAGE, transferred to nitrocellulose membrane followed by staining with Ponceau S, and the strip containing the fusion protein was cut out. The strip was blocked in 3% (w/v) dried nonfat milk in PBS, and crude rabbit serum was incubated with the strip for 2 h at 4°C. The strip was washed 5 times with PBS and antibodies were eluted using 0.1 M glycine pH 2.2.

**Differential centrifugation**

Arabidopsis plants were homogenized in 0.3 M sucrose, 100 mM Tris-Cl pH 7.5, 1 mM EDTA, 0.1 mM PMSF. The homogenate was centrifuged at 1,000 x g for 10 min at 4°C to remove cell debris. The supernatant was centrifuged at 20,000 x g, the supernatant removed and further centrifuged at 100,000 x g. The TCA-precipitated supernatant after
centrifugation at 100,000 x g and pellets after centrifugation at 20,000 x g and 100,000 x g were analyzed by immunoblotting with TNO1 and SYP61 antibodies (Sanderfoot et al., 2001a).

**Extraction of TNO1 from membranes**

Total membrane fractions were isolated from 2 week old seedlings by centrifugation at 125,000 x g and treated with 1% (v/v) Triton X-100, 2 M urea, 1 M NaCl or 0.1 M Na₂CO₃, followed by pelleting of non-solubilized proteins at 125,000 x g (Phan et al., 2008). Pellets and TCA-precipitated supernatants were analyzed by immunoblotting with TNO1 and SYP41 antibodies.

**Intercellular fluid (ICF) extraction**

ICF was extracted as described (Neuhaus et al., 1991). One gram of Arabidopsis leaves were infiltrated under vacuum in 50 mM sodium citrate pH 5.5. After infiltration, surface dried leaves were rolled with filter paper and inserted into a 10 mL syringe. The syringe was inserted into a 50 mL conical tube, which was centrifuged at 1000 x g for 10 min at 10°C. ICF was collected in the bottom of the tube, and ICF-depleted leaves after centrifugation were ground in 50 mM sodium citrate for the control. ICF and ICF-depleted protein extract were analyzed by immunoblotting using anti-AALP (1:1000; Ahmed et al., 2000), anti-RD21 (1:1000; Hayashi et al., 2001), anti-invertase (1:3000; Rojo et al., 2003), anti-ChlI (1:2000; Adhikari et al., 2009) and anti-PEX11d (1:1000; Orth et al., 2007).

**Preparation of vacuoles**
Leaf protoplasts were generated as described (Sheen, 2002). After harvesting protoplasts, vacuole isolation was performed as described (Robert et al., 2007). After adding 6 mL of pre-warmed lysis buffer (0.2 M mannitol, 10% (w/v) ficoll, 1 mM EDTA and 5 mM Na phosphate pH 8.0) at 42°C, lysed protoplasts were transferred to clear tubes for a SW41 swinging bucket rotor (Beckman Coulter, Brea, CA) and overlayed with 3 mL of 4% (w/v) ficoll (by mixing lysis and vacuole buffer) and 1 mL of vacuole buffer (0.45 M mannitol, 2 mM EDTA and 5 mM Na phosphate pH 7.5) followed by centrifugation at 20,000 rpm for 50 min at 10°C. Vacuoles were recovered from the interface between 4% (w/v) ficoll and vacuole buffer.

**Immunofluorescence**

Three or four day old Arabidopsis seedlings grown on MS solid medium were immunolabeled with a protocol slightly modified from Phan et al. (2008). Seedlings used were ST-GFP transgenic plants (Boevink et al., 1998), VHAa1-GFP transgenic plants (Dettmer et al., 2006) and YFP-RHA1 transgenic plants (Preuss et al., 2004) to label Golgi, TGN and PVC, respectively.

For protoplast immunofluorescence, protoplasts were fixed in 3% (w/v) paraformaldehyde in MTSB with 0.4 M sorbitol for 30 min and washed twice with MTSB with 0.4 M sorbitol. Fixed protoplasts were mounted onto slides and dried for 1 h. Protoplasts were permeabilized using permeabilization buffer (0.5% (v/v) NP40, 10% (v/v) DMSO in MTSB) for 30 min in a moist chamber followed by washing 3 times using MTSB. Samples were blocked in 3% BSA in MTSB for 2 h, and primary antibodies in 3% (w/v) BSA in
MTSB were added. After 3 h incubation, protoplasts were washed 3 times using MTSB and treated with conjugated secondary antibody in 3% (w/v) BSA in MTSB for 50 min followed by washing 5 times with MTSB. Anti-TNO1 (1:25), anti-SYP41 (1:50) and anti-SYP61 (1:50) were used as primary antibodies. Alexa Fluor® 594-conjugated goat anti-rabbit IgG (1:250) was used as secondary antibody.

Fluorescent signals were viewed with a confocal laser scanning microscope (Leica SP5, Leica Microsystems, Exton, PA, USA). Excitation and emission wavelengths of GFP were 488/500 nm and those of Alexa Fluor® 594 were 590/600 nm.

**Salt, ionic and osmotic stresses, and drug treatment**

For stress treatment, seedlings were grown on MS solid medium for 5 days and transferred to MS solid medium containing 130 mM NaCl, 17 mM LiCl, 140 mM KCl or 300 mM mannitol, followed by growth for an additional 10 days (Zhu et al., 2002).

For BFA treatment, seedlings were grown on MS solid medium for 4 days and transferred to MS liquid medium containing 50 μM BFA or DMSO as carrier control for 5 h. Seedlings were washed twice with MS liquid medium and incubated in MS liquid medium for recovery. BFA treatment of protoplasts was performed as above for 2 h with the same concentration of BFA.
Supplemental Material

Supplementary Fig 1. Sequence analysis of \textit{TNO1}.

Supplementary Fig 2. TNO1 domain prediction.

Supplementary Fig 3. Expression pattern of \textit{TNO1} by microarray analysis.

Supplementary Fig 4. Salt/ osmotic stress responsive gene expression in wild-type and \textit{tno1} mutant.
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Figure legends

Figure 1. Identification of TNO1.

A. Identification of TNO1 in SYP41 immunoprecipitate.

Detergent-solubilized Arabidopsis suspension cell extracts were applied to a column containing resin cross-linked to either immobilized SYP41 antibodies (I) or SYP41 pre-immune serum (P). The eluates were analyzed by SDS-PAGE and silver staining. p200, p67, p35 and p15 indicate the mobility of protein bands, when compared with molecular weight markers.

B. Co-immunoprecipitation of TNO1 with SYP41.

Immunoprecipitations were performed from detergent-solubilized Arabidopsis membrane extracts using SYP41 antibodies (I) and pre-immune serum (P). Immunoprecipitates were analyzed by immunoblotting with antibodies against SYP41 or TNO1.

C. Expression of TNO1 mRNA in Arabidopsis.

RT-PCR was performed using cDNA from different tissues of Arabidopsis using TNO1- and SYP41- specific primers. 18S RNA was used as a control. R; root, RL; rosette leaves, CL; cauline leaves, FL; flower, ST; stem.

Figure 2. Membrane association of TNO1.

A-B. Detection of TNO1 using TNO1 antibody.
Total protein extract (T), pellet (P), and soluble proteins (S) from Arabidopsis seedlings were separated by SDS-PAGE and analyzed by immunoblotting using affinity-purified TNO1 antibody (A) or TNO1 pre-immune serum (B). Position of molecular markers is shown at left.

C. Differential centrifugation.

Total Arabidopsis extracts (T) from 2 week old seedlings were centrifuged sequentially at 20,000 x g and 100,000 x g, resulting in a 20,000 x g pellet (P20), 100,000 x g pellet (P100) and soluble fractions (S), followed by immunoblotting using TNO1 and SYP61 antibodies

D. Membrane association test.

Total membrane pellets from Arabidopsis seedlings were resuspended in 1% (v/v) Triton X-100, 2 M urea, 1 M NaCl or 0.1 M Na2CO3. Suspensions were re-centrifuged at 125,000 x g to separate solubilized fractions (S) and insoluble fractions (P), which were analyzed by immunoblotting using TNO1 and SYP41 antibodies.

Figure 3. TNO1 is localized to the TGN.

A. TNO1 antibodies specifically label punctate structures.

Four day old wild type and tno1 mutant seedlings were fixed and processed for immunofluorescence using TNO1 antibody (left panels) and DIC (right panels).

B-D. TNO1 co-localizes with VHAa1 at the TGN.
Four day old RHA1-YFP (PVC; B), ST-GFP (Golgi; C), and VHAa1-GFP (TGN; D) transgenic Arabidopsis seedlings were fixed and immunolabeled using TNO1 antibody. Scale bars represent 10 μm.

**Figure 4. Identification of a homozygous tno1 knockout mutant.**

**A. T-DNA insertion site in tno1 knockout mutant.**

The tno1 mutant has a complex T-DNA insertion in its third exon. Left border (LBa1), TNO1 internal left (LP) and right (RP) primers were used to identify the tno1 mutant. Arrow indicates the direction of each primer. Boxes and lines indicate exons and introns, respectively.

**B. Identification of homozygous tno1 knockout mutant.**

PCR from wild-type and tno1 mutant genomic DNA was performed using LP (TNO1 internal left)/ RP (TNO1 internal right) primers and LBa1 primer from the T-DNA, producing a 900 bp band from LP and RP primers and a 450 bp band from LBa1 and either LP or RP primers.

**C. Lack of TNO1 in tno1 mutant.**

Membrane extracts from wild-type (WT) and tno1 mutant plants were analyzed by SDS-PAGE and immunoblotting using TNO1 antibody. Position of molecular markers is shown at left.
D. Expression of TNO1 in tno1 complemented line.

Membrane extracts from wild-type (WT) and tno1/TNO1 promoter::TNO1 plants (COM) were analyzed by SDS-PAGE and immunoblotting using TNO1 antibody.

Figure 5. tno1 mutant is sensitive to salt, ionic and osmotic stresses.

A. Wild-type, tno1 mutant and TNO1 complemented seedlings were grown vertically on MS solid medium for 4 days after germination. WT, Wild type; KO, tno1 KO mutant; COM, tno1/TNO1 promoter::TNO1 transgenic lines.

B. Root length was measured 2 and 4 days after germination on MS solid medium. Error bars represent standard deviation (n = 30 for each genotype). WT, Wild type; KO, tno1 KO mutant; COM, tno1/TNO1 promoter::TNO1 transgenic lines.

C-F. Wild-type, tno1 mutant and TNO1 complemented seedlings were grown vertically on MS solid medium for 5 days, transferred to MS plates containing 130 mM NaCl (A), 140 mM KCl (B), 17mM LiCl (C) or 300 mM mannitol (D) and grown inverted. Images were taken after 1 week. WT, Wild type; KO, tno1 KO mutant; COM, tno1/TNO1 promoter::TNO1 transgenic lines.

G-J. Root growth under salt, ionic and osmotic stress conditions. Seeds were germinated on MS plates containing 130 mM NaCl (E), 140 mM KCl (F), 17 mM LiCl (G) or 300 mM mannitol (H) and the root length was measured at 4 and 8 days after germination. Error bars
represent standard deviation (n = 18 for each genotype). WT, Wild type; KO, tno1 KO mutant; COM, tno1/TNO1 promoter::TNO1 transgenic lines.

Figure 6. Mis-localization of SYP61 in tno1 mutant.

A. Co-localization of SYP61 or SYP41 with VHAa1-GFP in wild-type and tno1 mutant.

Protoplasts were isolated from leaves of VHAa1-GFP transgenic wild-type plants and VHAa1-GFP transgenic tno1 mutants and fixed, followed by immunofluorescence labeling using SYP61 or SYP41 antibodies. Scale bars represent 10 µm.

B. Quantification of co-localization of SYP61 and SYP41 with VHAa1-GFP in wild-type and tno1 mutant.

Percentage of co-localization of SYP61 or SYP41 with VHAa1 was determined. Error bars represent standard deviation (n=17). Results shown are an average of three independent experiments. The asterisk shows a statistically significant difference between wild-type and tno1 mutant (t-test, p = 0.00011).

Figure 7. Secretion of vacuolar proteins in the tno1 mutant.

A. Comparison of vacuolar proteins in wild-type and tno1 mutant.
Vacuoles were isolated from leaves of WT and tno1 mutant and analyzed by immunoblotting using AALP, RD21, and invertase antibodies. Amount of vacuolar proteins loaded is shown on the top.

**B. Secretion of vacuolar proteins in tno1 mutant.**

Intercellular wash fluids were harvested from wild type (WT), tno1 mutant (KO), and TNO1 complemented line (COM) and analyzed by SDS-PAGE and immunoblotting using AALP, RD21, invertase, ChlI and PEX11d antibodies. ChlI and PEX11d were used to show equal cell breakage. ICF, intercellular wash fluid; TOT-ICF, extract after collection of ICF.

**Figure 8. Delayed formation of a BFA compartment in tno1 mutant and localization of TNO1 upon BFA treatment.**

**A-D. VHAa1-GFP in wild-type and tno1 mutant without BFA treatment**

TGN is labeled with VHAa1-GFP in roots of wild-type (A) and tno1 mutant (B), and in cotyledons of wild-type (C) and tno1 mutant (D).

**E-K. The formation of BFA compartment in wild-type and tno1 mutant**

Wild-type and tno1 mutant seedlings were treated with 50 µg/ml BFA in liquid medium for either 1 hour (E-H) or 5 hours (K). For the recovery, BFA treated seedlings were washed twice with the same medium and incubated for 1 hour (I and J). Formation of the BFA compartments in roots of wild type (E) and tno1 mutant (F), and in cotyledons of wild-type
(G) was observed after 1 hour BFA treatment. Formation of BFA compartment in cotyledons of tno1 mutant was not observed after 1 hour BFA treatment (H), but was seen after 5 hour BFA treatment (K). Recovery from BFA treatment in cotyledons of wild-type (I) and tno1 KO mutant (J) was indistinguishable.

**L-O. Localization of TNO1 after BFA treatment.**

VHAa1-GFP transgenic seedlings were treated with 50 µg/ml of BFA in liquid medium for 2 hours and fixed, followed by immunofluorescence using TNO1 antibody. Arrows indicate the BFA compartment and arrow heads indicate small punctate structures, presumably TGN. Scale bars represent 10 µm.
Table 1. Primer sequences. The restriction sites used for cloning are underlined.

| Primer name          | Direction | Sequence                                           |
|----------------------|-----------|----------------------------------------------------|
| **TNO1 cDNA**        | Forward   | 5’-GGTTGGGATCGTATTTAGATAATG-3’                     |
|                      | Reverse   | 5’-GTAGTAGTTTATGAGAGACC-3’                        |
| **TNO1 internal**    | Forward   | 5’-TTGACCGACACCTGGCTGGTACA-3’                      |
|                      | Reverse   | 5’-GCAAATGACATCCACGTCCTATCAGAG-3’                  |
| **T-DNA left border-a1** |          | 5’-TGGTTTCACGTAATGGGGCCATCG-3’                     |
| **TNO1 promoter**    | Forward   | 5’–ATCAGTGCGAACAAAAATCAGTCAATT-3’                  |
|                      | Reverse   | 5’-GCGGCCGCTATCTAAATACGATCC-3’                     |
| **genomic TNO1**     | Forward   | 5’-GCGGCCGCGTGCAGAAGGATG-3’                        |
|                      | Reverse   | 5’-TTCTGGTCACCGATTCATGAGCAG-3’                     |
| **SYP41**            | Forward   | 5’-GGATCCTCAGAGGAGGATCAGTTGCTGTTT-3’               |
|                      | Reverse   | 5’-GGATCCTCAGAGGAGGATCAGTTGCTGTTT-3’               |
| **DREB2A**           | Forward   | 5’-GGAGATGGCGAGTTATGATC-3’                         |
|                      | Reverse   | 5’-TATGGTTCACGATCCAGGAA-3’                         |
| **SOS1**             | Forward   | 5’-ATGACGACTGTAAATCGACGCGACGA-3’                    |
|                      | Reverse   | 5’-TGCAAAACACACTGAGATAAAAT-3’                      |
| **RD29**             | Forward   | 5’-ATGGATGAAACAGAGGACCCACCAC-3’                     |
|                      | Reverse   | 5’-CCATTCAGGTTTCACTGTCAT-3’                        |
| **SYP61**            | Forward   | 5’-GGATCCTCAGGAGGACCGTCCAGACGATCC-3’               |
|                      | Reverse   | 5’-GGATCCTTATATCATCATCATCAT-3’                     |
