An Arabidopsis Prenylated Rab Acceptor 1 Isoform, AtPRA1.B6, Displays Differential Inhibitory Effects on Anterograde Trafficking of Proteins at the Endoplasmic Reticulum

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Prenylated Rab acceptors (PRAs), members of the Ypt-interacting protein family of small membrane proteins, are thought to aid the targeting of prenylated Rabs to their respective endomembrane compartments. In plants, the Arabidopsis (Arabidopsis thaliana) PRA1 family contains 19 members that display varying degrees of sequence homology to animal PRA1 and localize to the endoplasmic reticulum (ER) and/or endosomes. However, the exact role of these proteins remains to be fully characterized. In this study, the effect of AtPRA1.B6, a member of the AtPRA1 family, on the anterograde trafficking of proteins targeted to various endomembrane compartments was investigated. High levels of AtPRA1.B6 resulted in differential inhibition of coat protein complex II vesicle-mediated anterograde trafficking. The trafficking of the vacuolar proteins sporamin:GFP (for green fluorescent protein) and AALP:GFP, the secretory protein invertase:GFP, and the plasma membrane proteins FMP:GFP and H+ATPase:GFP was inhibited in a dose-dependent manner, while the trafficking of the Golgi-localized proteins ST:GFP and KAM1(C):mRFP was not affected. Conversely, in RNA interference plants displaying lower levels of AtPRA1.B6 transcripts, the trafficking efficiency of sporamin:GFP and AALP:GFP to the vacuole was increased. Localization and N-glycan pattern analyses of cargo proteins revealed that AtPRA1.B6-mediated inhibition of anterograde trafficking occurs at the ER. In addition, AtPRA1.B6 levels were controlled by cellular processes, including 26S proteasome-mediated proteolysis. Based on these results, we propose that AtPRA1.B6 is a negative regulator of coat protein complex II vesicle-mediated anterograde trafficking for a subset of proteins at the ER.

Many small integral membrane proteins are known to play roles in the various steps of protein trafficking (Emery et al., 2000; Compton and Behrend, 2006). The Ypt-interacting protein (YIP) family displays an ability for binding Rabs/Ypts (Shishева et al., 1994; Wada et al., 1997; Yang et al., 1998; Liang and Li, 2000; Figueroa et al., 2001; Calero et al., 2002; Calero and Collins, 2002; Pfeffer and Aizvazian, 2004; Spang, 2004). The YIP family members, containing multiple transmembrane domains ranging from two to five, are divided into seven subfamilies (Yip1–Yip6 and Yif1) based on sequence homology (Pfeffer and Aizvazian, 2004).

Among the YIP family members, the biological role of Yip3p/PRA1 (for prenylated Rab acceptor 1) has been studied most extensively. In animal cells, PRA1 localizes to the Golgi apparatus and endosomes, and it interacts with multiple Rab proteins as well as the GDP dissociation inhibitor (GDI). Additionally, PRA1 activates the dissociation of prenylated Rabs from the Rab/GDI complex, facilitating the association of prenylated Rabs with target membranes. This indicates that PRA1/Yip3 plays a role in targeting prenylated Rabs to their specific compartments by acting as a GDI displacement factor (Abdul-Ghani et al., 2001; Sivars et al., 2003; Seabra and Wasmeyer, 2004). PRA1 also interacts with the SNARE protein VAMP2 and may play a role in regulating vesicle fusion to target membranes. In contrast to PRA1 in animal cells, Yip3p, the ortholog of animal PRA1, appears to be involved in a different physiological process. In yeast, Yip3p localizes primarily to the Golgi, with a minor portion localizing to the endoplasmic reticulum (ER). The localization of Rab proteins was not perturbed in yip3Δ. Furthermore, overexpression of Yip3p causes ER membrane proliferation, a well-known phenotype resulting from the inhibition of anterograde trafficking at the ER (Kaiser and Schekman, 1990). These results raise the possibility that Yip3p plays a role in the biogenesis of coat protein complex II (COP II) vesicles.
at the ER in yeast (Geng et al., 2005). Thus, the biological role of PRA1 has not been fully characterized.

The biological role of PRA2 is even less known. PRA2 belongs to the Yip6 subfamily and localizes to the ER (Abdul-Ghani et al., 2001; Pfeffer and Aivazian, 2004). PRA2/PRA2F expression levels are elevated in various cancer cells, and the encoded proteins are enriched in synaptic vesicles in neuronal cells (Koomoa et al., 2008; Borsics et al., 2010). In neuronal cells, Yip6b and JM4, also members of the Yip6 subfamily, are involved in regulating the ER exit of the neuron-specific neurotransmitter transporters (Ruggiero et al., 2008). Yip6b interacts specifically with EAAC1, resulting in its retention in the ER.

Similar to animal and yeast cells, plant cells also contain a large number of small membrane proteins that display varying degrees of sequence homology to animal and yeast YIP proteins. In Arabidopsis (Arabidopsis thaliana), members of the AtPRA1 family have been studied for their expression and subcellular localization (Alvim Kamei et al., 2008). Upon expression in transgenic plants, specific GFP-tagged AtPRA1 family members localized to the ER and/or endosomes. Additionally, AtPRA1.B6, a member of the AtPRA1 family, was recently demonstrated to localize primarily to the Golgi apparatus. However, at high expression levels, AtPRA1.B6 localizes to the ER and the Golgi apparatus (Jung et al., 2011). A PRA1 homolog in rice (Oryza sativa), OsPRA1, interacts with OsRab7 and localizes to the prevacuolar compartment. Thus, OsPRA1 is believed to play a role in protein trafficking to the vacuole (Heo et al., 2010).

In this study, the effect of AtPRA1.B6 on the trafficking of cargo proteins targeted to various endomembrane compartments was investigated. As AtPRA1.B6 levels increased due to ectopic expression, the anterograde trafficking of a subset of proteins was inhibited at the ER in a protein level-dependent manner. Conversely, when AtPRA1.B6 levels were reduced using an RNA interference (RNAi) approach, the anterograde trafficking of vacuolar proteins was enhanced.

RESULTS

HA::AtPRA1.B6 Inhibits the Trafficking of Vacuolar Proteins, Sporamin::GFP, and AALP::GFP

Previously, AtPRA1.B6 expressed with an N-terminal hemagglutinin (HA) tag was demonstrated to localize primarily to the Golgi apparatus (Jung et al., 2011). In addition, when HA::AtPRA1.B6 was expressed at high levels, it localized to the ER and the Golgi apparatus. Yeast Yip3, the likely ortholog of AtPRA1, exhibits similar localization patterns, with a primary localization to the Golgi apparatus and a minor portion to the ER (Geng et al., 2005). To gain insight into the physiological role of AtPRA1.B6, the effect of high expression levels of AtPRA1.B6 on anterograde trafficking of various cargo proteins was examined. In a previous study, overexpression of Yip3 in yeast resulted in an inhibition of anterograde trafficking (Geng et al., 2005). A similar observation was made when Yip6b/GTRAP3-18 was expressed in animal cells (Ruggiero et al., 2008). In initial investigations, the utilized proteins were destined for the central vacuole. For example, sporamin::GFP, a chimera of the sweet potato (Ipomoea batatas) protein sporamin and GFP, is targeted to the vacuole, where it is processed to a 30-kD protein (Kim et al., 2001). Increasing amounts of HA::AtPRA1.B6 along with sporamin::GFP were cotransformed into protoplasts, and protein extracts were used in western-blot analysis using anti-GFP antibody to determine the trafficking efficiency. The ratio of processed proteins to the total amount of expressed proteins (processed proteins plus full-length proteins) was used to assess the trafficking efficiency. The trafficking efficiency of sporamin::GFP decreased gradually with increasing amounts of HA::AtPRA1.B6 (Fig. 1A), indicating that vacuolar trafficking was inhibited by HA::AtPRA1.B6 in a dose-dependent manner. The expression of HA::AtPRA1.B6, as determined by anti-HA antibody, was proportional to the amount of introduced plasmid DNA.

To further confirm that AtPRA1.B6 inhibits vacuolar trafficking, a second vacuolar protein, AALP::GFP, was analyzed. As a chimeric protein consisting of Arabidopsis aleurain-like protein (AALP) and GFP, this protein is targeted to the central vacuole, where it is processed proteolytically to produce a 30-kD protein (Sohn et al., 2003). AALP::GFP and HA::AtPRA1.B6 or empty vector (R6) were introduced into protoplasts, and these proteins were detected by western-blot analysis using anti-GFP and anti-HA antibodies. In the presence of cotransformed HA::AtPRA1.B6, the amount of processed AALP::GFP protein was reduced significantly compared with the control transformed with empty vector R6 (Fig. 1B). These data confirm that HA::AtPRA1.B6 inhibits vacuolar trafficking.

To obtain independent evidence for inhibition, the localization of vacuolar cargo proteins was examined in the presence of HA::AtPRA1.B6. HA::AtPRA1.B6 or R6 was cotransformed into protoplasts together with sporamin::GFP or AALP::GFP, and the localization of these cargo proteins was examined using a fluorescence microscope. In control protoplasts transformed with R6, both sporamin::GFP and AALP::GFP produced the vacuolar pattern in a majority of the protoplasts (Fig. 1C, panels a and e), and a minor population of protoplasts displayed a network pattern alone or both the vacuolar and network patterns. The vacuolar pattern indicates that sporamin::GFP and AALP::GFP were trafficked to the central vacuole. However, in the presence of HA::AtPRA1.B6, both sporamin::GFP and AALP::GFP primarily produced the network pattern with a minor portion of the vacuolar pattern (Fig. 1C, panels c and g). This confirms that AtPRA1.B6 inhibits the vacuolar trafficking of sporamin::GFP and AALP::GFP.

To eliminate the possibility that the inhibition of vacuolar trafficking by HA::AtPRA1.B6 was caused by
high levels of HA:AtPRA1.B6 produced from the transformed HA:AtPRA1.B6 plasmid DNA in protoplasts, transgenic plants harboring HAx3:AtPRA1.B6 were examined. Transgenic plants with HAx3:AtPRA1.B6 were generated previously (Jung et al., 2011). AtPRA1.B6 was tagged with a trimeric HA tag (HAx3) and placed under the control of the dexamethasone-inducible promoter (Aoyama and Chua, 1997; Jung et al., 2011). Transgenic plants containing only a single copy of HAx3:AtPRA1.B6 (HAx3:AtPRA1.B6 plants) were grown on dexamethasone-containing plates, and the HAx3:AtPRA1.B6 levels were analyzed by western blotting using anti-HA antibody. The protein levels increased in a dose-dependent manner when plants were incubated on plates supplemented with varying concentrations of dexamethasone (Jung et al., 2011). The HAx3:AtPRA1.B6 level in transgenic plants was significantly lower than the HA:AtPRA1.B6 level in protoplasts transformed with 2 μg of HA:AtPRA1.B6 plasmid DNA (Supplemental Fig. S1). Since AtPRA1.B6 in transgenic plants and protoplasts has trimeric and monomeric HA tags, respectively, the

Figure 1. HA:AtPRA1.B6 expressed transiently in protoplasts inhibits the trafficking of sporamin:GFP and AALP:GFP in a dose-dependent manner. A, Inhibition of vacuolar trafficking of sporamin:GFP to the vacuole by HA:AtPRA1.B6 in a dose-dependent manner. Sporamin:GFP was cotransformed into protoplasts with the indicated amounts of HA:AtPRA1.B6 or R6, and protein extracts from protoplasts were analyzed by western-blot analysis using anti-GFP and anti-HA antibodies. Trafficking efficiency of sporamin:GFP was determined by the signal intensity of the processed form over the total amount of expressed proteins. Three independent trafficking experiments were performed. Error bars indicate SD (n = 3). HA:AtPRA1.B6 or R6. AALP:GFP was cotransformed with HA:AtPRA1.B6 or R6 into protoplasts, and protein extracts from protoplasts were analyzed by western-blot analysis using anti-GFP and anti-HA antibodies. In order to measure the trafficking efficiency of AALP:GFP, the amount of the processed form was quantified using software equipped to LAS3000 and expressed as a relative value to the total amount of protein. Error bars indicate SD (n = 3). C, Image analysis of protein trafficking inhibition by HA:AtPRA1.B6. Sporamin:GFP or AALP:GFP was introduced into protoplasts together with HA:AtPRA1.B6 or R6, and the localization of cargo proteins was examined. Three independent transformation experiments were performed for each sample. More than 100 protoplasts were examined to determine their localization patterns. Red signals show the autofluorescence of chlorophyll (CH). Bars = 20 μm.
difference in the protein levels between HAx3:AtPRA1.B6 in transgenic plants and HA:AtPRA1.B6 in protoplasts would be greater than the difference in the immunoblot band intensity. Subsequently, whether HAx3:AtPRA1.B6 produced in transgenic plants is able to inhibit the trafficking of proteins was investigated. HAx3:AtPRA1.B6 expression was induced with dexamethasone using two approaches, before and after protoplast preparation. Protoplasts from dexamethasone-treated and untreated HAx3:AtPRA1.B6 plants were incubated in the absence and presence of dexamethasone after transformation with cargo constructs, respectively. The trafficking of proteins to the vacuole was examined by western-blot analysis using anti-GFP. The trafficking efficiency of sporamin:GFP and AALP:GFP to the vacuole was significantly reduced in the presence of HAx3:AtPRA1.B6 that had been induced before protoplasting (Fig. 2). Similarly, the vacuolar trafficking of AALP:GFP was inhibited by HAx3:AtPRA1.B6 that had been produced in protoplasts at the same time with AALP:GFP (Supplemental Fig. S2). The HAx3:AtPRA1.B6 protein level in these samples was confirmed by western-blot analysis using anti-HA antibody. The results indicate that the stable expression of HAx3:AtPRA1.B6 in transgenic plants also inhibits the anterograde trafficking of vacuolar cargoes.

**HA:AtPRA1.B6 Inhibits the Trafficking of Secretory and Plasma Membrane Proteins**

Next, we examined whether AtPRA1.B6 inhibits the trafficking of secretory and plasma membrane proteins. Invertase:GFP was selected as a secretory protein. Invertase:GFP was contransformed with AtPRA1.B6 or R6 into protoplasts, and the localization of invertase:GFP was examined. Chloroplasts (CH) were detected by red autofluorescence of chlorophyll. Bars = 20 μm. B, Western-blot analysis. Invertase:GFP was cotransformed with HA:AtPRA1.B6 or R6, and the protein extracts from protoplasts and incubation medium were analyzed by western-blot analysis using anti-GFP and anti-HA. To quantify trafficking efficiency, the band intensity was determined using software equipped to LAS3000. Trafficking efficiency was determined by the relative amount of secreted invertase:GFP over the total expressed proteins (proteins in both the medium and protoplasts). In order to measure the trafficking efficiency of invertase:GFP, the amount of secreted protein was quantified using software equipped to LAS3000 and expressed as a relative value to the total amount of protein. Error bars indicate SD (n = 3). R6, Empty vector; PRA1, HA:AtPRA1.B6; M, protein extracts from the incubation medium; P, protoplast extracts. C, Inhibition of invertase:GFP trafficking by HAx3:AtPRA1.B6 expressed in transgenic plants. Protoplasts from pTA or HAx3:AtPRA1.B6 transgenic plants treated with dexamethasone (30 μM) for 1 d were transformed with invertase:GFP, and trafficking efficiency was examined by western-blot analysis using anti-GFP antibody. Expression of HAx3:AtPRA1.B6 was detected with anti-HA antibody. Proteins from the incubation medium were included in the analysis. Actin detected with anti-actin antibody was used as a loading control. The asterisk indicates a 30-kD nonspecific band detected by anti-HA antibody.

**Figure 2.** HAx3:AtPRA1.B6 expressed stably in transgenic plants inhibits the trafficking of sporamin:GFP and AALP:GFP. Protoplasts were prepared from HAx3:AtPRA1.B6 or pTA transgenic plants treated with dexamethasone for 1 d and transformed with sporamin:GFP or AALP:GFP. Trafficking of sporamin:GFP and AALP:GFP was examined 24 h after transformation by western-blot analysis using anti-GFP. Expression of HAx3:AtPRA1.B6 was examined by western blotting using anti-HA antibody. pTA, pTA plants; PRA1, HAx3:AtPRA1.B6 plants.

**Figure 3.** HA:AtPRA1.B6 inhibits trafficking of the secretory protein invertase:GFP. A, Inhibition of invertase:GFP trafficking by HA:AtPRA1.B6. Invertase:GFP was cotransformed with AtPRA1.B6 or R6 into protoplasts, and the localization of invertase:GFP was examined. Chloroplasts (CH) were detected by red autofluorescence of chlorophyll. Bars = 20 μm. B, Western-blot analysis. Invertase:GFP was cotransformed with HA:AtPRA1.B6 or R6, and the protein extracts from protoplasts and incubation medium were analyzed by western-blot analysis using anti-GFP and anti-HA. To quantify trafficking efficiency, the band intensity was determined using software equipped to LAS3000. Trafficking efficiency was determined by the relative amount of secreted invertase:GFP over the total expressed proteins (proteins in both the medium and protoplasts). In order to measure the trafficking efficiency of invertase:GFP, the amount of secreted protein was quantified using software equipped to LAS3000 and expressed as a relative value to the total amount of protein. Error bars indicate SD (n = 3). R6, Empty vector; PRA1, HA:AtPRA1.B6; M, protein extracts from the incubation medium; P, protoplast extracts. C, Inhibition of invertase:GFP trafficking by HAx3:AtPRA1.B6 expressed in transgenic plants. Protoplasts from pTA or HAx3:AtPRA1.B6 transgenic plants treated with dexamethasone (30 μM) for 1 d were transformed with invertase:GFP, and trafficking efficiency was examined by western-blot analysis using anti-GFP antibody. Expression of HAx3:AtPRA1.B6 was detected with anti-HA antibody. Proteins from the incubation medium were included in the analysis. Actin detected with anti-actin antibody was used as a loading control. The asterisk indicates a 30-kD nonspecific band detected by anti-HA antibody.
signal in the protoplasts was likely a result of the efficient secretion of invertase:GFP into the medium. However, when coexpressed with HA:AtPRA1.B6, invertase:GFP produced the network pattern in the majority of protoplasts (Fig. 3A, panel c), indicating that HA:AtPRA1.B6 inhibits the secretion of invertase:GFP to the medium. To confirm this finding at the biochemical level, protein extracts were prepared from protoplasts and analyzed by western blotting using anti-GFP antibody. Proteins from the incubation medium were included in the analysis. In control protoplasts transformed with R6, 89% of the invertase:GFP was secreted into the medium (Fig. 3B). However, when coexpressed with HA:AtPRA1.B6, 57% of the invertase:GFP was secreted into the medium. These results confirm that HA:AtPRA1.B6 inhibits the anterograde trafficking of secretory proteins. To eliminate the possibility that an excess of transiently expressed AtPRA1.B6 in protoplasts caused the inhibition of invertase:GFP secretion, protoplasts were prepared from HAX3:AtPRA1.B6 plants that had been treated with dexamethasone for 1 d and transformed with invertase:GFP. Subsequently, the secretion of invertase:GFP was examined by western-blot analysis using anti-GFP antibody. As a control, protoplasts from plants harboring the empty expression vector pTA (pTA plants) were included. The amount of invertase:GFP in the medium was decreased to lower levels in comparison with the control protoplasts from pTA plants (Fig. 3C). This confirms that AtPRA1.B6 inhibits the trafficking of invertase:GFP.

Next, the trafficking of proteins to the plasma membrane was examined using H+-ATPase:GFP and PMP:GFP. H+-ATPase:GFP, a chimeric protein between plasma membrane H+-ATPase and GFP, is localized to the plasma membrane (Kim et al., 2001). Recently, plasma membrane protein (PMP):GFP, a chimeric protein generated using the N-terminal 40-amino acid segment containing the transmembrane domain of At1g53610, a protein predicted to localize to the plasma membrane, and GFP was shown to localize to the plasma membrane (Lee et al., 2011). These reporter protein constructs were introduced into protoplasts together with HA:AtPRA1.B6 or R6, and their localization was examined using a fluorescence microscope. In control protoplasts transformed with R6, both proteins produced a ring pattern, indicating that they were targeted to the plasma membrane (Fig. 4). In contrast, when coexpressed with HA:AtPRA1.B6, the protoplasts that had been transformed with H+-ATPase:GFP or PMP:GFP produced various localization patterns, such as aggregation, network, and plasma membrane patterns (Fig. 4). In the presence of HA:AtPRA1.B6, the plasma membrane pattern of H+-ATPase:GFP and PMP:GFP was reduced to 67% and 50%, respectively, from 97%. This suggests that HA:AtPRA1.B6 inhibits the trafficking of these proteins to the plasma membrane. We examined whether H+-ATPase:GFP and

Figure 4. HA:AtPRA1.B6 inhibits trafficking of plasma membrane proteins H+-ATPase:GFP and PMP:GFP. H+-ATPase:GFP (A) or PMP:GFP (B) was introduced into protoplasts together with empty expression vector R6, HA:AtPra1.B6, or Sar-1[H74L], and localization of the plasma membrane proteins was examined. To quantify the localization pattern, transformation was performed three times for each sample, and each time more than 200 transformed protoplasts were analyzed to determine the localization. The plasma membrane pattern was expressed as a relative value to the total number of transformed protoplasts. Error bars indicate SD (n = 3). PRA1, HA:AtPRA1.B6; CH, chloroplast. Bars = 20 μm.
HA:AtPRA1.B6 Does Not Inhibit the Trafficking of Proteins Localized to the Golgi Apparatus

The effect of AtPRA1.B6 on the trafficking of Golgi-localized proteins was also investigated. ST:GFP, a chimeric protein containing rat sialyltransferase and GFP, has been shown to localize to the Golgi apparatus (Wee et al., 1998; Kim et al., 2001). In this study, ST:GFP was introduced into protoplasts together with HA:AtPRA1.B6 or R6, and the localization of ST:GFP was examined. Surprisingly, ST:GFP produced a typical punctate staining pattern in protoplasts transformed with HA:AtPRA1.B6, similar to the control protoplasts transformed with R6 (Fig. 5A, panels a and c). To examine whether the ST:GFP-positive punctate staining represented the Golgi apparatus, the localization of ST:GFP was compared with that of endogenous γ-COP, a COP1 component localized to the Golgi apparatus (Pimpl et al., 2000). The localization of ST:GFP and γ-COP was determined directly by GFP fluorescence and immunohistochemistry using anti-γ-COP antibody (Pimpl et al., 2000), respectively. In both the presence and absence of HA:AtPRA1.B6, the endogenous γ-COP and ST:GFP produced punctate staining patterns (Fig. 5A, panels e, f, i, and j). Moreover, the punctate patterns closely overlapped one another (Fig. 5A, panels g and k), indicating that HA:AtPRA1.B6 does not inhibit the trafficking of ST:GFP to the Golgi apparatus. The production of HA:AtPRA1.B6 in transformed protoplasts was confirmed by western-blot analysis using anti-HA antibody (Fig. 5B).

To confirm this result at the biochemical level, endoglycosidase H (Endo H) sensitivity of the N-glycans of ST:GFP was utilized. The N-glycans of ER-localized proteins are sensitive to Endo H, whereas the N-glycans of Golgi-localized proteins are resistant to Endo H (Frigerio et al., 1998). Protein extracts from protoplasts transformed with ST:GFP were treated with or without Endo H and analyzed by western blotting using anti-GFP antibody. Both in the presence and absence of HA:AtPRA1.B6, the majority of ST:GFP was resistant to Endo H (Fig. 5C), thus confirming that ST:GFP localizes to the Golgi apparatus. Furthermore, HA:xAtPRA1.B6 expressed in transgenic plants also did not inhibit the trafficking of ST:GFP to the Golgi apparatus (Supplemental Fig. S3).

To determine whether this result is specific to ST:GFP, another Golgi reporter protein was utilized. KAM1
DC):mRFP, a chimera between the Golgi-localized C-terminal deletion construct of Katamari1 (KAM1) and monomeric red fluorescent protein (mRFP; Tamura et al., 2005), is targeted to the cis-Golgi. KAM1(DC):mRFP was introduced into protoplasts together with HA:AtPRA1.B6 or R6, and the localization of KAM1(DC):mRFP was examined. In both the presence and absence of HA:AtPRA1.B6, KAM1(DC):mRFP produced the punctate staining pattern (Fig. 6A, panels a and c), suggesting localization to the Golgi apparatus. To confirm that KAM1(DC):mRFP localizes to the Golgi apparatus even in the presence of HA:AtPRA1.B6, KAM1(DC):mRFP and ST:GFP were introduced into protoplasts together with HA:AtPRA1.B6 or R6 and the localization of the proteins was examined. Again, both KAM1(DC):mRFP and ST:GFP colocalized to the punctate stains regardless of HA:AtPRA1.B6 expression (Fig. 6A, panels e–g and i–k). These results indicate that HA:AtPRA1.B6 does not inhibit the trafficking of KAM1(DC):mRFP to the Golgi apparatus.

To eliminate the possibility that HA:AtPRA1.B6 inhibits both KAM1(DC):mRFP and ST:GFP and causes them to accumulate in the ER as punctate stains, as observed with Sar-1:RFP at the ER exit site, protoplasts were prepared from transgenic plants expressing ST:GFP (Lee et al., 2002). These protoplasts were cotransformed with KAM1(DC):mRFP and HA:AtPRA1.B6 or R6, and the localization of these proteins was examined. In both the presence and absence of HA:AtPRA1.B6, KAM1(DC):mRFP colocalized with stably expressed ST:GFP at punctate stains (Fig. 6B, panels a–c and e–g), indicating that HA:AtPRA1.B6 does not inhibit the trafficking of KAM1(DC):mRFP to the Golgi apparatus. To eliminate the possibility that HA:AtPRA1.B6 inhibits both KAM1(DC):mRFP and ST:GFP and causes them to accumulate in the ER as punctate stains, as observed with Sar-1:RFP at the ER exit site, protoplasts were prepared from transgenic plants expressing ST:GFP (Lee et al., 2002). These protoplasts were cotransformed with KAM1(DC):mRFP and HA:AtPRA1.B6 or R6, and the localization of these proteins was examined. In both the presence and absence of HA:AtPRA1.B6, KAM1(DC):mRFP colocalized with stably expressed ST:GFP at punctate stains (Fig. 6B, panels a–c and e–g), indicating that HA:AtPRA1.B6 does not inhibit the trafficking of KAM1(DC):mRFP to the Golgi apparatus.

Figure 6. HA:AtPRA1.B6 does not inhibit the trafficking of KAM1(DC):mRFP to the Golgi apparatus. A and B, Localization of KAM1(DC):mRFP to the Golgi apparatus upon coexpressing HA:AtPRA1.B6. Protoplasts from wild-type plants (A) or ST:GFP transgenic plants (B) were transformed with the indicated constructs, and the localization of these proteins was examined directly by RFP or GFP fluorescence. In each transformation, 10 μg of HA:AtPRA1.B6 was introduced. Bars = 20 μm. Three independent transformation experiments were performed for each sample, and more than 60 protoplasts were analyzed for the localization each time. C, Differential inhibition of anterograde trafficking by coexpressing HA:AtPRA1.B6. Protoplasts were cotransformed with three plasmids, HA:AtPRA1.B6 (10 μg), invertase:GFP (5 μg), and KAM1(DC):mRFP (5 μg), and the localization of these proteins was examined. Bars = 20 μm. Three independent transformation experiments were performed, and more than 30 protoplasts were examined to determine the localization.

Figure 7. HA:AtPRA1.B6 inhibits ER-to-Golgi trafficking of PMP:GFP-glyNC. Protoplasts were cotransformed with PMP:GFP-glyNC, incubated with (+) or without (−) tunicamycin, and analyzed by western blotting using anti-GFP. Protoplasts were cotransformed with PMP:GFP-glyNC (5 μg) and HA:AtPRA1.B6 (10 μg) or R6 (10 μg). Protein extracts from the transformed protoplasts were treated with (+) or without (−) Endo H and analyzed by western blotting using anti-HA and anti-GFP antibodies. In order to measure the trafficking efficiency of PMP:GFP-glyNC to the Golgi apparatus, the amount of the Endo H-resistant form was quantified using software equipped to LAS3000 and expressed as a relative value to the total amount of protein. Error bars indicate SD (n = 3). gly, Glycosylated form; un-gly, unglycosylated form; R6, empty vector; PRA1, HA:AtPRA1.B6.
trafficking of KAM1(ΔC):mRFP to the Golgi apparatus. These results clearly demonstrate that AtPRA1.B6 does not inhibit the trafficking of Golgi-resident proteins.

To further confirm the differential effect of AtPRA1.B6 on anterograde trafficking and to eliminate the possibility that the lack of inhibitory effect on Golgi-localized proteins is caused by low levels of HA:AtPRA1.B6 in a subpopulation of transformed protoplasts, the trafficking of these proteins was examined in protoplasts cotransformed with three constructs, KAM1(ΔC):mRFP, invertase:GFP, and HA:AtPRA1.B6. GFP and RFP signals of invertase:GFP and KAM1(ΔC):mRFP produced the network pattern and the punctate staining pattern, respectively, in a single protoplast (Fig. 6C). This clearly demonstrates that AtPRA1.B6 inhibits the trafficking of invertase:GFP but not of Golgi-localized KAM1(ΔC):mRFP.

**HA:AtPRA1.B6 Inhibits Anterograde Trafficking at the ER**

The results shown in Figure 6 prompted an examination of where the AtPRA1.B6-mediated inhibition of anterograde trafficking occurs. If the AtPRA1.B6-mediated inhibition occurs at the post-Golgi compartment, AtPRA1.B6 would not inhibit the Golgi-localized proteins. To define which step is inhibited by AtPRA1.B6 during anterograde trafficking, the Endo H sensitivity of the N-glycans of cargo proteins was used (Frigerio et al., 1998; Park et al., 2004). PMP:GFP-glyNC was examined. Protoplasts incubated with or without tunicamycin were incubated with or without tunicamycin. In the presence of HA:AtPRA1.B6, the majority of PMP:GFP-glyNC was resistant to Endo H (Fig. 7, lane 2 in right panel), consistent with the image analysis (Fig. 4, panel e). In contrast, in the presence of HA:AtPRA1.B6, the majority of PMP:GFP-glyNC was sensitive to Endo H (Fig. 7, lane 4 in right panel), indicating that PMP:GFP-glyNC localized to the ER. These results suggest that AtPRA1.B6 inhibits anterograde trafficking of plasma membrane proteins at the ER. In addition, the results were supported by the accumulation of vacuolar proteins in the ER in the presence of HA:AtPRA1.B6 (Fig. 1C, panels c and g).

**PMP:GFP-glyNC from tunicamycin-treated protoplasts migrated faster than that from tunicamycin-untreated protoplasts (Fig. 7, left panel), confirming that PMP:GFP-glyNC is N-glycosylated.** Next, PMP:GFP-glyNC was introduced into protoplasts together with HA:AtPRA1.B6 or R6. Subsequently, proteins from transformed protoplasts were treated with or without Endo H and analyzed by western blotting using anti-GFP antibody. In the absence of HA:AtPRA1.B6, the majority of PMP:GFP-glyNC was resistant to Endo H (Fig. 7, lane 2 in right panel), consistent with the image analysis (Fig. 4, panel e). In contrast, in the presence of HA:AtPRA1.B6, the majority of PMP:GFP-glyNC was sensitive to Endo H (Fig. 7, lane 4 in right panel), indicating that PMP:GFP-glyNC localized to the ER. These results suggest that AtPRA1.B6 inhibits anterograde trafficking of plasma membrane proteins at the ER. In addition, the results were supported by the accumulation of vacuolar proteins in the ER in the presence of HA:AtPRA1.B6 (Fig. 1C, panels c and g).

**The ER-Localized C-Terminal Deletion Mutant ΔC1, But Not the Prevacuolar Compartment-Localized N-Terminal Deletion Mutant ΔN2, Inhibits Anterograde Trafficking of Sporamin:GFP and Invertase:GFP**

Two AtPRA1.B6 deletion mutants, ΔC1 and ΔN2, which have a deletion of the C-terminal 37 and N-terminal 36 amino acid residues of AtPRA1.B6, localize to the ER and prevacuolar compartment, respectively (Jung et al., 2011). The trafficking of proteins to the vacuole or apoplast was examined in the presence of these mutants. Sporamin:GFP or invertase:GFP was cotransformed into protoplasts together with HA:AtPRA1.B6, HA:ΔC1, HA:ΔN2, or R6, and their trafficking efficiency was determined by western blot analysis using anti-GFP antibody. In the case of invertase:GFP, proteins from the incubation medium were
included in the analysis. HA:ΔC1, but not HA:ΔN2, inhibited the trafficking of sporamin:GFP and invertase:GFP (Fig. 8), as observed with wild-type HA:AtPRA1.B6. These results indicate that the localization of HA:AtPRA1.B6 to the ER is important for the inhibition. The expression of HA:AtPRA1.B6, HA:ΔC1, and HA:ΔN2 was confirmed by western-blot analysis using anti-HA antibody.

COPII Vesicles Are Involved in Anterograde Trafficking Regardless of Whether the ER-to-Golgi Trafficking Is Inhibited by AtPRA1.B6

In anterograde trafficking from the ER to the Golgi apparatus, one group of proteins was subjected to AtPRA1.B6-mediated inhibition and the other group of proteins was not. To investigate the differential inhibition of anterograde trafficking by AtPRA1.B6, any differences in the trafficking pathways due to the groups of proteins were examined. COPII vesicles are involved in the ER-to-Golgi trafficking in plant cells. Accordingly, cargo proteins (AALP:GFP, sporamin:GFP, invertase:GFP, and PMP:GFP-glyNC) subject to AtPRA1.B6-mediated trafficking inhibition at the ER were examined to determine whether they were transported from the ER to the Golgi apparatus by COPII vesicles. Protoplasts were transformed with AALP:GFP, sporamin:GFP, invertase:GFP, and PMP:GFP-glyNC, together with Sar-1[H74L], a dominant negative Sar-1 mutant that inhibits anterograde trafficking from the ER (Takeuchi et al., 2000; Phillipson et al., 2001; Sohn et al., 2003), and the localization of these proteins was examined by fluorescence microscopy and western-blot analysis using anti-GFP antibody. All of these proteins, AALP:GFP, sporamin:GFP, invertase:GFP, and PMP:GFP-glyNC, produced a network pattern (Fig. 9A, panels a–d). Additionally, in the presence of Sar-1[H74L], the majority of AALP:GFP and sporamin:GFP was detected as precursors and invertase:GFP was detected primarily in the protoplasts, with almost no secretion into the medium (Fig. 9B). In the case of PMP:GFP-glyNC, the majority of the protein was detected as Endo H-sensitive forms. These results confirmed that all these cargo proteins are transported by COPII vesicles.
Previously, ST:GFP was reported to be transported to the Golgi apparatus in protoplasts of leaf tissues (Batoko et al., 2000). To confirm this, protoplasts were transformed with ST:GFP together with Sar-1[H74L], and the localization of ST:GFP was examined. In the presence of Sar-1[H74L], ST:GFP produced a network pattern (Fig. 9A, panel e), an indication of ER localization. This confirms that ST:GFP is transported in a COPII-dependent manner.

Figure 10. Trafficking efficiency of vacuolar proteins is increased in AtPRA1.B6 RNAi plants. A, qRT-PCR analysis of AtPRA1.B6 transcript levels in RNAi plants. Total RNA was obtained from RNAi plants and used for qRT-PCR analysis with gene-specific primers. As a negative control, AtPRA1.D, a homolog of AtPRA1.B6, was included. Actin levels were used as an internal control. WT, Wild-type plants; RNAi, AtPRA1.B6 RNAi plants. Error bars indicate SD (n = 3). B, Trafficking efficiency in RNAi plants. Protoplasts from two independent RNAi plants (lines 10 and 14) were transformed with sporamin:GFP or AALP:GFP, and their trafficking to the vacuole was examined by western-blot analysis using anti-GFP antibody. The trafficking efficiencies of AALP:GFP and sporamin:GFP were quantified in wild-type and RNAi plants. The amounts of the processed forms were expressed as relative values to the total amount of proteins. Error bars indicate SD (n = 3). Spo:GFP, Sporamin:GFP.

AtPRA1.B6 Levels Are Decreased by 26S Proteasome-Dependent Degradation

As anterograde trafficking of a subset of proteins was shown to be sensitive to the AtPRA1.B6 level, the intriguing possibility that the AtPRA1.B6 levels are subject to regulation in plant cells was investigated. In cells, protein levels can be lowered by 26S proteasome-mediated proteolysis (Smalle and Vierstra, 2004). To examine whether AtPRA1.B6 proteins are degraded by the 26S proteasome, the effect of MG132, an inhibitor of the 26S proteasome (Lee and Goldberg, 1998), on the level of AtPRA1.B6 in protoplasts was determined. Protoplasts transformed with HA:AtPRA1.B6 were treated with MG132 or dimethyl sulfoxide (DMSO) 12 h after transformation, followed by an additional incubation of 12 or 24 h. Protein levels were examined by western-blot analysis using anti-HA antibody and compared at three time points (0, 12, and 24 h) after MG132 or DMSO treatment. In the DMSO-treated protoplasts, the HA:AtPRA1.B6 levels rose to a peak at 12 h and then declined to a lower level at 24 h (Fig. 11). In contrast, in MG132-treated protoplasts, the HA:AtPRA1.B6 levels rose to a peak at 12 h and then declined to a lower level at 24 h (Fig. 11). As a control, the level of ER-localized GKX, a chimeric fusion protein containing the KKXX motif for ER retrieval from the Golgi complex, was examined as a cargo protein marker (Benghezal et al., 2000; Lee et al., 2009). The GKX level was not affected by MG132 treatment, indicating that 26S proteasome-dependent proteolysis is specific to HA:AtPRA1.B6. The loading control Lhcb4, detected with anti-Lhcb4 antibody, was at an equal level in all samples. In addition, public microarray data revealed low levels of AtPRA1.B6 on anterograde trafficking was investigated. RNAi plants were generated using a 317-bp fragment of AtPRA1.B6 (Wesley et al., 2001), as atpra1.b6 mutant plants were not available. Two independent RNAi lines (lines 10 and 14) were selected, and the AtPRA1.B6 transcript levels were examined by quantitative reverse transcription (qRT)-PCR using gene-specific primers. Figure 10A reveals that the transcript levels of AtPRA1.B6 were specifically decreased in RNAi plants. The transcript level of AtPRA1.D, a homolog and member of the AtPRA1 family used as a negative control, was not affected. Next, the protein trafficking efficiency of vacuolar proteins was examined in protoplasts prepared from the RNAi plants. The vacuolar trafficking efficiency of AALP:GFP was increased in protoplasts prepared from two independent lines of AtPRA1.B6 RNAi plants compared with that in wild-type plants (Fig. 10B). Similarly, the vacuolar trafficking efficiency of sporamin:GFP was also enhanced in AtPRA1.B6 RNAi plants, indicating that lower levels of AtPRA1.B6 result in an increase in anterograde trafficking efficiency.

Previously, ST:GFP was reported to be transported to the Golgi apparatus in protoplasts of leaf tissues (Batoko et al., 2000). To confirm this, protoplasts were transformed with ST:GFP together with Sar-1[H74L], and the localization of ST:GFP was examined. In the presence of Sar-1[H74L], ST:GFP produced a network pattern (Fig. 9A, panel e), an indication of ER localization. This confirms that ST:GFP is transported in a COPII-dependent manner.

RNAi Plants Expressing Low Levels of AtPRA1.B6 Exhibit Enhanced Vacuolar Trafficking

As high levels of AtPRA1.B6 inhibit anterograde trafficking of a subset of cargo proteins, the effect of
that expression of AtPRA1.B6 is induced by multiple environmental stresses (http://www.genevestigator.com). Taken together, these results strongly suggest that AtPRA1.B6 levels are regulated by cellular processes including 26S proteasome-mediated proteolysis in plant cells.

**DISCUSSION**

This study provides evidence that AtPRA1.B6 functions as a negative regulator of the anterograde trafficking of a subset of proteins at the ER. The anterograde trafficking efficiency was inversely correlated with the protein levels of AtPRA1.B6. For two vacuolar proteins (sporamin:GFP and AALP:GFP), an apoplast-secreted protein (invertase:GFP), and two plasma membrane proteins (H^+-ATPase:GFP and PMP:GFP), overexpression of AtPRA1.B6 inhibits the anterograde trafficking. In contrast, suppression of AtPRA1.B6 in RNAi plants resulted in an increase in trafficking efficiency. The inhibition of anterograde trafficking by high levels of AtPRA1.B6 homologs is not unique to plants. Similarly, overexpression of yeast and animal homologs, Yip3p and Yip6b, respectively, inhibits ER-to-Golgi transport in yeast and animal cells (Geng et al., 2005; Ruggiero et al., 2008). In addition, the overexpression of JMY/Yip6a suppresses the trafficking of the CC chemokine receptor 5 to the cell surface (Schweneker et al., 2005). To demonstrate the effect of AtPRA1.B6 levels on protein trafficking, this study employed two artificial approaches, ectopic overexpression and RNAi-mediated suppression, to increase and decrease AtPRA1.B6 levels, respectively. This hypothesis requires the AtPRA1.B6 levels to be controlled by cellular processes. Indeed, AtPRA1.B6 levels are down-regulated by the 26S proteasome. Moreover, under certain conditions, AtPRA1.B6 levels may be altered by biotic or abiotic stresses, according to microarray data in the public database (http://www.genevestigator.com).

The AtPRA1.B6-mediated inhibition of anterograde trafficking occurs at the ER. This conclusion is based on the fact that the cargo proteins inhibited by AtPRA1.B6 accumulated in the ER, as determined by the Endo H sensitivity of N-glycans and the localization of cargo proteins. In support of this finding, the C-terminal deletion mutant ΔC1 that localized primarily to the ER, but not the prevacuolar compartment-localized ΔN2 (Jung et al., 2011), also inhibited the anterograde trafficking of vacuolar proteins. This raises the intriguing question of how the Golgi-localized AtPRA1.B6 is able to function as a negative regulator of anterograde trafficking at the ER. One simple explanation is that the mislocalization of AtPRA1.B6 to the ER, resulting from overexpression, causes anterograde trafficking at the ER. Another possibility is that although the majority of AtPRA1.B6 localizes to the Golgi apparatus, a small amount also localizes to the ER. The ER-localized AtPRA1.B6 may not be easily detected at normal expression levels but may become visible at high levels, as observed in the case of overexpression. It is possible that the ER-localized AtPRA1.B6 functions as a negative regulator of anterograde trafficking of a subset of proteins. This second explanation is favored. Indeed, a minor portion of Yip3p localizes to the ER, in addition to the major portion localized to the Golgi apparatus in yeast. Similarly, overexpression of Yip3p causes inhibition of anterograde trafficking at the ER (Geng et al., 2005).

Despite the fact that AtPRA1.B6-mediated inhibition of trafficking occurs at the ER, trafficking of ST:GFP and KAM1(ΔC):mRFP to the Golgi apparatus was not affected by AtPRA1.B6. These data indicate that AtPRA1.B6-mediated inhibition is specific to a subset of cargo proteins that are transported by the Golgi-dependent anterograde trafficking pathway. In addition, these results argue strongly against the possibility that inhibition is caused by the nonspecific effects of HA:AtPRA1.B6 overexpression. Furthermore, the inhibitory effect of AtPRA1.B6 on anterograde trafficking appears to differ from that of SNARE SYP31 and SYP81 overexpression. SNARE overexpression inhibits the trafficking of not only secreted α-amylase but also the Golgi-localized proteins ST:GFP and Man:GFP (Bubeck et al., 2008). However, the mechanism by which Golgi-localized proteins escape the inhibition is not clearly understood. As reported previously with other vacuolar and secretory proteins in plant cells (Phillipson et al., 2001; daSilva et al., 2004), all of the

**Figure 11.** AtPRA1.B6 protein levels are lowered by 26S proteasome-mediated degradation. Protoplasts transformed with HA:AtPRA1.B6 or GKX as a negative control were treated with DMSO or MG132 12 h after transformation, followed by additional incubation of 12 or 24 h, and protein extracts were analyzed by western-blot analysis using anti-HA antibody. As a loading control, Lhcb4 was detected with anti-Lhcb4 antibody. PRA1, HA:AtPRA1.B6.
cargo proteins were transported by COPII vesicles regardless of whether their trafficking was inhibited by AtPRA1.B6 or not. Thus, one possible explanation for the differential inhibition is that plant cells may contain multiple types of COPII vesicles that are differentially used depending on the cargo proteins. Indeed, in animal cells and yeast cells, multiple types of COPII vesicles exist (Tang et al., 1999; Wendeler et al., 2007). Among COPII vesicle components, Sec24 plays a critical role in selecting cargo proteins for the COPII vesicle (Miller et al., 2003). In yeast cells, the deletion of LST1, encoding an isoform of Sec24, inhibits the trafficking of Pma1p from the ER but does not affect the trafficking of other proteins (Roberg et al., 1999). In animal cells, Wendeler et al. (2007) reported the differential effects of Sec24 isoform-specific silencing on the transport of membrane proteins. Similarly, in Arabidopsis, multiple isoforms of Sec24 exist, supporting the possibility that multiple types of COPII vesicles also exist in plant cells. Consistent with this inference, mutations in each of these Sec24 isoforms resulted in different phenotypes (Faso et al., 2009). Additionally, Arabidopsis contains two Sar-1 isoforms that exhibit differences in their localizations and effects on protein secretion. Again, the presence of two Sar-1 isoforms supports the inference that multiple COPII vesicles exist in plants (Hanton et al., 2008). However, it is currently unknown whether these multiple Sec24 and Sar-1 isoforms account for the differential inhibition of anterograde trafficking by AtPRA1.B6. The differential inhibitory effect of AtPRA1.B6 can also be observed if the COPII-dependent exit of cargo proteins from the ER is mediated by multiple pathways. Indeed, certain cargoes, such as GONST1, CASP, and KAT, exit the ER in a sorting signal-dependent manner, whereas α-amylase is secreted from the ER by the default or bulk flow pathway (Denecke et al., 1990; Phillipson et al., 2001; Hanton et al., 2005; Sieben et al., 2008).

PRA1 is proposed to play a role in targeting prenylated Rabs to the Golgi apparatus and endosomes by catalyzing their dissociation from Rab/GDI complexes in the cytoplasm (Sivars et al., 2003). Similarly, Arabidopsis PRA1 homologs have been shown to interact with Rabs (Alvim Kamei et al., 2008). Thus, AtPRA1.B6 may regulate the behavior of Rab proteins involved in COPII vesicle formation in a cargo-specific manner. For this reason, high levels of AtPRA1.B6 may inhibit the activity of Rabs. In animal cells, PRA1 inhibits the extraction of membrane-bound Rab GTPase by GDI1 (Hutt et al., 2000), which in turn may affect the activity of Rabs. Additionally, Yip6b/GTRAP3-18, a member of the Yip6 subfamily that includes PRA2, has an inhibitory effect on Rab1, a protein involved in ER-to-Golgi trafficking. Interestingly, the inhibitory effect of Yip6b/GTRAB3-18 can be reversed by an excess of Rab1 (Maier et al., 2009). In yeast cells, Yip3p also forms a complex with Ypt1p, a protein involved in ER-to-Golgi trafficking (Geng et al., 2005). However, the overexpression or deletion of Yip3p does not affect the behavior of Rab proteins (Geng et al., 2005), raising the possibility that the inhibitory effect of Yip3p on protein trafficking may occur through a mechanism that does not require Rab proteins. Originally, Yip3p was detected as a binding partner of Yip1p, an integral membrane protein and a member of the Yip1 subfamily localized to the Golgi apparatus (Otte et al., 2001; Calero and Collins, 2002; Pfeffer and Aivazian, 2004). Yip1p, in conjunction with Yif1p, a member of the Yif1 subfamily in yeast, plays an essential role in COPII vesicle biogenesis. In the absence of Yip1p, COPII vesicle budding does not occur and cargo accumulates in the ER (Heidtman et al., 2003). In fact, Yip3p has been recently identified as a component of COPII vesicles (Chen et al., 2004). Furthermore, overexpression of Yip3p causes ER membrane proliferation, likely due to the blockage of COPII vesicle budding (Geng et al., 2005). However, it is not known whether AtPRA1.B6 is a component of COPII vesicles or is involved in COPII vesicle budding in plant cells. Further study is necessary to understand the detailed mechanism of AtPRA1.B6 action in protein trafficking in plant cells.

MATERIALS AND METHODS

Growth of Plants

Arabidopsis (Arabidopsis thaliana ecotype Columbia) plants were grown on B5 plates in a culture room under 70% relative humidity and a 16-h-light/8-h-dark cycle. A third set of plants were grown in soil in a greenhouse under 70% relative humidity and a 16-h-light/8-h-dark cycle. A third set of plants were grown on B5 plates supplemented with dexamethasone (30 μM). Leaf tissues obtained from 15- to 18-d-old plants were used immediately for protoplast preparation, as described previously (Jin et al., 2001).

Generation of AtPRA1.B6 RNAi Plants and qRT-PCR

To generate AtPRA1.B6 RNAi transgenic plants, a 317-bp fragment (nucleotide positions 1–317) of the AtPRA1.B6 coding region was amplified by PCR using primers 5'-ATGGCTTCTCCTCTCTCCCATC-3' and 5'-GCTCTAGAATGTCGCATAGAAATCAAAC-3'. The PCR product was ligated to a pUC-based plasmid PMP:GFP as a template. The PCR product was ligated to the RNAi vector (Wesley et al., 2001). The construct was introduced into plants by the floral dip method (Clough and Bent, 1998).

To examine the transcript levels of AtPRA1.B6, total RNA was prepared using an RNAqueous Kit (Ambion) with a TURBO DNA-Free Kit (Applied Biosystems). cDNA was synthesized using a high-capacity cDNA Reverse Transcription Kit (Applied Biosystems). qRT-PCR was performed on the Step One Plus Real Time PCR System (Applied Biosystems). Plasmid DNA containing the corresponding cDNAs was used as a template to generate a calibration curve. The primers used for PCR were as follows: 5'-CCACTTCCGACTACTCTCTCCTGATCAA-3' and 5'-CCGGTACGAGCATCAGAAACT-3' for AtPRA1.B6, and 5'-GGATGATCTTGTGAGTGACGATCT-3' and 5'-ACACCGCCAGGAGGTAAGA-3' for AtPRA1.D.

Construction of Plasmids

Various AtPRA1.B6 full-length and deletion constructs were reported previously (Jung et al., 2011). PMP cDNA (At1g53610) was amplified using the primers 5'-CGGGATCCACCCGTTTCTGAGGAGGAAACTATTCTCTCCCATC-3' and 5'-GCTCTAGAATGTCGCATAGAAATCAAAC-3'. The PCR product was ligated to PMP:GFP as a template. The PCR product was ligated to a pUC-based plasmid.
expression vector containing the 3S cauliflower mosaic virus promoter and nos terminator. All PCR products were confirmed by sequencing.

Transient Expression, Immunofluorescence Staining, and Microscopy
Plasmids were purified using Qiagen columns and introduced into Arabidopsis leaf cell protoplasts by polyethylene glycol-mediated transformation (Jin et al., 2001; Kim et al., 2001). Protoplasts were prepared from leaf tissues of wild-type, STGFP, or HA3x:AtPRA1.B6 plants (Lee et al., 2002; Jung et al., 2011). Before preparing protoplasts, the HA3x:AtPRA1.B6 plants were treated with desamethasone (30 μM) for 1 d. In addition, protoplasts from desamethasone-untreated HA3x:AtPRA1.B6 plants were incubated with desamethasone (30 μM) after transformation. The expression of these constructs was monitored at various time points after transformation. Images of GFP fluorescence were obtained from intact protoplasts placed on glass slides and covered with a coverslip. For immunostaining, protoplasts on coverslips were fixed with 4% (v/v) paraformaldehyde, as described previously (Frigero et al., 1996; Park et al., 2004). Fixed protoplasts were labeled with anti-GFP antibody (Pimpl et al., 2000). Cells were washed with buffer (10 mM Tris, pH 7.4, 0.9% [w/v] NaCl, 0.25% [w/v] gelatin, 0.02% [w/v] SDS, and 0.1% [v/v] Triton X-100), and secondary immunostaining was performed for 3 h using tetramethylrhodamine-5-(and-6)-isothiocyanate-labeled goat anti-rabbit antibodies (Molecular Probes). In immunostained protoplasts were mounted in medium (120 mM Tris, pH 8.4, and 30% glycerol) containing Mowiol 4-88 (Calbiochem).

Images were obtained using a fluorescence microscope (Axioplan 2; Carl Zeiss) equipped with a 40× objective (Plan-NEOFULUR) and a cooled CCD camera (Senicam; PCO Imaging) at 20°C. Two filter sets were used: XF116 (exciter, 474AF20; dichroic, 500DRLP; emitter, 510AF23) and XF117 (exciter, 540AF30; dichroic, 585ALP [Omega Optical]). Images were processed with Adobe Photoshop (version 7.0).

Western-Blot Analysis of HA-Tagged AtPRA1.B6 Expressed in Protoplasts and Transgenic Plants
Transformed protoplasts were harvested at appropriate time points after transformation and resuspended in lysis buffer (250 mM Suc, 25 mM HEPES-NaOH [pH 7.4], 150 mM NaCl, 3 mM EDTA, 2 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitor cocktail [Roche Diagnostics]). All PCR products were confirmed by sequencing.

Endo H Digestion
For Endo H treatment, transformed protoplast extracts were denatured by adding 100 μL of 2× denaturation buffer (1% SDS and 2% β-mercaptoethanol) and, subsequently, boiling for 10 min. After cooling to 22°C, 100 μL of 2× reaction buffer (100 mM sodium citrate, pH 5.5) was added. The sample (100 μL) was mixed with 1 μL of Endo H (1 unit μL−1). Digestions were conducted for 1 h at 37°C and were terminated by the addition of 1× SDS-PAGE loading buffer. Control samples were treated in the same way but without Endo H.

Supplemental Data
The following materials are available in the online version of this article. The Supplemental Figure S1. Protein levels of HA:AtPRA1.B6 in protoplasts and HA3x:AtPRA1.B6 in transgenic plants.

Supplemental Figure S2. Trafficking of AALP:GFP to the vacuole is inhibited by HA3x:AtPRA1.B6 induced in protoplasts.

Supplemental Figure S3. Trafficking of STGFP to the Golgi apparatus is inhibited by HA3x:AtPRA1.B6 induced in protoplasts.

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