Actin Filaments Play a Critical Role in Vacuolar Trafficking at the Golgi Complex in Plant Cells

Hyeran Kim,* Misoon Park,* Soo Jin Kim, and Inhwan Hwang*ab1

*a Division of Molecular and Life Sciences, Pohang University of Science and Technology, Pohang, 790-784, Korea
b Center for Plant Intracellular Trafficking, Pohang University of Science and Technology, Pohang, 790-784, Korea

Actin filaments are thought to play an important role in intracellular trafficking in various eukaryotic cells. However, their involvement in intracellular trafficking in plant cells has not been clearly demonstrated. Here, we investigated the roles actin filaments play in intracellular trafficking in plant cells using latrunculin B (Lat B), an inhibitor of actin filament assembly, or actin mutants that disrupt actin filaments when overexpressed. Lat B and actin2 mutant overexpression inhibited the trafficking of two vacuolar reporter proteins, sporamin:green fluorescent protein (GFP) and Arabidopsis thaliana aleurain-like protein:GFP, to the central vacule; instead, a punctate staining pattern was observed. Colocalization experiments with various marker proteins indicated that these punctate stains corresponded to the Golgi complex. The A. thaliana vacuolar sorting receptor VSR-At, which mainly localizes to the prevacuolar compartment, also accumulated at the Golgi complex in the presence of Lat B. However, Lat B had no effect on the endoplasmic reticulum (ER) to Golgi trafficking of sialyltransferase or retrograde Golgi to ER trafficking. Lat B also failed to influence the Golgi to plasma membrane trafficking of H+-ATPase:GFP or the secretion of invertase:GFP. Based on these observations, we propose that actin filaments play a critical role in the trafficking of proteins from the Golgi complex to the central vacule.

INTRODUCTION

In eukaryotic cells, a large number of proteins are targeted after translation to specific organelles by a process called intracellular trafficking. This targeting process is quite complex and involves many different pathways depending on the organelle involved (Rothman, 1994; Hawes et al., 1999; Jahn and Südhof, 1999). Cytoskeletal components such as actin filaments and microtubules play important roles in intracellular trafficking. It is generally assumed that the cytoskeleton acts like a network of tracks for the movement of vesicles between cellular compartments (Simon and Pon, 1996; Huang et al., 1999; Nebenführ and Staehelin, 2001). In addition, reorganization of actin filaments mediated by small GTPases such as ADP ribosylation factor (Arf) and Rho is required for the generation of endocytic vesicles and the docking at and fusion of secretory granules with the plasma membrane in animal cells (Wendland et al., 1998; Goode et al., 2001; Musch et al., 2001; Ridley, 2001). In addition, many actin binding proteins interact either directly or indirectly with proteins involved in endocytosis (Jeng and Welch, 2001; Schafer et al., 2002). For example, the F-actin binding protein Abp1 interacts with dynamin through the SH3 domain (Kessels et al., 2001), and dynamin 2 interacts directly with cortactin, an actin binding protein, at the plasma membrane (Schafer et al., 2002). Actin filaments also have been shown to be important in trafficking through the Golgi apparatus (Godi et al., 1998; Hirschberg et al., 1998; De Matteis and Morrow, 2000; Wu et al., 2000; Fucini et al., 2002). In yeast (Saccharomyces cerevisiae) cells, actin filaments are critical for polarized secretion (Johnston et al., 1991; Govindan et al., 1995; Schott et al., 1999; Zhang et al., 2001). Similarly, actin filaments are involved in trafficking of apical and basolateral proteins from the trans-Golgi network (TGN) in certain animal cell types (Musch et al., 2001). Expression of a dominant-negative mutant of Cdc24, a member of the Rho GTP binding protein subfamily and a key modulator of cortical actin, inhibited the exit of basolateral proteins from the TGN (Musch et al., 2001). In addition, Valderrama et al. (2001) reported that in mammalian cells, retrograde trafficking from the Golgi apparatus to the endoplasmic reticulum (ER) is inhibited by latrunculin B (Lat B), an inhibitor of actin filament assembly.

The large amount of data described above was largely derived from yeast and animal cells, but actin filaments also are thought to be important for vesicle trafficking in plant cells (Mollenhauer and Morre, 1976; Picton and Steer, 1981, 1983; Nebenführ and Staehelin, 2001; Waller et al., 2002). One of the clearest lines of evidence for this is the role actin filaments play in pollen tube growth and root tips (Picton and Steer, 1981, 1983; reviewed in Taylor and Hepler, 1997; Gibbon et al., 1999; Vidali et al., 2001). In particular, at the tip of the pollen tube, exocytic vesicles are thought to travel along actin filaments that run parallel to the direction of the pollen tube. In addition, cytochalasin D and Lat B treatment inhibited brefeldin A (BFA)–induced intracellular PIN1 accumulation as well as its relocation to the plasma membrane when BFA was washed out (Geldner et al., 2001; Baluska et al., 2002). Furthermore, actin filaments play a role in...
fluid-phase endocytosis or internalization of sterol from the plant cell plasma membrane (Grebe et al., 2003; Baluska et al., 2004). Thus, actin filaments appear to be involved in intracellular trafficking in plant cells. However, the exact molecular mechanism by which this occurs is not clearly understood. Recently, the trafficking in plant cells. However, the exact molecular mechanism by which this occurs is not clearly understood. Recently, the Arabidopsis thaliana actin binding protein AtSH3P1 was shown to colocalize with clathrin at various locations (Lam et al., 2002), which further supports the idea that actin filaments are involved in intracellular trafficking.

In this study, we investigated the involvement of actin filaments in various steps of anterograde trafficking in plant cells. To do this, we examined the trafficking of newly synthesized marker proteins to various final destinations in protoplasts, including the Golgi apparatus, the plasma membrane, and the central vacuole, in the presence of Lat B. We present evidence that actin filaments play a critical role in vacuolar trafficking of proteins from the Golgi complex to the prevacuolar compartment (PVC) but do not participate in the trafficking of plasma membrane or secretion of proteins.

RESULTS

Lat B Inhibits Trafficking of Two Reporter Proteins to the Central Vacuole

To investigate the roles played by actin filaments during intracellular trafficking, we examined the effect of Lat B, an agent known to disassemble the actin filaments (Spector et al., 1983), on the trafficking of vacuolar cargo proteins. As an experimental system, we used protoplasts derived from Arabidopsis leaf tissues. First, we determined the effective concentration of Lat B needed to disassemble the actin filaments in these protoplasts because the Lat B concentrations used in previous studies with plant cells varied greatly from a few nanomoles per liter to several hundred micromoles per liter (Vidali et al., 2001; Brandizzi et al., 2002; Saint-Jore et al., 2002). Thus, protoplasts were treated with varying Lat B concentrations, after which protein extracts were prepared and fractionated by low-speed centrifugation into pellet and soluble fractions, which contain the filamentous and soluble forms of actin, respectively (Abe and Davies, 1995). The actin in these fractions was detected by protein gel blot analysis using an anti-actin antibody. In the absence of Lat B, the majority of actin was detected in the pellet fraction (Figure 1A, top, lanes 1 to 3). In the presence of up to 1 μM Lat B, the majority of actin was still detected in the pellet fraction, although the amount of the soluble form did gradually increase as the Lat B concentration rose (Figure 1A, top, lanes 4 to 12). By contrast, at 10 μM Lat B, ~50% of the actin was detected in the soluble fraction (Figure 1A, top, lanes 13 to 15), which indicates that 10 μM Lat B may be the appropriate concentration to use with Arabidopsis leaf protoplasts. As a control for the fractionation, we detected the ER-localized chaperone binding protein (BiP) by protein gel blot analysis using an anti-BiP antibody. The majority of BiP was detected in the soluble fraction (Figure 1A, bottom), indicating that BiP is not pelleted by this low-speed centrifugation, and this fractionation pattern of BiP was not affected by up to 10 μM Lat B.

To obtain independent evidence that the actin filaments are disassembled by 10 μM Lat B, we visualized the actin filaments by transforming the protoplasts with GFP:talin, which expresses a chimeric protein consisting of green fluorescent protein (GFP) and the actin binding domain of talin (Kost et al., 1998). Fluorescence microscopy of the protoplasts showed that in the absence of Lat B, GFP:talin revealed numerous networks that indicate actin networks (Figure 1B, panel a). By contrast, at 10 μM Lat B, GFP:talin showed a diffuse pattern (Figure 1B, panel c), indicating that the actin filaments are disassembled.

Previous studies have shown that Lat B in a nanomolar concentration is sufficient to disassemble the actin filaments in pollen tubes (Vidali et al., 2001), whereas 25 and 265 μM were used with tobacco (Nicotiana tabacum) leaf and BY-2 cells, respectively (Brandizzi et al., 2002; Saint-Jore et al., 2002). Thus, although the Lat B concentration needed to disassemble the actin filaments in Arabidopsis leaf protoplasts is much higher than that used with pollen tubes, it is similar to that used with tobacco leaf cells.

Next, we examined whether two vacuolar reporter proteins, namely, GFP-fused sporamin (Spo:GFP) and Arabidopsis aleurain-like protein (AALP::GFP), are targeted to the central vacuole in the presence of Lat B. Sporamin is a tuber protein of sweet potato (Ipomoea batatas), whereas AALP is a Cys protease homologous to barley (Hordeum vulgare) aleurain; both have been shown to be targeted to the central vacuole in both their native form and as part of a GFP fusion protein (Matsuoka

Figure 1. Effect of Lat B Treatment on the Actin Filaments in Arabidopsis Protoplasts.

(A) Concentrations of Lat B needed to disassemble the protoplast actin filaments. Protoplasts obtained from Arabidopsis leaf tissues were treated with varying concentrations (10 nM to 10 μM) of Lat B for 24 h. Subsequently, protein extracts were prepared, fractionated into pellet and soluble fractions by centrifugation, and subjected to protein gel blot analysis with anti-actin and anti-BiP antibodies. T, total; S, soluble fraction; P, pellet fraction.

(B) Effect of 10 μM Lat B on actin filament architecture as shown by GFP:talin fluorescence. Protoplasts transformed with GFP:talin were incubated in the presence of Lat B (+); panels c and d) and absence (Lat B –); panels a and b) of Lat B (10 μM), and the pattern of GFP:talin fluorescence was examined by fluorescence microscopy. CH, chlorophyll. Bar = 20 μm.
et al., 1995; Ahmed et al., 2000; Jin et al., 2001; Sohn et al., 2003). Protoplasts transformed with Spo:GFP or AALP:GFP were incubated with Lat B, and the localization of the GFP signal was examined at various time points after transformation. In the absence of Lat B, both reporter proteins were targeted to the central vacuole (Figure 2A, panels a, f, and k). We also observed Spo:GFP distributed in an ER pattern (Figure 2A, panels e and g), which suggests that the Spo:GFP in a fraction of the transformed protoplasts had not yet been transported to the central vacuole at this time point (Jin et al., 2001; Kim et al., 2001). In the presence of Lat B, however, the majority of protoplasts transformed with AALP:GFP displayed a novel punctate staining pattern (Figure 2A, panel b), and the number of protoplasts with a GFP-positive central vacuole was significantly reduced. This also was true for the Spo:GFP-transformed protoplasts. In the presence of Lat B, notably, the Spo:GFP-transformed protoplasts showed three new patterns, namely, a punctate staining pattern alone (Figure 2A, panel i), a punctate staining pattern together with the ER pattern (Figure 2A, panel h), and a punctate staining pattern together with the vacuolar pattern (Figure 2A, panel j), in addition to the two typical ER and vacuolar patterns. Furthermore, when we examined the effect of Lat B on the trafficking of Spo:GFP and AALP:GFP in tobacco leaf cell protoplasts, we obtained similar results (data not shown), which indicates that Lat B may have a similar effect on vacuolar trafficking in other plant cells.

We next determined how many Spo:GFP-transformed protoplasts had vacuolar versus punctate GFP-staining patterns. As shown in Figure 2B, in the absence of Lat B, 31 and 58% of the Spo:GFP-transformed protoplasts had GFP-positive central vacuoles at 24 and 48 h, respectively. By contrast, in the presence of Lat B, 7 and 29% of the protoplasts had the vacuolar staining pattern at 24 and 48 h, respectively, indicating significantly reduced vacuolar trafficking of Spo:GFP. Moreover, in the presence of Lat B, 67 and 47% of the protoplasts had a punctate GFP-staining pattern at 24 and 48 h, respectively, whereas this staining pattern was absent in the control protoplasts. In the presence of Lat B, the ER-staining pattern of Spo:GFP was reduced to 29% from 69%. The reason for this reduction is not clearly understood at the moment. At the least, this is in part because of the way by which we counted the protoplasts; a small fraction of protoplasts that has the punctate staining pattern together with the ER pattern was counted as the punctate staining pattern. These results strongly suggest that Lat B inhibits the vacuolar trafficking of Spo:GFP and AALP:GFP.

**Figure 2.** Inhibition of the Vacuolar Trafficking of AALP:GFP and Spo:GFP by Lat B.

(A) Phenotypic analysis of the effect of Lat B on AALP:GFP and Spo:GFP localization. Protoplasts transformed with AALP:GFP or Spo:GFP were incubated in the presence [Lat B (†); panels b to d and g to k] and absence [Lat B (‡); panels a, e, and f] of Lat B (10 μM), and the localization of AALP:GFP and Spo:GFP was examined 24 and 48 h after transformation, respectively. The red images show chlorophyll autofluorescence. CH, chloroplasts. Bar = 20 μm.

(B) Quantification of the Lat B-altered distribution patterns of Spo:GFP. Protoplasts were counted based on the Spo:GFP distribution patterns in the presence [Lat B (†)] and absence [Lat B (‡)] of Lat B (10 μM) 24 and 48 h after transformation. In each experiment, >100 transformed cells were counted, and three independent experiments were performed to obtain means and SD. Representatives of the ER (network pattern), punctate staining, and vacuolar patterns of Spo:GFP are shown in panels g, i, and k of Figure 2A, respectively. Note that a small fraction of protoplasts showing the punctate staining pattern together with the ER pattern or the vacuolar pattern was counted as the punctate staining pattern because the punctate staining pattern was considered to reflect the inhibition of the vacuolar trafficking of Spo:GFP.

**Traffic of Vacuolar Reporter Proteins Is Inhibited by Overexpressing the Actin Mutants actin2[D13K] and actin2[D13Q]**

While the above results suggest that Lat B interferes with vacuolar trafficking, this method suffers from the possibility that prolonged exposure to Lat B may have had a side effect on various cellular processes in protoplasts that indirectly affect the vacuolar trafficking of cargo proteins. To obtain independent evidence for actin filament involvement in vacuolar trafficking, we sought to interfere with the normal function of actin filaments by overexpressing actin mutants. In yeast cells, expression of
actin2[D11K] or actin2[D11Q] results in a dominant lethal phenotype (Johannes and Gallwitz, 1991). The D11 residue in yeast actin1 is surface exposed and is thought to interact with divalent metal ions or nucleotides (Johannes and Gallwitz, 1991; Wertman et al., 1992). Arabidopsis actin2 is the most abundant actin isoform in Arabidopsis vegetative tissues (Ringli et al., 2002). Thus, we substituted the Asp 13 (D13) residue of Arabidopsis actin2 with Lys (K) or Gln (Q) to generate mutants equivalent to yeast actin1[D11K] or actin1[D11Q]. In addition, the actin2 proteins were tagged with the small epitope hemagglutinin (HA) to distinguish the introduced molecules from the endogenous acts. First, we examined the effect of overexpressing proteins were tagged with the small epitope hemagglutinin (HA) to distinguish the introduced molecules from the endogenous acts. First, we examined the effect of overexpressing actin2[D13K]:HA, actin2[D13Q]:HA, or wild-type actin2:HA on the morphology of actin filaments in protoplasts. The actin filaments were visualized by cotransforming the cells with GFP:talin (Kost et al., 1998). As shown in Figure 3A (panel a), protoplasts transformed with wild-type actin2 showed numerous actin filaments in a staining pattern that was nearly identical to that of protoplasts expressing GFP:talin alone (data not shown). Thus, overexpression of wild-type actin2 does not affect morphology of actin filaments. By contrast, overexpression of actin2[D13K] or actin2[D13Q] in the protoplasts resulted in a diffuse staining pattern of GFP:talin (Figure 3A, panels c and e), which strongly suggests that transiently expressed actin2[D13K] and actin2[D13Q] disrupt endogenous actin filaments. Approximately 46% of the protoplasts transformed with actin2[D13K] showed a diffuse staining pattern of GFP:talin (Figure 3B). The expression of the introduced actin2 proteins was confirmed by protein gel blot analysis using a monoclonal anti-HA antibody (Figure 3E). Thus, actin2[D13K] or actin2[D13Q] overexpression may be used in place of Lat B to disrupt actin filaments.

We next examined the vacuolar trafficking of Spo:GFP and AALP:GFP in the presence of actin2[D13K] or actin2[D13Q] overexpression. As expected, overexpression of wild-type actin2 did not affect the staining pattern of Spo:GFP and AALP:GFP (data not shown). The percentage of wild-type actin2-transformed protoplasts that showed the vacuolar staining pattern, which indicates the vacuolar trafficking efficiency of Spo:GFP, was 22, 46, and 53% at 24, 36, and 48 h after transformation, respectively, which was nearly the same as when the protoplasts were only transformed with Spo:GFP (Figures 2B and 3D). However, actin2[D13K] or actin2[D13Q] overexpression produced a punctate staining pattern for Spo:GFP and AALP:GFP (Figure 3C), and the vacuolar trafficking efficiency of Spo:GFP was greatly reduced (Figure 3D). Furthermore, the degree to which the actin2 mutants inhibited vacuolar trafficking was nearly comparable to that of Lat B. This was rather unexpected because the percentage of protoplasts showing the diffuse actin pattern in the presence of an actin2 mutant was approximately half of that observed upon Lat B treatment. One possible explanation is that actin2 mutants may be incorporated into actin filaments as actin subunits, and these actin filaments with the mutants may not be functional, even though they are morphologically filamentous forms. To examine this possibility, protoplasts were transformed with actin2[D13K]:HA or actin2[D13Q]:HA, after which protein extracts were prepared and fractionated by low-speed centrifugation into pellet and soluble fractions, which contain the filamentous and soluble forms of actin, respectively (Abe and Davies, 1995). The actin2 mutants in these fractions were detected by protein gel blot analysis using an anti-HA antibody. Approximately 30% of actin2 mutants was detected in the pellet fraction (Figure 3F), which indicates that a portion of actin2 mutants may be incorporated into the actin filaments. Together, these results further support the notion that actin filaments play a critical role in the trafficking of vacuolar proteins in plant cells.

Spo:GFP and AALP:GFP Accumulate in the Golgi Complex in the Presence of Lat B

Next, we performed colocalization experiments with marker proteins to identify the organelle in which vacuolar cargo proteins accumulate in the presence of Lat B. Because vacuolar proteins are transported to the central vacuole through the Golgi apparatus and the PVC, we reasoned that the punctate staining pattern may correspond to either of these organelles. First, we examined the colocalization in Lat B–treated protoplasts of AALP:GFP and rat sialyltransferase (ST) tagged with HA because ST has been shown to localize to the Golgi complex in plant cells (Boevink et al., 1998; Wee et al., 1998; Lee et al., 2002; reviewed in Nebenführ et al., 2002). The majority of the AALP:GFP in the punctate stains colocalized with the ST:HA signals at the Golgi complex (Figure 4A). Interestingly, however, a portion of the punctate stains of AALP:GFP did not exactly overlap the ST:HA molecules, rather, they colocalized side by side. This indicates that in the presence of Lat B, AALP:GFP may localize at the Golgi complex but at a slightly different location within the Golgi complex than that of ST:HA, which is thought to mainly localize at the trans–Golgi (Wee et al., 1998; reviewed in Nebenführ et al., 2002).

To examine the localization further, we compared the localization in Lat B–treated protoplasts of AALP:GFP with that of AtVTI1a tagged with HA (AtVTI1a:HA). A previous study showed that AtVTI1a, a v-SNARE, and its T7-tagged form localize at the TGN and the PVC and are thought to travel from the TGN to the PVC together with vesicles (Zheng et al., 1999). Unlike when ST:HA was coexpressed with AALP:GFP, the green punctate staining pattern of AALP:GFP more closely overlapped the red punctate staining pattern of AtVTI1a:HA that was detected by an anti-HA antibody (Figure 4B). Thus, these results strongly suggest that AALP:GFP accumulates at the Golgi complex, and quite possibly at the TGN. Furthermore, >90% of AtVTI1a-positive punctate stains closely overlapped with AALP:GFP signals, which was rather unexpected because AtVTI1a is distributed in equal measure to both the TGN and the PVC (Zheng et al., 1999). One possible reason for this is that, like Spo:GFP and AALP:GFP, AtVTI1a:HA may not travel to the PVC in the presence of Lat B.

To obtain additional evidence for localization of vacuolar proteins in the Golgi complex in the presence of Lat B, we compared the localization in Lat B–treated protoplasts of AALP:GFP and endogenous vacuolar sorting receptor (VSR-At)/A. thaliana epidermal growth factor receptor-like protein 1 (At(AtELP1)), VSR-At(AtELP1), the vacuolar sorting receptor of Arabidopsis, localizes to both the TGN and the PVC although its primary residence is the PVC (Ahmed et al., 1997; Li et al., 2002).
Figure 3. Actin2 Mutant Overexpression Inhibits the Trafficking of Vacuolar Proteins.

(A) Disruption of actin filaments by actin2 mutant overexpression. GFP:talin was introduced into protoplasts together with actin2 (panels a and b), actin2[D13K] (panels c and d), or actin2[D13Q] (panels e and f), and the green fluorescence of GFP:talin was observed. Scale bar = 20 μm. Filamentous patterns [A], panel a) and diffuse pattern [A], panels c and e) are shown.

(B) Quantification of the effects of actin2 mutant overexpression on actin morphology. In each experiment, >100 transformed protoplasts were counted for GFP:talin staining patterns, and three independent experiments were performed to calculate means and sds.

(C) Punctate pattern of reporter proteins in the presence of overexpressed actin2 mutants. Protoplasts were transformed with the indicated constructs, and the localization of the reporter proteins was examined 48 h later. Note that the GFP-staining patterns of AALP:GFP (panels a, b, e, and f) and Spo:GFP (panels c, d, g, and h) in protoplasts overexpressing actin2 mutants were nearly identical to those in the Lat B–treated protoplasts. Only the typical punctate staining patterns are shown here to simplify the presentation. Scale bar = 20 μm.

(D) Vacuolar trafficking efficiency in the presence of actin2[D13K] and actin2[D13Q]. To estimate the vacuolar trafficking efficiency, protoplasts with the
Surprisingly, the majority of the green punctate stains also overlapped the red punctate stains of endogenous VSR-At-(AtELP1) that was detected with an anti-VSR-At antibody (Figure 4C). That AALP:GFP colocalizes with both ST:HA and VSR-At is rather contradictory because ST:HA localizes to the Golgi complex (Boevink et al., 1998; Wee et al., 1998; Lee et al., 2002), whereas VSR-At localizes primarily to the PVC (Li et al., 2002). VSR is thought to travel to the PVC from the TGN (Ahmed et al., 1997; Li et al., 2002). Thus, one possible explanation for this is that, like AtVTI1a:HA, VSR-At(AtELP1) may not travel to the PVC in the presence of Lat B.

To address this possibility, we first examined the distribution of VSR-At in Arabidopsis leaf protoplasts in the absence of Lat B. We used AtSYP21 (AtPEP12p) as another PVC marker (da Silva Conceicao et al., 1997) and compared the localization of VSR-At with HA-tagged AtSYP21 (AtPEP12p) or the Golgi-localizing ST:GFP protein. As shown in Figure 5A, the majority of the VSR-At–positive speckles overlapped the AtSYP21:HA–positive punctate stains (panel b), whereas a minor portion overlapped the ST:GFP–positive punctate stains (panel f). When these colocalization patterns were quantified, ~70% of the VSR-At–positive speckles overlapped AtSYP21:HA and 30% overlapped with ST:GFP. Thus, the majority of VSR-At normally localizes at the PVC, similar to what has been observed in root cells, albeit to a lesser degree (Li et al., 2002). Next, we examined the degree to which ST:GFP and VSR-At overlap in the presence of Lat B. As shown in Figure 5B, the majority of VSR-At colocalized with ST:GFP, with only a minor portion of VSR-At–positive speckles failing to colocalize with ST:GFP. When the overlap was quantified, 66% of the VSR-At–positive speckles closely overlapped the ST:GFP speckles in the presence of Lat B, in contrast with 30% in the absence of Lat B. Thus, in the presence of Lat B, the overlap between ST:GFP and VSR-At increased by approximately twofold, indicating that Lat B also affects the localization of VSR-At. These results suggest that in the presence of Lat B, VSR-At may not be able to leave the TGN, similar to AtVTI1a:HA, which in turn results in the accumulation of both VSR-At and AALP:GFP in the TGN.

To confirm the specificity of the antibodies we used to detect endogenous VSR-At and transiently expressed AtSYP21:HA, we performed protein gel blot analysis using proteins extracts prepared from AtSYP21:HA-transformed protoplasts. As shown in Figure 5C, the anti-VSR and anti-HA antibodies were highly specific for VSR-At and AtSYP21:HA (AtPEP12p:HA), respectively.

**Vacuolar Reporter Proteins Cofractionate with VSR-At in the Presence of Lat B**

To obtain additional evidence for the inhibition of vacuolar trafficking by Lat B, we analyzed the localization of the reporter proteins after fractionation of the transformed and Lat B–treated cells. A previous study demonstrated that vacuoles...
can be separated from the nonvacuolar fraction by ultracentrifugation using a Ficoll step gradient (Jiang and Rogers, 1998). Thus, Lat B–treated and untreated AALP:GFP-transformed protoplasts were loaded on top of a step gradient consisting of 4, 12, and 15% Ficoll and fractionated by ultracentrifugation. When the Lat B–untreated AALP:GFP-transformed protoplasts were fractionated, the proteolytically processed form of AALP:GFP, which is thought to be generated in the Golgi or post-Golgi compartments (Sohn et al., 2003), was detected in the vacuole-containing top fraction, whereas the unprocessed form was present in the pellet fraction (data not shown). However, Lat B–treated AALP:GFP-transformed protoplasts gave the same fractionation pattern (data not shown), although Lat B–treated AALP:GFP-transformed protoplasts showed different GFP staining patterns from that of untreated samples. This suggests that the Ficoll step gradient cannot separate AALP:GFP-positive Lat B–induced speckles from the vacuoles. Because the AALP:GFP punctate-staining compartment appeared to cofractionate with the vacuoles in the top fraction, we modified the gradient to include 1, 2, and 3% Ficoll layers on top of the 4% layer. The Lat B–treated and untreated protoplasts were loaded on top of the modified gradient and fractionated by ultracentrifugation. Using a hemocytometer, we determined the number of vacuoles in each fraction and found that ~50% of the vacuoles were present in the top fraction, with this percentage gradually decreasing with

Figure 5. Effect of Lat B on the Distribution of VSR-At(AtELP1).

(A) and (B) Localization patterns of VSR-At in the presence and absence of Lat B, respectively, are shown. Protoplasts were transformed with the indicated constructs, incubated in the absence (A) and presence (B) of Lat B (10 μM), and fixed with paraformaldehyde 24 h after transformation. To detect endogenous VSR-At, the fixed protoplasts were stained with an anti-AtVSR antibody followed by a Cy3-labeled anti-rabbit IgG antibody. To detect AtSYP21:HA, fixed cells were stained with an anti-HA antibody followed by a fluorescein isothiocyanate–labeled anti-rat IgG antibody. ST:GFP was directly observed through its green fluorescence. The arrows and arrowheads indicate an overlap and nonoverlapping, respectively, between AtSYP21 and VSR-At. To quantify the overlapping signals, the number of specks that indicated colocalization of VSR-At with ST:GFP or VSR-At with AtSYP21:HA was counted. More than 40 protoplasts were counted in each experiment, and at least three independent experiments were performed to obtain means and sd. Bar = 20 μm.

(C) Specificity of antibodies. Protein extracts were prepared from protoplasts transformed with AtSYP21:HA and subjected to protein gel blot analysis with an anti-VSR antibody and an anti-HA antibody. C, control serum; A, anti-VSR-At antibody; U, untransformed protoplasts; T, protoplasts transformed with AtSYP21:HA.
increasing Ficoll concentrations (Figure 6B). Lat B treatment did not affect the distribution of the vacuoles in the gradient (data not shown). Next, we examined the distribution of endogenous VSR-At in Lat B–treated and untreated untransformed protoplasts. In the absence of Lat B, VSR-At was widely distributed from the top to the 3% fraction, with a slightly higher amount in the 2% fraction (Figures 6A and 6B). However, the distribution pattern of VSR-At in the presence of Lat B was quite different because 60% of the VSR-At had accumulated in the 1% fraction (Figures 6A and 6B). This result confirms that Lat B alters the subcellular distribution of VSR-At. Furthermore, these results suggest that the 1% fraction may contain the Lat B–induced speckles.

We then used protein gel blot analysis to assess the respective presence of AALP:GFP and Spo:GFP in the AALP:GFP- and Spo:GFP-transformed protoplast fractions that were obtained using the modified gradient. As shown in Figures 6A and 6B, in the absence of Lat B, 42 and 46% of the processed forms of AALP:GFP and Spo:GFP, respectively, were present in the top fraction and gradually decreased as the Ficoll concentration increased, indicating that these reporter proteins were transported to the central vacuole. By contrast, in the presence of Lat B, the amounts of the proteolytically processed forms of AALP:GFP and Spo:GFP in the top fraction were reduced to 35 and 30%, respectively, whereas the 1% fraction contained 53% of the AALP:GFP and 64% of the Spo:GFP, suggesting that Lat B inhibits the transport of these proteins to the central vacuole.

Next, we examined the localization of endogenous AALP in untransformed Lat B–treated and untreated protoplasts. In the absence of Lat B, 43% of the endogenous AALP was found in the top fraction, and this amount gradually decreased as the Ficoll concentration increased (Figures 6A and 6B), as was observed with the GFP reporter proteins. By contrast, in the presence of Lat B, the highest amount (45%) of AALP was found in the 1% fraction but not in the top fraction, which indicates that Lat B also affects the trafficking of endogenous AALP. However, the fact that the amount of AALP present in the 1% fraction is higher than that present in the vacuole was rather surprising because endogenous AALP is likely present in the vacuole even before Lat B treatment. To explain the lower level of AALP in the top fraction than that in the 1% fraction upon Lat B treatment, we postulated that the amount of AALP in the vacuole is rapidly reduced without continuous supply of newly synthesized AALP. To address this question, we examined the turnover rate of endogenous AALP by measuring the endogenous AALP levels in the presence and absence of cycloheximide, an inhibitor of protein synthesis. After treating protoplasts with cycloheximide for 24 h, the amount of AALP was reduced to 30% of that in the control protoplasts (data not shown), which indicates that AALP has to be continually supplied to the vacuole to maintain its levels there. These results clearly demonstrate that in the presence of Lat B, the majority of vacuolar proteins are not transported to the central vacuole; rather, they accumulate in the 1% fraction, even though they are processed into smaller forms. In addition, these results demonstrate that in the presence of Lat B, proteins destined to go to the central vacuole cofractionate...
with the vacuolar sorting receptor VSR-At in the gradient, which further confirms the immunohistochemistry result that showed the majority of VSR-At colocalizes with Spo:GFP in a punctate staining pattern.

Lat B Does Not Affect the Trafficking of Reporter Proteins from the ER to the Golgi or from the Golgi Apparatus to the Plasma Membrane

We examined the effects of Lat B on other trafficking pathways using ST:GFP and H⁺-ATPase:GFP as reporter proteins for the trafficking to the Golgi and plasma membrane, respectively (Boevink et al., 1998; Wee et al., 1998; Jin et al., 2001; Kim et al., 2001). As shown in Figure 7A, ST:GFP appeared in a punctate staining pattern in the presence (panel f) and absence (panel b) of Lat B, indicating that Lat B does not affect its trafficking from the ER to the Golgi apparatus. To unequivocally demonstrate that this punctate staining pattern represents the Golgi apparatus, the protoplasts displaying this pattern were treated with BFA, a chemical agent known to disrupt the Golgi apparatus (Driouich et al., 1993; Boevink et al., 1999; Lee et al., 2002; Nebenführ et al., 2002). As shown in Figure 7A, both in the presence (panel h) and absence (panel d) of Lat B, BFA treatment generated a network pattern of the green fluorescent signal of ST:GFP that closely overlapped the red fluorescent signal of BiP:red fluorescent protein (RFP), a marker protein that localizes to the ER (Kim et al., 2001). This result indicates that BFA induces the relocation of ST:GFP to the ER both in the presence and absence of Lat B. This is consistent with the results from a previous study that showed that trafficking of cargo proteins from the ER to the Golgi or from the Golgi to the ER is not inhibited by Lat B (Brandizzi et al., 2002; Saint-Jore et al., 2002). In addition, because BFA treatment can induce the relocation of ST:GFP from the Golgi apparatus to the ER, actin filaments may not be required for retrograde trafficking in plant cells. This contrasts with what has been observed in animal cells, namely, that Lat B inhibits retrograde trafficking from the Golgi apparatus to the ER (Valderrama et al., 2001).

Next, we investigated the trafficking of H⁺-ATPase:GFP to the plasma membrane in the presence of Lat B. H⁺-ATPase:GFP is thought to be targeted to the plasma membrane through the Golgi apparatus because BFA treatment inhibits this targeting (Lee et al., 2002; Lefebvre et al., 2004). As shown in Figure 7B, the H⁺-ATPase:GFP distribution pattern was the same in the presence or absence of Lat B, which indicates that Lat B treatment does not affect the trafficking of H⁺-ATPase:GFP to the plasma membrane. To confirm this, we estimated the percentage of transformed protoplasts showing the plasma membrane localization of H⁺-ATPase:GFP. More than 90% of the H⁺-ATPase:GFP–transformed protoplasts showed GFP signals at the plasma membrane in the presence or absence of Lat B, confirming that Lat B does not affect this trafficking (data not shown). Together, these results suggest that actin filaments may not play a role in the trafficking from the ER to the Golgi apparatus or from the Golgi apparatus to the plasma membrane in leaf protoplasts.

Lat B Does Not Affect the Extracellular Secretion of Invertase Tagged with GFP

To further understand the roles actin filaments play in intracellular trafficking, we examined the effect of Lat B and actin2[D13K] on the secretory pathway. As a reporter protein for the secretory pathway, we selected a secreted form of invertase (Tymowska-Lalanne and Kreis, 1998) and tagged it with GFP at its C terminus (Sohn et al., 2003). Protoplasts were transformed with invertase:GFP together with RFP, a cytosolic protein that was included as a control for the secretion. The localization of the green and red fluorescent signals was examined at various time points after transformation. In both the presence and absence of Lat B, the transformed protoplasts did not show detectable levels of green fluorescent signals, although strong red fluorescent signals were readily observed (Figure 8A). However, the green fluorescent signals of invertase:GFP were readily detected in the protoplasts when the trafficking of invertase:GFP was inhibited by coexpressing AtArf11[S31N], a dominant-negative mutant of AtArf1 that is known to inhibit anterograde trafficking from the ER to the Golgi complex (Figure 8A, panel d; Lee et al., 2002; Takeuchi et al., 2003).
In the presence of Lat B or overexpressed actin2 mutants, both vacuolar reporter proteins examined, AALP:GFP and Spo:GFP, presented a punctate staining pattern. AALP:GFP also colocalized closely with AtVTI1a:HA and VSR-At in this punctate staining pattern. Both AtVTI1a and VSR-At localize at both the TGN and the PVC, although the degree of their distribution to these two organelles differs; AtVTI1a is distributed in equal measure to the TGN and the PVC, whereas VSR-At mainly localizes to the PVC in Arabidopsis leaf protoplasts (in this study) and root cells (Ahmed et al., 1997; Li et al., 2002). Interestingly, the distribution of both AtVTI1a and VSR-At was also altered in the presence of Lat B.

DISCUSSION

In this study, we demonstrated that actin filaments play a critical role in vacuolar trafficking of proteins in Arabidopsis leaf protoplasts. This was shown by disrupting the actin filaments in two ways, namely, by treating the protoplasts with Lat B, which is an inhibitor of actin filament assembly, or by overexpressing actin2[D13K] and actin2[D13Q]. In yeast cells, the equivalent actin mutants actin1[D11K] and actin1[D11Q] result in a dominant lethal phenotype (Johannes and Gallwitz, 1991). It is not yet clear how actin2[D13K] or actin2[D13Q] overexpression disrupts actin filaments, although the D11 residue in yeast actin1 (equivalent to D13 in Arabidopsis actin2) is thought to be involved in the formation of a hydrophilic pocket that either accommodates the calcium ion that binds to the β- and γ-phosphates of the bound ATP or binds bivalent metal ions or nucleotides (Johannes and Gallwitz, 1991; Wertman et al., 1992). Both Lat B treatment and the overexpression of actin2 mutants inhibited the trafficking of vacuolar cargo proteins.

In the presence of Lat B or overexpressed actin2 mutants, both vacuolar reporter proteins examined, AALP:GFP and Spo:GFP, presented a punctate staining pattern. AALP:GFP also colocalized closely with AtVTI1a:HA and VSR-At in this punctate staining pattern. Both AtVTI1a and VSR-At localize at both the TGN and the PVC, although the degree of their distribution to these two organelles differs; AtVTI1a is distributed in equal measure to the TGN and the PVC, whereas VSR-At mainly localizes to the PVC in Arabidopsis leaf protoplasts (in this study) and root cells (Ahmed et al., 1997; Li et al., 2002). Interestingly, the distribution of both AtVTI1a and VSR-At was also altered in the presence of Lat B.
For example, although ~30% of the VSR-At–positive punctate stains colocalized with the Golgi marker ST:GFP in Arabidopsis leaf protoplasts in the absence of Lat B, 66% overlapped with ST:GFP in the presence of Lat B. VSR is thought to be involved in the sorting of soluble vacuolar proteins with N-terminal propeptide sequences such as the NPIR motif, and it travels from the TGN to the PVC together with vacuolar cargo proteins, releases the cargo proteins to the PVC, and then returns to the TGN (Ahmed et al., 1997; Humair et al., 2001; Li et al., 2002). Lat B treatment also altered the distribution of AtVT1a:HA, which is thought to travel from the TGN to the PVC together with vesicles, to a predominant TGN localization. Thus, it is possible that both VSR-At and AtVT1a:HA may not leave the TGN in the presence of Lat B. This is consistent with the observed accumulation of AALP::GFP and Spo::GFP in the Golgi complex in the presence of Lat B. The Ficoll gradient fractionation experiments that showed cofractionation upon Lat B treatment of vacuolar cargo proteins and VSR-At in the 1% fraction but not in the vacuole fraction further support this possibility.

The exact mechanism by which actin filaments affect the vacuolar trafficking at the Golgi complex is not presently known. There are several possible scenarios that can be postulated based on the results shown here along with the known roles of actin filaments in various cellular processes. One possibility is that actin filaments may function as tracks for vesicles derived from the TGN that are destined to go to the central vacuole. Actin filaments play such a role during pollen tube growth (Taylor and Hepler, 1997; Gibbon et al., 1999; Vidali et al., 2001). In plant cells such as root tip cells and the pollen tube, cytochalasin D inhibits vesicle transport from the Golgi complex and causes vesicle accumulation around the Golgi complex (Mollenhauer and Morre, 1976; Picton and Steer, 1981, 1983), which leads to suggestion that exocytic vesicles carrying materials facilitating the polar growth of the pollen tube tip travel along actin filaments. However, our study here using Arabidopsis protoplasts does not support this possibility because vacuolar proteins accumulated in the Golgi complex in the presence of Lat B, as evidenced by their colocalization with AtVT1a::HA and VSR-At, which indicates that vacuolar proteins cannot leave the Golgi complex in the presence of Lat B. Another possibility, which we favor, is that actin filaments may play a role in the recruitment of proteins involved in vesicle formation and/or release at the Golgi complex. Supporting this notion is an early observation made by Shannon et al. (1984) showing that vesicle formation at the Golgi complex is a cytochalasin D–sensitive process. At present, it is not clear which proteins are involved in this process at the Golgi complex in plant cells. One of these may be the Arabidopsis dynamin-like protein DRP 2A (ADL6), which we have shown to play a critical role in the trafficking of Spo::GFP at the TGN in plant cells (Jin et al., 2001). In animal cells, dynamin that is involved in vesicle formation at the plasma membrane and the TGN binds to actin filaments (Schafer et al., 2002; Sever, 2002). Actin filaments may guide dynamin to specific target membranes. Cortactin, an actin binding protein that contains a well-defined SH3 domain, associates with the Pro-rich domain of dynamin through its SH3 domain (McNiven et al., 2000). In plant cells, AtSH3P3, an Arabidopsis protein with an SH3 domain, binds to DRP2A (ADL6) through its Pro-rich domain (Lam et al., 2002), although it is not known whether AtSH3P3 binds to actin filaments as well. In addition, actin and HiP1R are necessary for the clathrin-coated vesicle budding from the TGN needed for lysosome biogenesis in animal cells (Carreno et al., 2004). It is likely that many more proteins are involved in vesicle formation and/or release from the TGN, as observed at the plasma membrane during endocytosis in animal cells (Higgins and McMahon, 2002; Merrifield et al., 2002). In the absence of actin filaments, some of these proteins needed for vesicle formation and/or release at the TGN may not be efficiently recruited to the TGN, which in turn results in the accumulation of vacuolar cargo proteins as well as the cargo receptor and v-SNARE in the Golgi complex, as is observed in the presence of Lat B.

In contrast with vacuolar trafficking, trafficking of transiently expressed ST:GFP between the ER and the Golgi complex does not appear to involve actin filaments, as has been observed previously in tobacco leaf cells or BY-2 cells (Brandizzi et al., 2002; Saint-Jore et al., 2002). In addition, the trafficking of H+-ATPase::GFP and invertase::GFP, which served as plasma membrane and secretory pathway reporter proteins, respectively, was unaffected by the presence of Lat B in Arabidopsis protoplasts. These results suggest that actin filaments may not be involved in these pathways in leaf cell protoplasts. However, we cannot completely rule out the possibility that a requirement for actin filaments in these pathways in protoplasts of leaf cells is circumvented by the use of alternative pathways. The failure of Lat B to disrupt the trafficking of H+-ATPase::GFP to the plasma membrane contrasts with previous studies showing that actin filaments are involved in the trafficking of PIN and H+-ATPase to the plasma membrane in plant root cells (Geldner et al., 2001; Muday and DeLong, 2001; Baluska et al., 2002). One way to explain this is that in polarized cells or cells undergoing polarized cell elongation, trafficking of proteins to the polar apex or the cell poles may depend on the actin filaments (Vidali and Hepler, 2001; Waller et al., 2002), whereas the trafficking of proteins such as H+-ATPase::GFP and invertase::GFP in leaf protoplasts (in this study) and PMA4::GFP in BY2 cells (Lefebvre et al., 2004) occurs in a nonpolarized fashion and thus may not require the presence of intact actin filaments. However, it remains possible that certain proteins may be transported to the plasma membrane in an actin-dependent fashion even in protoplasts. Further studies are required to elucidate in greater detail the actin filament-dependent and -independent pathways that operate in plant cells.

In conclusion, we have shown that actin filaments play a critical role in the trafficking of soluble cargo proteins to the central (lytic) vacuole in protoplasts obtained from plant leaf cells. By contrast, actin filaments may not be necessary or their role may be limited in the trafficking that occurs between the ER and the Golgi complex and between the Golgi complex and the plasma membrane in leaf protoplasts. This also appears to be true for the secretion of proteins by leaf protoplasts. However, further studies are needed before we can fully understand the exact roles actin filaments play in the various protein trafficking pathways in plant cells. In addition, it is not known whether the actin filaments participate in the secretion of the carbohydrate precursors of the cell wall that are known to be produced in plant cells by the Golgi complex (Moore et al., 1991; Gibeaut and Carpita, 1994).
METHODS

Growth of Plants

*Arabidopsis thaliana* (ecotype Columbia) and tobacco (*Nicotiana tabacum*) plants were grown on B5 plates (Sigma-Aldrich, St. Louis, MO) in a growth chamber. Leaf tissues were harvested from the plants (10- to 14-old Arabidopsis, 3- to 4-week-old tobacco) and immediately used for protoplast isolation as described previously (Jin et al., 2001).

Construction of Plasmids

Actin2 cDNA (accession number NM_112764) was PCR amplified from a cDNA library using the primers Act-5 (5'-CATATGATGGCTGAGGCT-GATGATATTCA-3') and Act-3 (5'-CTCGAGTTAGAAACATTTCCTGT-GAACGATT-3'). Actin2[D13K] and actin2[D13Q] also were generated by PCR using the specific primers 5'-ATTCACCAAATCGTGTTAAG-AATGTTACC-3' and 5'-ATTCACCAAATCGTGTTCAQAAATGTACC-3', respectively. To tag the wild-type and mutant forms of actin2 with HA at the C terminus, the PCR products were digested with *SalI* and *BamH1* and ligated into a pUC-based HA-tagging vector digested with *SalI* and *BamH1*. The pUC-based HA-tagging vector consists of the 35S promoter of *CaMV* mosaic virus, a multicloning site, the HA epitope, and the poly-A signal. Onl. was constructed as described previously (Kost et al., 1998). To construct ATSP21 (AIP12p) tagged with HA, ATSP21 was PCR amplified from an Arabidopsis cDNA library using two specific primers: 5'-CACAAAGAGTAAAGAAGAACAATGAGTGACGCGTTTGATG-3' and 5'-GGATCCTGGTGAATTTGACGATCC-3'. The PCR product was confirmed and ligated vector to the HA tagging vector.

Fractionation of Filamentous and Soluble Actins from Protoplast Extracts

Protoplasts were treated with varying concentrations of Lat B for 24 and then lysed gently in a buffer (5 mM Hepes, pH 7.0, 100 mM KCl, 400 mM KI, 2 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM sodium vanadate, and 1 μg/mL leupeptin) by repeated freeze and thaw cycles (Abe and Davies, 1995). The lysates were then fractionated into soluble and pellet fractions by centrifugation at 2500g at 4°C for 15 min. The pellet fractions were resuspended to the original volume of the lysis buffer. These soluble and pellet fractions were analyzed by protein gel blot using anti-actin (CN, Aurora, OH) and anti-BiP antibodies.

Protein Preparation and Protein Gel Blot Analysis

To prepare cell extracts from protoplasts, transformed protoplasts were subjected to repeated freeze and thaw cycles in lysis buffer (150 mM NaCl, 20 mM Tris-Cl, pH 7.5, 1 mM EDTA, 1 mM EGTA, 3 mM MgCl₂, 0.1 mg/mL antipain, 2 mg/mL aprotinin, 0.1 mg/mL E-64, 0.1 mg/mL leupeptin, 10 mg/mL pepstatin, and 1 mM phenylmethylsulfonyl fluoride) and then centrifuged at 7000g at 4°C for 5 min. The protein aggregates were precipitated by centrifugation at 10,000g at 4°C for 5 min. The protein aggregates were dissolved in the lysis buffer in a volume that corresponds to the volume used to prepare total protoplast proteins. Protein gel blot analysis was performed using anti-RFP (a gift of E. Kim, Korea Advanced Institute of Science and Technology, Daejeon, Korea) and anti-HA (Roche Diagnostics, Mannheim, Germany) antibodies as described previously (Jin et al., 2001).

To determine the turnover rate of endogenous aleurain, protoplasts transformed with GFP were incubated in the presence of cycloheximide (100 μg/mL), a protein translation inhibitor. Protein extracts were prepared from the protoplasts at various time points and analyzed by protein gel blot analysis using anti-GFP and anti-aleurain antibodies. The turnover rate of endogenous aleurain was determined by measuring the amount of endogenous aleurain detected by the anti-aleurain antibody in the presence of cycloheximide. The inhibition of translation by cycloheximide was confirmed by measuring the amount of newly synthesized GFP.

Fractionation of Protoplasts by a Ficoll Step Gradient

The central vacuole was purified according to the method described by Jiang and Rogers (1998). Briefly, the transformed protoplasts were loaded onto a Ficoll step gradient consisting of 4, 12, and 15% Ficoll and subjected to ultracentrifugation at 150,000g for 2 h. The top and pellet fractions, which contain the intact vacuoles and nonvacuolar endomembrane compartments, respectively, were collected separately from the gradient. In addition, protoplasts were fractionated using a much finer step gradient consisting of 1, 2, 3, 4, 12, and 15% Ficoll. Fractions (1 mL each) were separately collected from these steps, and proteins from these fractions were concentrated by TCA precipitation and analyzed by protein gel blot analysis using anti-GFP (Clontech, Palo Alto, CA), anti-VRP (Li et al., 2002), and anti-aleurain antibodies (Jiang and Rogers, 1998).

Transient Expression and in Vivo Targeting of Reporter Cargo Proteins

Plasmids were introduced by polyethylene glycol-mediated transformation (Jin et al., 2001; Lee et al., 2002) of *Arabidopsis* protoplasts that had been prepared from leaf tissues. To observe the effects of various chemicals on trafficking, they were added to the incubation medium immediately after transformation except when indicated otherwise. The expression of the fusion constructs was monitored at various time points after transformation, and images were captured with a cooled CCD camera and a Zeiss Axiosplan fluorescence microscope (Jena, Germany). The filter sets used were XF116 (exciter, 474AF20; dichroic, 500DRLP; emitter, 585ALP; Omega, Brattleboro, VT) for GFP, RFP, and chlorophyll autofluorescence, respectively. The data were then processed using Adobe Photoshop software (Mountain View, CA), and the images were rendered in pseudocolor (Jin et al., 2001).
was included with the Golgi pattern when the staining patterns of AALP:GFP were scored.

**Immunohistochemistry**

For immunohistochemistry, transformed protoplasts were placed onto poly-L-lys-coated glass slides and fixed with 3% paraformaldehyde in a fixing buffer (10 mM Hepes, pH 7.2, 154 mM NaCl, 125 mM CaCl₂, 2.5 mM maltose, 5 mM KCl) for 1 h at room temperature. The fixed cells were incubated with appropriate primary antibodies such as rat monoclonal anti-HA (Roche Diagnostics) or rabbit anti-VSR antibodies (Li et al., 2002) in TSW buffer (10 mM Tris-HCl, pH 7.4, 0.9% NaCl, 0.25% gelatin, 0.02% SDS, 0.1% Triton X-100) at 4°C overnight and then washed with TSW buffer three times. Subsequently, the cells were incubated with Cy3-conjugated goat anti-rabbit IgG (Sigma-Aldrich) or Cy3-conjugated anti-rat IgG (Zymed, San Francisco, CA) secondary antibodies in TSW buffer and then washed three times with TSW buffer. The images were captured using a CCD camera and a Zeiss Axioplan fluorescence microscope as described above.

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