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Trafficking of Phosphatidylinositol 3-Phosphate from the *trans*-Golgi Network to the Lumen of the Central Vacuole in Plant Cells

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Very limited information is available on the role of phosphatidylinositol 3-phosphate (PI[3]P) in vesicle trafficking in plant cells. To investigate the role of PI(3)P during the vesicle trafficking in plant cells, we exploited the PI(3)P-specific binding property of the endosome binding domain (EBD) (amino acids 1257 to 1411) of human early endosome antigen 1, which is involved in endosome fusion. When expressed transiently in Arabidopsis protoplasts, a green fluorescent protein (GFP):EBD fusion protein exhibited PI(3)P-dependent localization to various compartments—such as the *trans*-Golgi network, the prevacuolar compartment, the tonoplasts, and the vesicles in the vacuolar lumen—that varied with time. The internalized GFP:EBD eventually disappeared from the lumen. Deletion experiments revealed that the PI(3)P-dependent localization required the Rab5 binding motif in addition to the zinc finger motif. Overexpression of GFP:EBD inhibited vacuolar trafficking of sporamin but not trafficking of H^+-ATPase to the plasma membrane. On the basis of these results, we propose that the trafficking of GFP:EBD reflects that of PI(3)P and that PI(3)P synthesized at the *trans*-Golgi network is transported to the vacuole through the prevacuolar compartment for degradation in plant cells.

INTRODUCTION

Evidence suggests that phosphoinositides play a regulatory role in vesicle trafficking (Camilli et al., 1996; Corvera and Czech, 1998; Gary et al., 1998; Cremona et al., 1999; Leevers et al., 1999; Roth, 1999). Phosphatidylinositol 3-phosphate (PI[3]P) has been implicated in this process (Whitman et al., 1998; Corvera et al., 1999). Direct evidence for the role of PI(3)P in vesicle trafficking was obtained when the yeast *VPS34* gene, one of the genes involved in the vesicular protein sorting in yeast, was found to encode a PI3-kinase (Schu et al., 1993). Almost all PI3-kinase activity in yeast can be attributed to Vps34p (Stack et al., 1995; Wurmser and Emr, 1998). However, Vps34p requires a protein kinase, Vps15p, for its activation and membrane association (Stack et al., 1995). Also, a mammalian protein, p110, which is homologous to yeast Vps34p, has PI3-kinase activity and can transduce signals from tyrosine-phosphorylated receptors into a variety of intracellular responses (Volinia et al., 1995). However, this mammalian PI3-kinase, in association with an adapter protein p85, is able to phosphorylate other PI compounds such as PI(4)P and PI(4,5)P_2 at the D3 position of PI (Volinia et al., 1995; Panaretou et al., 1997). It is now clear that PI3-kinases play critical roles in various trafficking events, such as endocytosis of transferrin (Li et al., 1995), endosome fusion (Jones et al., 1998), vacuolar trafficking in yeast (Peterson et al., 1999), vesicle formation at the *trans*-Golgi network (TGN) (Hickinson et al., 1997; Jones and Howell, 1997; Jones et al., 1998), vacuole morphogenesis in *Schizosaccharomyces pombe* (Takegawa et al., 1995), and multivesicular body formation (Fernandez-Borja et al., 1999).

PI(3)P is likely to act in the regulation of various steps of vesicle trafficking through its binding proteins. In mammalian cells, early endosome antigen1 (EEA1) has been well characterized as a PI(3)P binding protein (Stenmark et al., 1996; Simonsen et al., 1998; Christoforidis et al., 1999; McBride et al., 1999). EEA1 binds specifically to PI(3)P via a Zn^{2+}-coordinating finger at its C terminus (Patki et al., 1997; Burd and Emr, 1998; Gaullier et al., 1998). A similar motif has been found in other proteins that bind to PI(3)P. These include Vps27p (Misra and Hurley, 1999), Vac1p (Tall et al., 1999), and Fab1p (Gary et al., 1998), all of which are implicated in intracellular trafficking. In addition, Hrs, a tyrosine kinase substrate, has the motif and binds to PI(3)P with high affinity and has been implicated in vesicular trafficking via early endosomes (Komada and Soriano, 1999). This motif has

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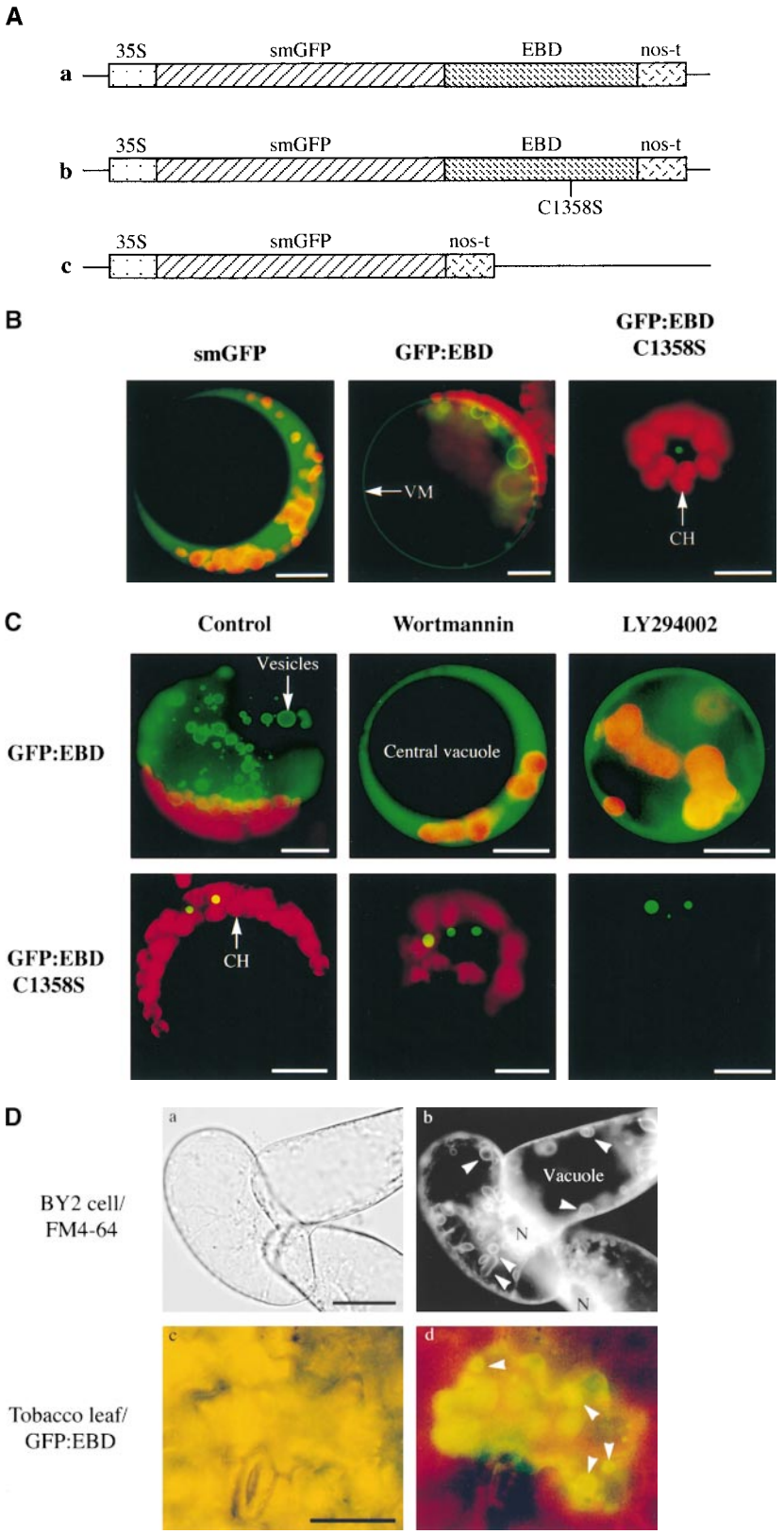

Figure 1. PI(3)P-Dependent Targeting of GFP:EBD in Plant Cells.

been dubbed a FYVE finger (Stenmark and Aasland, 1999). In EEA1, the FYVE motif is sufficient for binding to PI(3)P; however, it appears that the cooperation of the neighboring Rab binding motif is required for PI(3)P-dependent targeting of the protein to the endosome in vivo (Li et al., 1995; Callaghan et al., 1999; Pfeffer, 1999; Lawe et al., 2000).

In plant cells, Vps34p homologs have been isolated from Arabidopsis (Welters et al., 1994) and soybean (Hong and Verma, 1994). Transgenic Arabidopsis that expressed the *AtVPS34* gene in the antisense orientation showed abnormal development, which suggested that PI(3)P plays an important role in plants. Evidence for the role of PI(3)P in vesicle trafficking in plant cells was obtained by inhibition studies using wortmannin in BY2 cells (Matsuoka et al., 1995).

In this study, we addressed the question of whether PI(3)P plays a role in intracellular trafficking in plant cells. We used the endosome binding domain (amino acids 1257 to 1411) of EEA1 as an in vivo probe for PI(3)P in plant cells because the C-terminal endosome binding domain (EBD; amino acids 1257 to 1411) of human EEA1 was shown to be sufficient for binding of PI(3)P in vitro and could be directed to the endosome in a PI(3)P-dependent manner.

Our evidence shows that PI(3)P is present in various compartments of plant cells and is transported to vacuoles for degradation and that PI(3)P plays a role in vacuolar trafficking of sporamin in Arabidopsis.

RESULTS

EBD of Human EEA1 Exhibits PI(3)P-Dependent Localization in Plant Cells

To investigate whether PI(3)P plays a role in intracellular trafficking in plant cells, we used the PI(3)P-specific binding property of the FYVE domain as a molecular probe for PI(3)P. In this study, we used the C-terminal region of EEA1 (amino acids 1257 to 1411), that is, the EBD, consisting of the Rab5 binding and FYVE domains, because this region is sufficient for PI(3)P-dependent localization of the fused green fluorescent protein (GFP) to the endosome in vivo in animal cells (Stenmark et al., 1996). To visualize the EBD in plant cells, we constructed a fusion protein gene, *GFP:EBD* (Figure 1A). As a control, we constructed a similar GFP fusion protein gene that contained a mutant form of EBD (EBDC1358S) that does not bind to PI(3)P due to the mutation (C1358S) at the FYVE domain (Stenmark et al., 1996; Burd and Emr, 1998; Gaullier et al., 1998). These and soluble modified GFP (smGFP) (Davis and Vierstra, 1998) constructs were introduced into protoplasts prepared from 1- to 3-week-old whole Arabidopsis seedlings as described previously (Kang et al., 1998), and the fluorescence of transiently expressed GFP:EBD was examined 12 to 24 hr after transformation. As shown in Figure 1B, most of the green fluorescent signal from GFP:EBD was associated with the tonoplasts and within the central vacuoles (center), whereas in the control (GFP:EBDC1358S), only one or a few green fluorescent spots were observed in the cytoplasm (right). This is in sharp contrast to the pattern seen in animal cells, in which the mutant form was distributed uniformly in the cytosol. One possible explanation for this difference is that the mutation might have introduced a new localization signal in the molecule. The green fluorescent signal of smGFP was distributed uniformly in the cytosol as expected (left). To confirm that the fluorescent staining pattern of GFP:EBD was due to binding to PI(3)P at the FYVE domain of EBD in plant cells, we treated the protoplasts transformed with the *GFP:EBD* and *GFP:EBDC1358S* constructs with wortmannin (Ui et al., 1995) and LY294002 (Vlahos et al., 1994), which have been shown to be inhibitors for PI3-kinases in animal cells. As shown in Figure 1C, in the presence of both inhibitors, the fluorescence signals from GFP:EBD were distributed uniformly in the cytosol, whereas the inhibitors did

Figure 1. (continued).

(A) Scheme of various *EBD* constructs. EBD is the C-terminal region of EEA1 from amino acid residues 1257 to 1411, which contains the FYVE and Rab5 binding domains. **(a)** GFP:EBD; **(b)** GFP:EBDC1358S; **(c)** GFP. The 35S cauliflower mosaic virus promoter was used for expression of these constructs.

(B) Patterns of green fluorescent signals of proteins fused with GFP. Protoplasts prepared from whole Arabidopsis seedlings were transformed and examined 12 to 24 hr later. The red fluorescent signals of the chloroplasts are due to autofluorescence of chlorophyll. Yellow indicates overlap of red and green fluorescent signals. CH, chloroplasts; VM, vacuolar membrane.

(C) The effect of PI3-kinase inhibitors on the pattern of GFP signals. Protoplasts were incubated in the presence of wortmannin (10 μM) and LY294002 (100 μM) at room temperature, and the fluorescent pattern was examined 12 to 24 hr after transformation. The images are representative of at least three independent transformations for each construct and each condition. Note that some protoplasts do not contain chloroplasts because they originated from the root cells. CH, chloroplasts. Bars = 20 μm.

(D) Formation of similar types of vesicles in BY2 cells and tobacco leaf cells. The BY2 cells were incubated with FM4-64 (40 μM) for 5 min and then washed twice with Murashige and Skoog (1962) (MS) medium. The cells were examined under a fluorescent microscope at 20 to 30 hr after washing (**[a]** and **[b]**). Tobacco leaf tissues were bombarded using gold particles coated with *GFP:EBD* and incubated for 20 to 30 hr on MS plates after the bombardment. The cells were then examined under a fluorescent microscope (**[c]** and **[d]**). **(a)** and **(c)** show bright field images; **(b)** and **(d)** show fluorescent images. N, nucleus. Arrowheads indicate vesicles formed from the central vacuole. Bars = 20 μm.

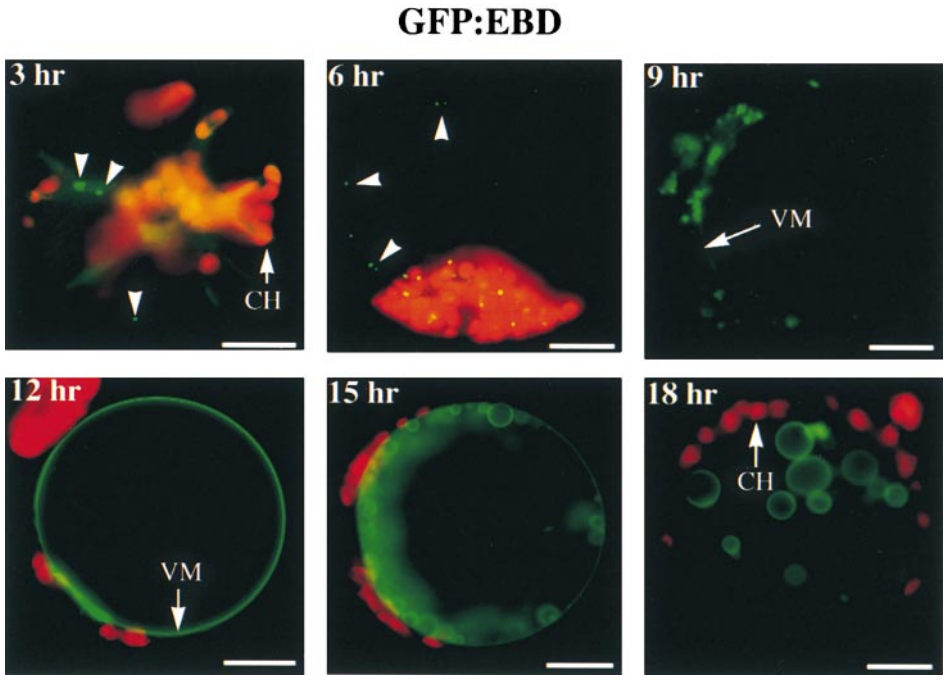

Figure 2. Temporal Expression Pattern of GFP:EBD.

Transformed protoplasts were examined at various times, as indicated. The whole population contained a mixture of protoplasts with different GFP patterns, especially between 6 to 12 hr after transformation. Each image represents the fluorescent pattern of the majority (~20 to 30%) of transformed protoplasts in the whole population at each time, from at least three independent transformation experiments. CH, chloroplasts; VM, vacuolar membrane.

not affect the fluorescence pattern of GFP:EBDC1358S. The behavior of GFP:EBD was very similar to that observed in animal cells in the presence of wortmannin, implying that these inhibitors affected production of PI(3)P in Arabidopsis protoplasts either directly or indirectly. These results strongly suggest that the FYVE domain of EEA1 may bind specifically to PI(3)P in plant cells, as has been shown in animal cells (Patki et al., 1997).

Next, we performed two experiments to exclude the possibility that the vacuolar targeting and vesicle formation at the central vacuole were caused artificially by expression of an animal protein (EBD). It has been shown that the lipophilic dye FM4-64 can be taken up efficiently by endocytosis and transported to the central vacuole in yeast cells (Vida and Emr, 1995). Therefore, we used FM4-64 to determine whether the dye can be used to visualize the tonoplast and the internalization process from the tonoplast into the lumen. When BY2 cells and Arabidopsis protoplasts were incubated with FM4-64, the dye was internalized into the lumen of the central vacuole by vesicles similar to those shown in Figure 1D (panels a and b) (data not shown for Arabidopsis protoplasts). In the second experiment, we introduced *GFP:EBD* into tobacco leaf cells by the particle bombardment method (Takeuchi et al., 1992) and examined the targeting of GFP:EBD in the leaf cells. As shown in Figure 1D (panels c and d), green fluorescent signals were accumulated at the central vacuole and vesicles similar to those seen in the protoplasts were formed from the central vacuole. Thus, these results strongly argue against the possibility of artifacts caused by experimental conditions.

PI(3)P-Dependent Trafficking of GFP:EBD to the Central Vacuole in Plant Cells

Protoplasts expressing GFP:EBD showed several different patterns of fluorescence that changed with time. Therefore, we examined in more detail the patterns of the fluorescent signals at various times after transformation. Green fluorescent signals were observed 4 to 6 hr after transformation. Initially, fluorescence was observed in the cytosol of protoplasts (Figure 2, top left). This pattern was replaced with numerous small punctate stains (Figure 2, top middle). Soon, the majority of protoplasts contained large fluorescent spots near the central vacuoles (Figure 2, top right). This was followed by localization of the GFP signal to the tonoplasts of the central vacuole, where the fusion protein was internalized into the vacuoles by numerous vesicles (Figure 2, bot-

tom). The green fluorescent vesicles present in the lumen disappeared with time. The internalization of the fusion protein by numerous vesicles is depicted more clearly in Figure 3. The z-sections of protoplasts show the numerous vesicles that were formed within the vacuole. Often, the vesicles contained few speckles. The disappearance of the fluorescent signals may be due to the degradation of the GFP:EBD protein by vacuolar proteases. The observed behavior of GFP:EBD in plant cells closely resembled the proposed turnover mechanism of PI(3)P in yeast, in which PI(3)P is transported to the vacuole by vesicle trafficking that requires Vam3p and Ypt7p and is then degraded in the vacuole lumen by lipases and phosphatases (Wurmser and Emr, 1998; Wurmser et al., 1999). Therefore, the results strongly suggest that PI(3)P is turned over in plant cells by transportation from the cytosol to the vacuoles.

The GFP:EBD Fusion Protein Is Initially Targeted to the TGN

The green fluorescent signal was present as numerous punctate stains in the cytosol 4 to 6 hr after transformation. One explanation for such localization is that GFP:EBD may be targeted to an organelle in which PI(3)P is being synthesized by a PI3-kinase. In yeast, PI(3)P has been shown to be synthesized in the Golgi and/or endosome (Stack et al., 1995). Also, at least one isoform of PI3-kinase is localized to the TGN in animal cells (Domin et al., 2000). To examine the localization of GFP:EBD, we attempted colocalization with the Golgi marker rat sialyltransferase (Munro, 1995; Wee et al., 1998) fused to red fluorescent protein (DsRed). First, we confirmed the localization of rat sialyltransferase:red fluorescent protein (ST:RFP) to the Golgi apparatus by cotransforming

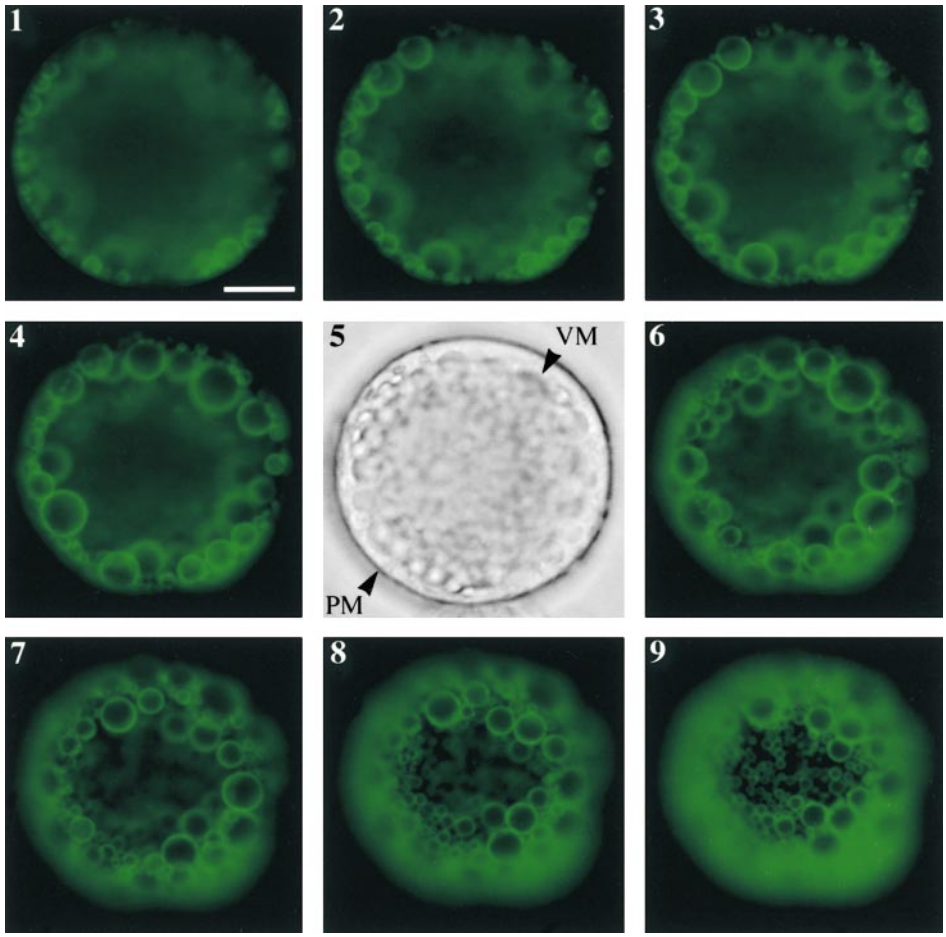

Figure 3. Internalization of GFP:EBD into the Lumen of Vacuoles by Numerous Vesicles.

Serial sections were obtained from the middle to the bottom of a protoplast through the *z* axis at 2-μm intervals. **(1)** and **(9)** show the middle and bottom views of the protoplast, respectively. PM, plasma membrane; VM, vacuolar membrane. Bar = 20 μm.

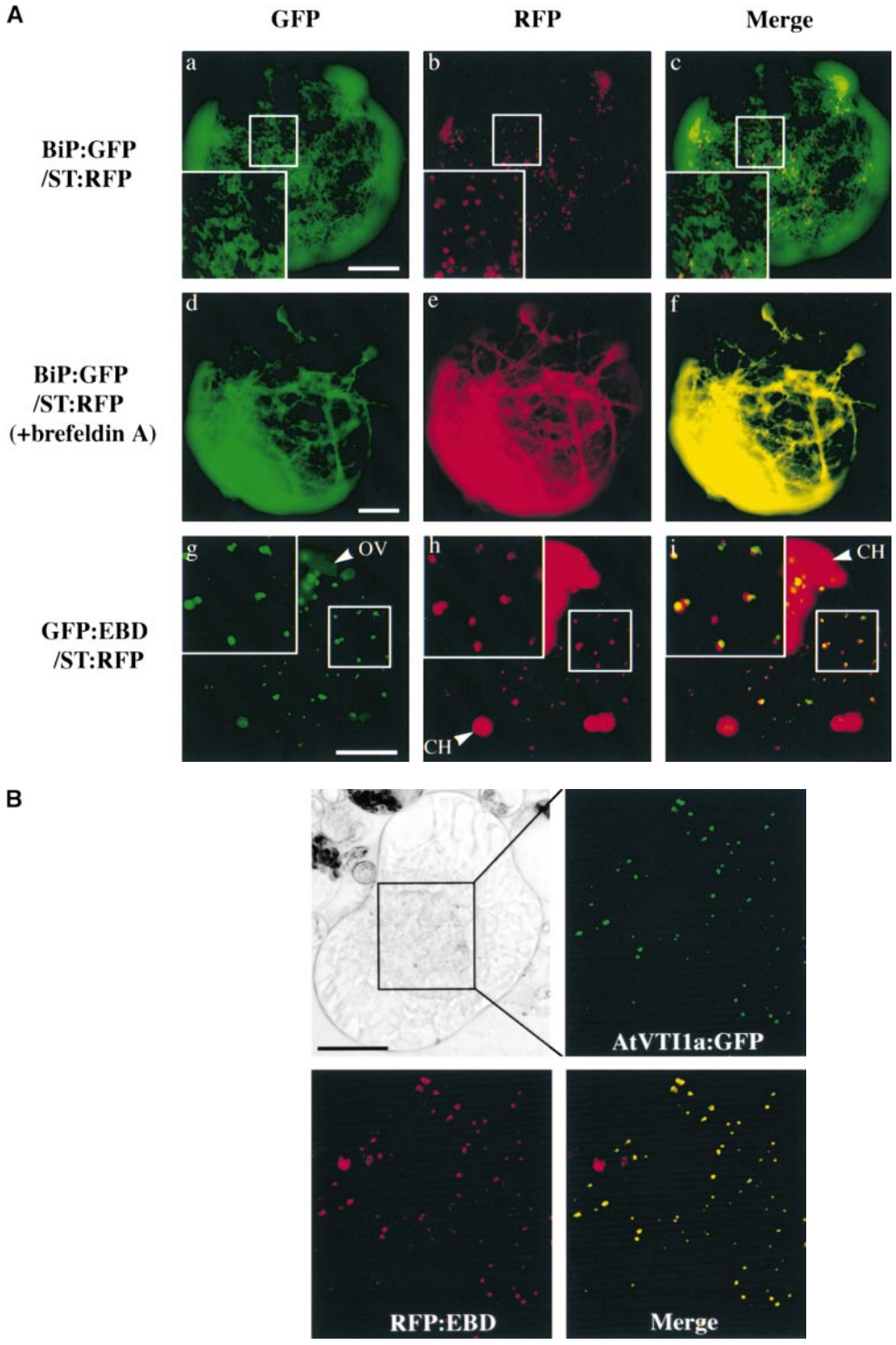

Figure 4. The GFP:EBD Fusion Protein Is Localized to the TGN Early after Transformation.

(A) Colocalization of GFP:EBD with ST. Protoplasts were cotransformed with *ST:RFP* and *BiP:GFP*, and the localization of ST and BiP was examined in the absence (**[a]** to **[c]**) or presence (**[d]** to **[f]**) of brefeldin A (30 μg/mL). Note the relocation and complete overlap of red fluorescent signals with green fluorescent signals. Protoplasts were cotransformed with *ST:RFP* and *GFP:EBD*, and the colocalization of red and green fluorescent signals was examined (**[g]** to **[i]**). The red fluorescent signals from the chlorophyll and red fluorescent protein are quite different from

the protoplasts with a fusion (BiP:GFP) between GFP and portions of the chaperone binding protein (BiP). BiP is an endoplasmic reticulum resident, and it is widely used as a marker of this compartment. The BiP:GFP fusion gives the typical punctate pattern indicative of the endoplasmic reticulum (Figure 4A, first and second rows). ST:RFP gave the pattern of punctate staining that was expected if the marker protein was localized at the Golgi apparatus (panel b) (Boevink et al., 1998; Wee et al., 1998). In the presence of brefeldin A, an agent that disrupts the Golgi apparatus (Fujiwara et al., 1988), the punctate staining pattern disappeared (panel e), and red fluorescent signals overlapped with the green fluorescent signals of BiP:GFP, thus giving yellow fluorescent signals (panel f). These results suggest that ST:RFP was localized at the Golgi apparatus, as shown previously (Munro, 1995; Wee et al., 1998). Next, we examined the localization of GFP:EBD in the protoplasts cotransformed with *ST:RFP* (Figure 4A, bottom row). Most of the green fluorescent signal of GFP:EBD overlapped with the red fluorescent signal of ST:RFP (panel i). Also, some signals were juxtaposed to each other (panel i), thus raising the possibility that GFP:EBD may be localized to the Golgi apparatus or to an organelle that is closely associated with the Golgi apparatus, such as the TGN. To obtain additional evidence for the localization, we performed colocalization of RFP:EBD with AtVTI1a:GFP, a fusion protein between GFP and AtVTI1a, a t-SNARE that is localized primarily to the TGN with a minor portion localized to the prevacuolar compartment in plant cells (Zheng et al., 1999). *RFP:EBD* was cotransformed with *AtVTI1a*:*GFP* into protoplasts. Most of the red and green fluorescent signals clearly overlapped each other (Figure 4B), which further supported the notion that EBD is targeted to the TGN 4 to 6 hr after transformation in plant cells.

At ~1 to 2 hr later, when the small punctate stains were visible, we started to observe protoplasts with several large fluorescent spots or a group of small punctate stains connected to each other (Figure 5A). The protoplasts with these structures were ~20 to 30% at 8 to 10 hr after transformation. At 9 hr after transformation, some of the protoplasts with this structure showed a weak fluorescent signal at the tonoplast, indicating that GFP:EBD started to accumulate at the tonoplast (Figure 2, top right). The latter structures (indicated by arrows in Figure 5A, panels 1 and 2) were clearly different from the small punctate stains seen at earlier times, indicating the possibility of another organelle. Therefore, we attempted colocalization of GFP:EBD with a marker protein for the prevacuolar compartment. We tagged a fusion protein, 491 (aleurain without the targeting signal motif, NPIR, fused to the C terminus of BP80), with DsRed (491:RFP) to use as a marker for the prevacuolar/multivesicular compartment (Jiang and Rogers, 1998). As shown in Figure 5B, the green fluorescent signals clearly overlapped with the red fluorescent signals of 491:RFP (arrowheads), indicating that the green fluorescent signals of the large spots are associated with the prevacuolar compartment. These results suggested that GFP:EBD was transported to the prevacuolar compartment from the TGN.

PI4-Kinase Is Involved in the PI(3)P-Dependent Trafficking to the Central Vacuoles

To enhance our understanding of the mechanism of the PI(3)P-dependent trafficking of GFP:EBD, we set out to determine whether PI4-kinase was involved in the trafficking in plant cells, because PI4-kinases have been proposed to play a role in intracellular trafficking in yeast and animal cells (Wiedemann et al., 1996; Hama et al., 1999). Also, Arabidopsis PI4-kinase β has been proposed to play a role in intracellular trafficking (Xue et al., 1999). We constructed various deletion mutants of the PI4-kinase β gene of Arabidopsis and cotransformed them with the *GFP:EBD* construct into protoplasts to determine whether any of these deletion mutants had a dominant negative effect on the transportation of GFP:EBD. As a control, a full-length construct was included in the experiment. After cotransformation, we compared the pattern of fluorescence among the population of transformed protoplasts at various times. Only the deletion mutant, LKU(PI4-Kβ), caused a significant retardation of the vacuolar trafficking of GFP:EBD (Figures 6A and 6B). The inhibition was pronounced early in the time course; however, the effect was lost eventually. These results suggest that PI4-kinase may be involved in the PI(3)P-dependent trafficking of GFP:EBD to the vacuole membranes.

The GFP:EBD Fusion Protein Inhibits Trafficking of Sporamin to the Central Vacuoles

Overexpression of the FYVE domain may deplete endogenous PI(3)P, or at least compete with an endogenous PI(3)P binding protein, which in turn may result in the inhibition of

Figure 4. (continued).

each other morphologically and can be distinguished easily. CH, chloroplasts; OV, fluorescent signals of chlorophyll that were detected by the GFP filter due to overexposure.

(B) Protoplasts were cotransformed with *AtVTI1a:GFP* and *RFP:EBD* and examined at various times after transformation. The photograph is representative of protoplasts at 4 to 6 hr after transformation. At least three independent transformation experiments were performed for each construct and each condition.

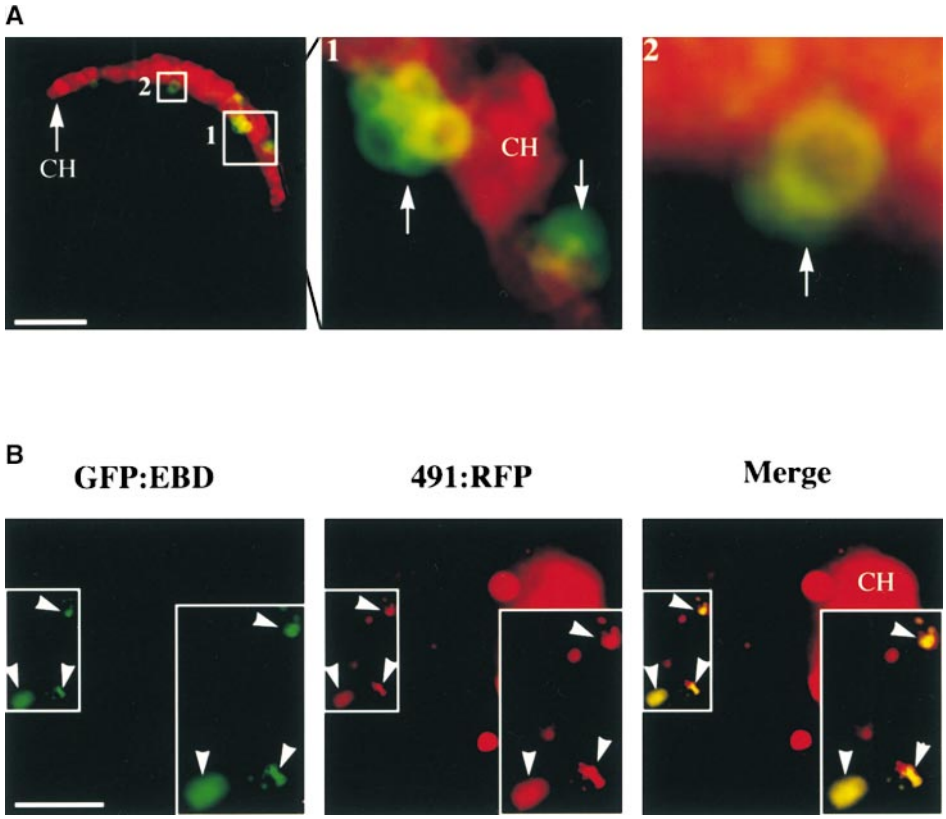

Figure 5. GFP:EBD Was Temporally Localized to the Prevacuolar Compartment.

(A) Protoplasts transformed with *GFP:EBD* had structures that appeared to be formed by the fusion of multiple vesicles (left). Enlarged images of boxes 1 and 2, respectively, are shown in the middle and at right.
(B) Protoplasts were cotransformed with *GFP:EBD* and *491:RFP*. Most of the green fluorescent signals clearly overlapped with the red fluorescent signals of 491 (arrowheads).
CH, chloroplasts.

PI(3)P-dependent trafficking in plant cells. To examine this possibility, an *EBD* construct was cotransformed with a reporter construct, *sporamin:GFP*, that is normally targeted to the large central vacuole (Figure 7A, panel d) (Matsuoka et al., 1995). Vacuolar localization of sporamin:GFP was monitored at various times, and the efficiency of targeting was calculated as the number of protoplasts containing a central vacuole with green fluorescent signals among total protoplasts expressing sporamin:GFP (Figure 7B). The targeting efficiency reached nearly 80% at 70 hr after transformation with *sporamin:GFP* alone or together with the EBDC1358S. In contrast to the control experiments, the efficiency of targeting was ~38%, when an *EBD* construct was cotransformed into protoplasts. To determine whether this inhibition was specific to the vacuolar trafficking of sporamin, we examined the effect of overexpression of EBD on the trafficking of a plasma membrane–type H^+-ATPase (DeWitt et al., 1996) as a GFP fusion protein. As shown in Figure 7A, in the presence of EBD there was no inhibition of trafficking of H^+-ATPase:GFP to the plasma membrane (Figure 7A, panel b), which indicates that the inhibition was specific to the trafficking of sporamin. This finding strongly suggests that PI(3)P is involved in the trafficking of sporamin to the central vacuole. However, it has been shown that sporamin is not missorted by wortmannin in BY2 cells (Matsuoka et al., 1995). Thus, we wanted to examine whether vacuolar trafficking of sporamin:GFP is inhibited by wortmannin in Arabidopsis protoplasts. In the presence of wortmannin, the green fluorescent signal of sporamin:GFP was accumulated in the cytosol as punctate stains instead of being localized at the central vacuole. Only less than 5% of transformed protoplasts showed targeting of sporamin:GFP to the central vacuole at 40 hr after transformation (Figure 7C). Another PI3-kinase inhibitor, LY294002, gave a similar result with wortmannin (data not shown). Furthermore, an AtVPS34 mutant that has a small deletion (30 amino acids) at the C-terminal kinase domain resulted in ~30 to 40% inhibition of sporamin:GFP targeting when cotransformed into

protoplasts (D.H. Kim and I. Hwang, unpublished data). Thus, these results further support the notion that PI(3)P may be involved in the vacuolar targeting of sporamin in Arabidopsis cells.

The Rab5 Binding Domain Is Required for the PI(3)P-Dependent Localization and Trafficking of GFP:EBD in Plant Cells

The C-terminal domain (amino acids 1257 to 1411) of EEA1, which was used as a molecular probe for PI(3)P, also contained the IQ (calmodulin binding) and Rab5 binding domains in addition to the zinc finger domain. The Rab5 binding domain is necessary for PI(3)P-dependent localization of EEA1 to the early endosome in animal cells (Lawe et al., 2000). Therefore, we investigated whether these neighboring domains contributed to the PI(3)P-dependent localization and transportation of EBD in plant cells. We generated two deletion constructs, *EBD1306* (amino acids 1306 to 1411) and *EBD1336* (amino acids 1336 to 1411), and fused them with *GFP* (Figure 8A). The *EBD1306* construct contained the zinc finger domain and the neighboring Rab5 binding domain, but not the IQ domain, whereas the *EBD1336* construct contained only the zinc finger domain. As shown in Figure 8B, the green fluorescent signal was distributed uniformly in the protoplasts transformed with *GFP:EBD1336*, whereas the protoplasts transformed with *GFP:EBD1306* showed a pattern of fluorescence identical to that of the original construct, *GFP:EBD*. These results indicate that in addition to

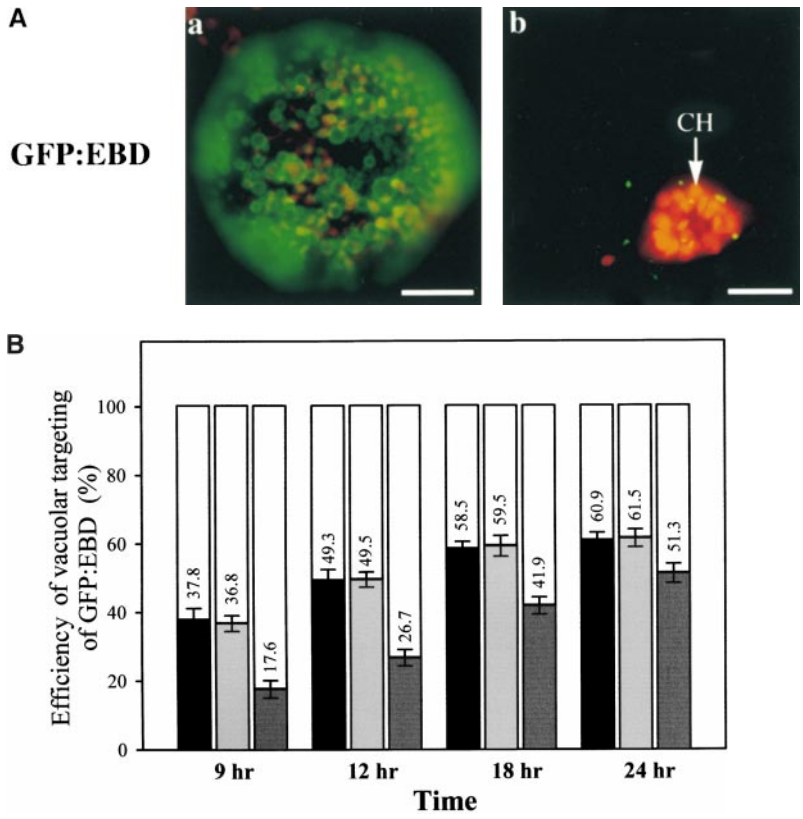

Figure 6. Overexpression of the LKU Domain of PI4-Kinase β Inhibits Transport of GFP:EBD to Vacuoles.

Protoplasts were transformed with *GFP:EBD* either alone or together with *Full*(*PI4-Kβ*) or *LKU*(*PI4-Kβ*), and the targeting efficiency of GFP:EBD to the vacuole was examined at various times.

(A) Typical GFP patterns of protoplasts with GFP:EBD targeted to vacuoles **(a)** and not targeted to vacuoles **(b)**. CH, chloroplasts. Bars = 20 μm.

(B) The number of protoplasts with the GFP pattern shown in **(A)** were counted from a whole population to give the efficiency of targeting. Also, protoplasts with punctate staining together with the vacuolar staining were counted as GFP:EBD targeted to the vacuoles. Three independent transformation experiments were performed, and each time 200 transformed protoplasts were counted. The first, second, and third columns at each time point indicate protoplasts transformed with *GFP:EBD* alone, *GFP:EBD* plus *Full*(*PI4-Kβ*), and *GFP:EBD* plus *LKU*(*PI4-Kβ*), respectively. The numbers indicate the percentage of protoplasts with GFP:EBD targeted to the vacuoles. Error bars indicate ±SD.

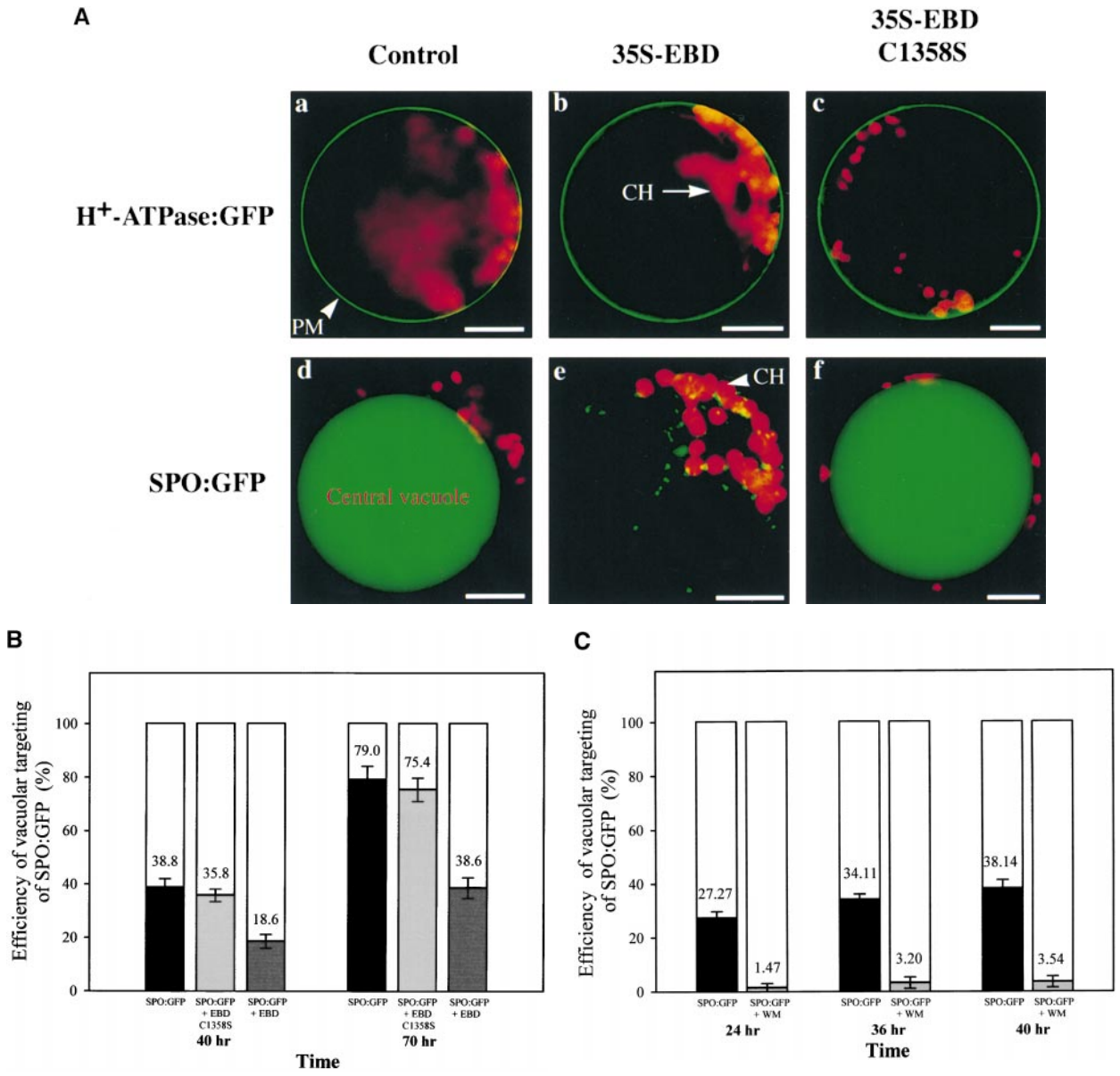

Figure 7. Inhibition of Vacuolar Targeting of Sporamin.

(A) Protoplasts were transformed with *H⁺-ATPase:GFP* alone **(a)**, with *35S-EBD* **(b)**, or with *35S-EBDC1358S* **(c)**, and examined 24 hr later. Also, protoplasts were transformed with *sporamin:GFP* (SPO:GFP) alone **(d)**, with *35S-EBD* **(e)**, or with *35S-EBDC1358S* **(f)**, and examined 40 and 70 hr later. Protoplasts with the GFP pattern observed in **(e)** and **(f)** were counted as sporamin:GFP not targeted to vacuoles and targeted to vacuoles, respectively. CH, chloroplasts; PM, plasma membrane. Bars = 20 μm.

(B) Efficiency of vacuolar targeting of sporamin:GFP. Protoplasts with vacuolar staining together with punctate staining were considered as sporamin:GFP targeted to the vacuoles. At least three independent transformation experiments were performed, and more than 200 transformed protoplasts were counted each time. The numbers indicate the percentage of protoplasts with sporamin:GFP targeted to the vacuoles.

(C) Inhibition of vacuolar targeting of sporamin:GFP by wortmannin. Protoplasts transformed with *sporamin:GFP* were incubated with 5 μM wortmannin (SPO:GFP + WM) at room temperature, and targeting efficiency was measured at various times. At least three independent transformation experiments were performed, and more than 200 transformed protoplasts were counted each time. Criterion for the inhibition was the accumulation of fluorescent punctate stains in the cytosol versus the accumulation of the green fluorescent signals in the central vacuole. The numbers indicate the percentage of protoplasts with sporamin:GFP targeted to the vacuoles. Error bars indicate ±SD.

the zinc finger domain, the Rab5 binding domain is necessary for the PI(3)P-dependent localization and transportation of GFP:EBD in plant cells, as has been reported for animal cells (Lawe et al., 2000).

DISCUSSION

PI(3)P-Dependent Binding of GFP:EBD in Plant Cells

The FYVE domain alone has been shown to be sufficient for PI(3)P-dependent binding in vitro and also can be targeted to the endosome in vivo in a PI(3)P-dependent manner when introduced into cells as a GFP fusion protein (Stenmark et al., 1996; Burd and Emr, 1998; Lawe et al., 2000). In this study, we constructed a similar GFP:EBD fusion protein using the endosome binding domain of human EEA1 and introduced the construct into protoplasts of Arabidopsis. We observed three different fluorescent patterns of the GFP:EBD fusion protein in protoplasts: green fluorescent signals were observed as numerous punctate stains in the cytosol, at the tonoplasts, and at vesicles within the central vacuoles. Inhibition studies using wortmannin and LY294002, which have been shown to be PI3-kinase inhibitors in animal cells, suggest that these staining patterns may be due to the binding of GFP:EBD to PI(3)P. In addition, the fact that GFP:EBDC1358S showed a completely different staining pattern supports the notion that the localization of GFP:EBD is dependent on PI(3)P as in animal cells. Interestingly, experiments with the deletion constructs *EBD1306* and *EBD1336* indicate that the neighboring Rab5 binding domain, but not the IQ domain, is necessary for PI(3)P-dependent localization of the fusion protein, as shown in animal cells (Lawe et al., 2000). Together, these results strongly suggest that PI(3)P is present in various organelles in plant cells and that the targeting mechanism of a protein with the EBD domain may be highly conserved in yeast, animal, and plant cells. In addition, these results imply that an endogenous Rab5 homolog of Arabidopsis may play a role in the localization of the fusion protein.

Transport of PI(3)P to the Vacuole for Degradation

The pattern of the green fluorescent signal of GFP:EBD that was expressed transiently in protoplasts changed with time. The signal was observed initially at the TGN and then at the prevacuolar compartment. The signal was then localized to the membrane of the central vacuole and finally in the lumen

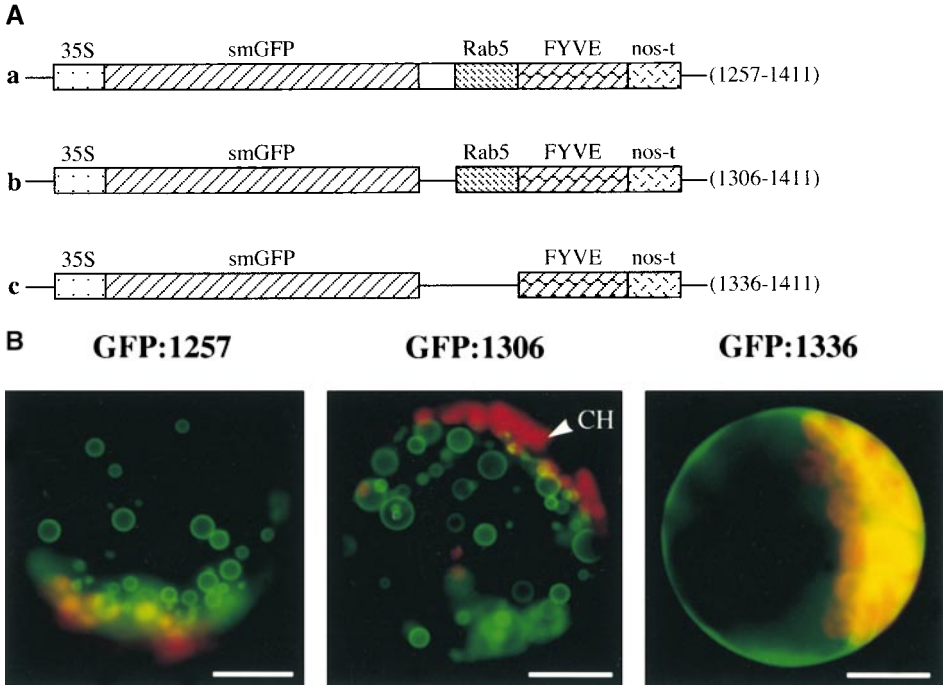

Figure 8. The Rab5 Binding Motif Is Necessary for PI(3)P-Dependent Targeting and Transport of GFP:EBD.

(A) Scheme of deletion constructs of EBD. FYVE and Rab5 indicate the zinc finger and Rab5 binding domains, respectively.
(B) Protoplasts were transformed with *GFP:EBD* (denoted as GFP:1257), *GFP:EBD1306* (GFP:1306), or *GFP:EBD1336* (GFP:1336). CH, chloroplasts. Bars = 20 μm.

of the central vacuole. Because GFP:EBD localization is dependent on the presence of PI(3)P, the differential localization of GFP:EBD likely reflects the localization of PI(3)P in plant cells. In yeast, PI(3)P is synthesized at the Golgi/endosome by Vps34p (Schu et al., 1993; Wurmser and Emr, 1998), which is recruited to the Golgi/endosome and activated by Vps15p (Stack et al., 1995). In Arabidopsis, AtVPS34, a homolog of yeast Vps34p, has been isolated and shown to play a critical role in plant development (Welters et al., 1994). However, it is not known whether AtVPS34 is responsible for biosynthesis of PI(3)P, which was identified by GFP:EBD. In the protoplasts transformed with *GFP:EBD*, one of the earliest patterns of fluorescence was the punctate stains at the TGN. Initial localization of GFP:EBD at the TGN may indicate that PI(3)P is synthesized by a PI3-kinase at the TGN in plant cells, as occurs in animal cells (Domin et al., 2000). Of course, it is also possible that PI(3)P synthesized at other places was transported to the TGN. However, we believe the first interpretation to be correct because this is the mechanism reported for other cells (Wurmser and Emr, 1998).

To prove unequivocally where PI(3)P is synthesized in plant cells, it is necessary to identify and localize the PI3-kinase involved. Once PI(3)P is synthesized at the Golgi/endosome, it is transported for turnover to vacuoles by a vesicle-trafficking process that requires Vam3p and Ypt7p in yeast (Wurmser and Emr, 1998). Degradation of PI(3)P in the vacuole is the primary turnover mechanism of PI(3)P in yeast, because blocking of vacuolar transport causes a several-fold increase in the PI(3)P level. Also, Fab1p, a PI(3)P 5-kinase localized at the endosome and the vacuolar membrane, is involved in the turnover of PI(3)P by phosphorylating the D5 position of the inositol ring (Gary et al., 1998; Odorizzi et al., 1998). In plant cells, the fluorescent signal of GFP:EBD that was initially localized to the TGN was subsequently observed to be associated with the prevacuolar compartment and with the central vacuole. These results suggest that the complex of GFP:EBD and PI(3)P was transported from the TGN through the prevacuolar compartment to the central vacuole, and finally internalized into the central vacuole by vesicles, as depicted in our model (Figure 9).

The behavior of GFP:EBD was very similar to the proposed turnover mechanism of PI(3)P in yeast (Wurmser and Emr, 1998; Wurmser et al., 1999), which suggests that the turnover mechanism may be highly conserved in yeast and plant cells. However, it is not known whether plant cells possess a homolog of yeast Fab1p (Wurmser et al., 1999) that downregulates the level of PI(3)P. Interestingly, the expression of LKU(PI4-Kβ), a deletion mutant of PI4-kinase β, causes retardation of the PI(3)P-dependent trafficking of GFP:EBD to the tonoplasts of the central vacuole, suggesting that PI(4)P may play a role in the trafficking.

PI(3)P-Dependent Vesicle Trafficking in Plant Cells

It is highly likely that overexpressed GFP:EBD may bind to a significant amount of PI(3)P, which results in inhibition of a process that requires PI(3)P in plant cells. The vacuolar trafficking of sporamin:GFP was inhibited by 50% when EBD

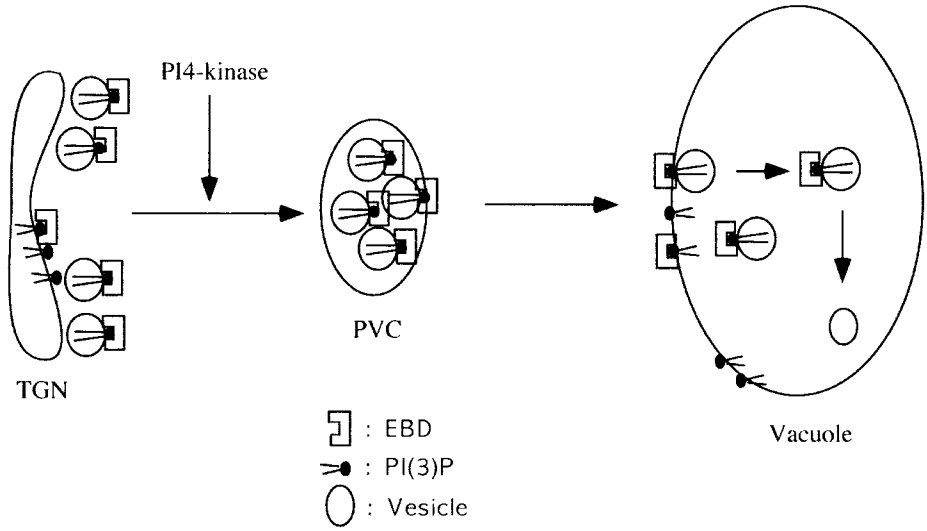

Figure 9. A Model for the PI(3)P-Dependent Trafficking of EBD of EEA1 in Plant Cells.

The EBD binds to PI(3)P at the TGN, and the EBD:PI(3)P complex is transported to the tonoplasts through the prevacuolar compartment by a PI4-kinase-dependent mechanism. The complex is then internalized into the lumen of the central vacuole for degradation by vacuolar hydrolyases. PVC, prevacuolar compartment.

was overexpressed in the protoplasts. Also, this inhibition was specific to the trafficking of sporamin, because targeting of H^+-ATPase:GFP to the plasma membrane was not affected under the same conditions. Interestingly, targeting of sporamin:GFP in the Arabidopsis protoplasts was also inhibited by wortmannin, which is in stark contrast to the previous results seen in the BY2 cells (Matsuoka et al., 1995), implying that there may be a difference between cell types in the inhibition. One possible explanation for the inhibition is competition for PI(3)P between the FYVE domain and the endogenous PI(3)P binding protein. However, we cannot exclude the possibility that other mechanisms, such as depletion of a Rab5 homolog by EBD, may be responsible for the inhibition. In fact, these two possibilities are not mutually exclusive, because Rab5 and PI(3)P work together to form a complex involved in endosome fusion in animal cells. EBD inhibits early endosome fusion by interfering with normal EEA1/SNARE complex formation (McBride et al., 1999). Therefore, EBD may also interfere with the formation of a similar complex containing PI(3)P and a Rab5 homolog in plant cells. Interestingly, analysis of Arabidopsis genomic sequences revealed a gene that potentially codes for a protein with the FYVE domain. Also, there are several Rab5 homologs in the Arabidopsis genome.

METHODS

Growth of Plants

Arabidopsis thaliana (ecotype Columbia) was grown on Murashige and Skoog (1962) plates at 20°C in a culture room with a 16-hr-light/8-hr-dark cycle. Arabidopsis seedlings used for preparation of protoplasts were grown in MS liquid medium with constant shaking (160 rpm) at 20°C under a 16-hr-light/8-hr-dark cycle.

In Vivo Targeting of Fusion Proteins

To generate a chimeric fusion construct of *GFP:EBD*, DNA that encoded the C-terminal fragment of human early endosome antigen 1 (EEA1; amino acid residues 1257 to 1411) was amplified by polymerase chain reaction (PCR) using primers 5′-GAATTCGTGGCAATCTAGTCAACGG-3′ and 5′-CTAATGTTAGTGTAATATTAC-3′, sequenced to confirm the nucleotide sequence, and ligated in frame to the C terminus of the green fluorescent protein (GFP) coding region without the terminator codon. The *FYVEC1358S* mutant (Stenmark et al., 1996) was also fused to soluble modified GFP (smGFP) to give *GFP:EBDC1358S*. To construct *GFP:EBD1306* and *GFP:EBD1336*, *EBD1306* and *EBD1336* were amplified by PCR using primers (1306, 5′-ATAACAATGCTTCAAACCAAAGTATTAGAA-3′ and 5′-ATAACAATGATCAAACATACACAAGCG-3′; 1336, 5′-CTAATGTTAGTGTAATATTAC-3′ and 5′-CTAATGTTAGTGTAATATTAC-3′) and ligated to the 3′ end of the gene encoding smGFP. To construct the reporter fusion genes, *AtVTI1a:GFP*, *491:RFP*, *H^+-ATPase:GFP*, *sporamin:GFP*, and *ST:RFP*, *DsRed1-1* or *GFP* were ligated in frame to DNA that encoded the C termini of *AtVTI1a*, *491*, *AHA2*, *sporamin B*, and sialyltransferase, respectively. To construct *BiP:GFP*, the Arabidopsis chaperone binding protein (*BiP*) gene (GenBank accession number D82817) was amplified by PCR from total cDNA using specific primers BiP5 (5′-TACGCAAAAGTTTCC-GAT-3′) and BiP3 (5′-CTAGAGCTCATCGTGAGA-3′). The N-terminal leader sequence (44 amino acid residues) and the C-terminal region (79 amino acid residues with the retrieval signal HDEL) were fused in frame to the N- and C-termini, respectively, of smGFP without the termination codon. All the chimeric GFP fusion constructs were placed under the control of the 35S promoter in a pUC vector for expression in protoplasts. Plasmids were purified using Qiagen (Valencia, CA) columns according to the manufacturer's protocol. The fusion constructs were introduced into Arabidopsis protoplasts prepared from whole seedlings by polyethylene glycol–mediated transformation (Kang et al., 1998). Expression of the fusion constructs was monitored at various times after transformation, and images were captured with a cooled charge-coupled device camera using a Zeiss (Jena, Germany) Axioplan fluorescence microscope. The filter sets used were XF116 (exciter, 474AF20; dichroic, 500DRLP; emitter, 510AF23) and XF137 (exciter, 540AF30; dichroic, 570DRLP; emitter, 585ALP) (Omega Optical, Brattleboro, VT) for green and red fluorescent proteins, respectively. Images were then processed using Adobe Photoshop (Mountain View, CA), and the images are in pseudocolor.

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Trafficking of Phosphatidylinositol 3-Phosphate from the *trans*-Golgi Network to the Lumen of the Central Vacuole in Plant Cells

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