An A/ENTH Domain-Containing Protein Functions as an Adaptor for Clathrin-Coated Vesicles on the Growing Cell Plate in Arabidopsis Root Cells

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Cytokinesis is the process of partitioning the cytoplasm of a dividing cell, thereby completing mitosis. Cytokinesis in the plant cell is achieved by the formation of a new cell wall between daughter nuclei using components carried in Golgi-derived vesicles that accumulate at the midplane of the phragmoplast and fuse to form the cell plate. Proteins that play major roles in the development of the cell plate in plant cells are not well defined. Here, we report that an AP180 amino-terminal homology/epsin amino-terminal homology domain-containing protein from Arabidopsis (Arabidopsis thaliana) is involved in clathrin-coated vesicle formation from the cell plate. Arabidopsis Epsin-like Clathrin Adaptor1 (ATECA1; AT2g01600) and its homologous proteins ATECA2 and ATECA4 localize to the growing cell plate in cells undergoing cytokinesis and also to the plasma membrane and endosomes in nondividing cells. ATECA1 (AT2g01600) does not localize to nascent cell plates but localizes at higher levels to expanding cell plates even after the cell plate fuses with the parental plasma membrane. The temporal and spatial localization patterns of ATECA1 overlap most closely with those of the clathrin light chain. In vitro protein interaction assays revealed that ATECA1 binds to the clathrin H chain via its carboxyl-terminal domain. These results suggest that these AP180 amino-terminal homology/epsin amino-terminal homology domain-containing proteins, ATECA1, ATECA2, and ATECA4, may function as adaptors of clathrin-coated vesicles budding from the cell plate.

Cytokinesis is the last stage of mitosis, in which the cytoplasm of a dividing cell is partitioned to daughter cells. Unlike animal cells, which divide by fission, plant cells employ a more complicated mechanism for cytokinesis: a phragmoplast, a cytokinetic organelle, is formed from the remains of spindle microtubules, and a new cell wall is generated at the midplane of the phragmoplast, thereby separating the cytoplasm (Jürgens, 2005). Secretory vesicles originating from the trans-Golgi network (TGN) are delivered to the division plane and fuse to each other via a homotypic fusion to form the cell plate (Mayer and Jürgens, 2004; Jürgens, 2005; Dhonukshe et al., 2006; Chow et al., 2008; Van Damme et al., 2008; Thiele et al., 2009; Reyes et al., 2011). The fused vesicles at the growing cell plate are then further processed via intermediate structures, such as tubulovesicular networks, to form planar fenestrated sheets (Otegui et al., 2001; Jürgens, 2005). Recent studies have revealed that many vesicle trafficking-related proteins are involved in cell plate formation. These proteins include the cytokinesis-specific t-SNARE KNOLLE (Lauber et al., 1997), a syntaxin-binding protein, KEULE (Assaad et al., 2001), AtSNAP33 (Heese et al., 2001), dynamin-related proteins (DRPs; Hong et al., 2003; Ito et al., 2012), ESCRT (for endosomal sorting complex required for transport) components, and proteins of the exocyst complex (Fendrych et al., 2010). These observations suggest that exocytosis and endocytosis play critical roles in cell plate formation.

Clathrin, composed of light and heavy chains, is one of the coat proteins involved in vesicle budding in multiple pathways (Robinson and Bonifacino, 2001; Hwang and Robinson, 2009). In addition to clathrin, a large number of accessory proteins are involved in clathrin-coated vesicle (CCV) formation (Robinson and Bonifacino, 2001; Chen et al., 2011). One of the accessory proteins is Epsin1, which has a unique domain at its N terminus that is shared by a group of proteins termed the epsin N-terminal homology (ENTH) domain-containing proteins (Chen et al., 1998). Another

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accessory protein, AP180, has a domain that displays high amino acid sequence similarity to the ENTH domain. The AP180 domain has also been identified in other proteins that are collectively called the AP180 N-terminal homology (ANTH) domain-containing proteins (Rosenthal et al., 1999; Drake et al., 2000; Mao et al., 2001; Wendland, 2002). Both ENTH and ANTH domains have the ability to bind inositol phospholipids in the membrane, most notably phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P$_2$] (Ford et al., 2001; Itoh et al., 2001). Thus, they are often grouped together as the A/ENTH domain. However, these two homologous domains have different structural features and lipid-binding properties. The ENTH domain has in front of α-helix 1 an N-terminal unstructured region that is converted to an α-helix on binding to PtdIns(4,5)P$_2$ in membranes and is thus referred to as H$_0$ (or α0; De Camilli et al., 2002; Ford et al., 2002; Legendre-Guillemin et al., 2004; Silkov et al., 2011). H$_0$ has been shown to be essential for membrane-binding and membrane-deforming activities of the ENTH domain (Ford et al., 2002; Stahelin et al., 2003; Yoon et al., 2010; Silkov et al., 2011). In contrast, the ANTH domain does not have the N-terminal H$_0$ (Ford et al., 2001; Itoh et al., 2001) and binds PtdIns(4,5)P$_2$ using α-helix 1, α-helix 2, and the loop between them. Consequently, the ANTH domain does not have the membrane-deforming activity (Ford et al., 2001; Itoh et al., 2001; Stahelin et al., 2003).

Among a large number of ANTH domain proteins, AP180 is involved in endocytosis in both animal and plant cells (Mao et al., 2001; Barth and Holstein, 2004). Among the ENTH domain proteins, Epsin1, Epsin2, and Epsin3 in animal cells and EN1 in yeast function in endocytosis (Chen et al., 1998; Aguilar et al., 2003), whereas EpsinR/clip1, yeast EN3, and plant AtEpsinR1 and AtEpsinR2 are involved in lysosomal/vacuolar trafficking at the TGN (Kalthoff et al., 2002; Chidambaram et al., 2004; Song et al., 2006; Lee et al., 2007). The Arabidopsis (Arabidopsis thaliana) genome encodes a large number of A/ENTH domain-containing proteins (Holstein and Oliviusson, 2005). However, their physiological roles and biochemical properties are not well understood. Only three proteins, AtEpsinR1, AtEpsinR2, and AtAP180, have been studied at the molecular and cellular levels (Barth and Holstein, 2004; Song et al., 2006; Lee et al., 2007). Recently, another A/ENTH domain protein (At2g01600) has been characterized for its membrane-binding mode and structural features (Silkov et al., 2011). The amino acid sequence of its N-terminal domain is closely related to the ANTH domain. However, unlike the typical ANTH domain, it contains the N-terminal H$_0$ and can thus induce vesicle tubulation, hence named the N-ANTH domain. Interestingly, the At2g01600 N-ANTH domain has higher affinity for phosphatidylinositol (3,4,5)-trisphosphate [PtdIns(3,4,5)P$_3$] than for PtdIns(4,5)P$_2$ (Silkov et al., 2011). The physiological roles of this unusual N-ANTH domain-containing protein, as well as other plant proteins with the N-ANTH domain, have not been elucidated. This study investigates A/ENTH domain-containing proteins of Arabidopsis, including Arabidopsis Epsin-like Clathrin Adaptor1 (AtECA1; At2g01600), by means of immunolocalization, live cell imaging, and in vitro protein-protein interaction assays. The results provide evidence that several A/ENTH domain-containing proteins, including At2g01600, localize to the growing cell plate and suggest that the proteins may serve as adaptors for clathrin in CCV-mediated endocytosis at the growing cell plate.

RESULTS

A/ENTH Domain-Containing Proteins Localize to the Plasma Membrane, Endosomes, and Cell Plate

The Arabidopsis genome encodes 22 proteins with an A/ENTH domain. Phylogenetic analysis revealed that they can be grouped into five to six subfamilies (Supplemental Fig. S1; Holstein and Oliviusson, 2005). To gain insight into the role of these proteins in plant cells, the cellular localization of Arabidopsis A/ENTH domain-containing proteins was determined. Initially, three genes were selected, At2g01600, At1g03050, and At2g25430, named AtECA1, AtECA2, and AtECA4, respectively. These were fused to the N terminus of sGFP for in vivo localization (Fig. 1A). The sGFP fusion constructs AtECA1:sGFP, AtECA2:sGFP, and AtECA4:sGFP were transformed into protoplasts from leaf tissues of Arabidopsis using polyethylene glycol-mediated transformation. H'-ATPase:mRFP (for red fluorescent protein) was cotransformed with the AtECA:sGFP constructs to provide a counterstain for the plasma membrane (Lee et al., 2002). In the protoplasts, all three sGFP fusion proteins closely overlapped with H'-ATPase:mRFP (Fig. 1B), indicating that AtECAs localize to the plasma membrane. Punctate spots were observed along the cell periphery, but it was not clear whether the spots were at the plasma membrane or in the cytosolic strands compressed between the central vacuole and the plasma membrane. These results contrast with the previous study of AtEpsinR1 and AtEpsinR2, which were clearly associated with cytosolic foci and thought to play roles in vacuolar trafficking pathways (Song et al., 2006; Lee et al., 2007). The current observations of AtECA: sGFP localization suggest that these proteins may be involved in plasma membrane-associated processes.

To confirm the plasma membrane localization of these proteins, transgenic plants transformed with the sGFP fusion constructs were generated. The expression of intact sGFP fusion proteins was verified by immunoblot analysis. An anti-GFP antibody detected 96-, 97-, and 100-kD polypeptides that match the expected sizes of AtECA1:sGFP, AtECA2:sGFP, and AtECA4:sGFP, respectively (Fig. 2A). Next, the in vivo localization of GFP proteins in the root tip was examined. GFP fluorescence was observed at the plasma membrane, endosomes, and in the cytosol in the transgenic root samples, with each reporter displaying different subcellular localization intensities. AtECA1:sGFP associated...
mostly with the plasma membrane, whereas AtECA4:sGFP was more densely populated at endosomes than at the plasma membrane. AtECA2:sGFP appeared primarily in the cytosol, with a weak signal at the plasma membrane and endosomes. In dividing cells, all these proteins accumulated at the cell plate; AtECA1:sGFP exhibited the strongest fluorescence signal at the cell plate, followed by AtECA4:sGFP and AtECA2:sGFP (Fig. 2D). We thus focused the ensuing investigation on AtECA1. To rule out the possibility that the expression of AtECA:sGFPs driven by the strong cauliflower mosaic virus (CaMV) 35S or cassava vein mosaic virus (CsVMV) promoter may perturb normal localization, AtECA1:sGFP was also expressed under the control of its native promoter to ensure that its localization pattern was identical to that observed with the CaMV 35S promoter (Fig. 2Bb).

The localization of endogenous AtECA1 was further examined with an anti-AtECA1 antibody raised against recombinant AtECA1 expressed in Escherichia coli (Supplemental Fig. S2B). When root tip tissues of wild-type plants were probed with the anti-AtECA1 antibody, AtECA1-specific fluorescence was observed at cytosolic puncta, the plasma membrane, and the cell plate, which is consistent with the localization pattern of AtECA1:sGFP (Fig. 2E). In transgenic AtECA1:sGFP plants, the anti-AtECA1-positive signals overlapped with GFP signals of AtECA1:sGFP, confirming that overexpressed AtECA1:sGFP and the endogenous AtECA1 have the same localization behaviors.

AtECA1 Localizes to the Plasma Membrane and Early Endosomes in Nondividing Cells

To test whether the AtECA:sGFP-positive cytosolic punctate spots correspond to endosomes, colocalization of AtECA1:sGFP and the lipophilic endocytic tracer FM4-64 was examined. The TGN functions as the early endosome (EE) in plant cells, and FM4-64 labels the TGN within several minutes (Bolte et al., 2004; Dettmer et al., 2006; Lam et al., 2007). Brefeldin A (BFA), a fungal compound known to inhibit Arf-GEF activity, causes an aggregation of endosomes, known as the BFA compartment (Satiat-Jeunemaitre et al., 1996). These properties of EEs were utilized to determine the identity of the AtECA1:sGFP-positive structures. Root tissues of AtECA1:sGFP plants were labeled with FM4-64, and localization was examined after 5 min. AtECA1:sGFP fluorescence overlapped with FM4-64 fluorescence at the endosomes and plasma membrane (Fig. 3A). When root tissues were treated with BFA (50 μM for 20 min), both AtECA1:sGFP and FM4-64-positive compartments were enriched in the BFA compartment (Fig. 3B), indicating that AtECA1:sGFP is associated with the EE.

Figure 1. AtECA:sGFP proteins localize primarily to the plasma membrane in protoplasts. A, Constructs used in this study. AtECA1 were fused with sGFP at the C terminus. Promoters for their expression included CaMV 35S, CsVMV, and AtECA1 native promoter (2.0-kb fragment from the –1 position). B, Localization of AtECA:sGFP fusion proteins in protoplasts. Protoplasts were transformed with AtECA1:sGFP, AtECA2:sGFP, or AtECA4:sGFP together with H+-ATPase:mRFP, a marker for the plasma membrane, by polyethylene glycol-mediated transformation, and their localization was examined using a fluorescence microscope. Bars = 20 μm.
To further investigate the identity of the AtECA1:sGFP compartment, the localization of AtECA1:sGFP was compared with the localization of known organelle proteins. The clathrin light chain (CLC) localizes to the TGN/EE and to the plasma membrane (Konopka et al., 2008; Ito et al., 2012). SYP43 is another TGN/EE marker protein. Plants expressing AtECA1:sGFP were crossed to plants expressing CLC:mOrange (Konopka et al., 2008) and to plants expressing monomeric RFP-tagged SYP43 (mRFP:SYP43; Ebine et al., 2008), and the subcellular localization of fluorescent proteins was examined. AtECA1:sGFP colocalized well with both TGN/EE markers (Fig. 4, A–F). However, AtECA1:sGFP did not colocalize with γ-COP, a component of COPI vesicles of the Golgi that was detected with an anti-γ-COP antibody (Pimpl et al., 2000). Neither did AtECA1:sGFP colocalize with prevacuolar compartment marker proteins, including mCherry:RabF2α/Rha1 (Lee et al., 2004; Geldner et al., 2009) and AtVSR (detected with an anti-VSR antibody; Li et al., 2002; Fig. 4, G–O). These results
show that AtECA1 localizes to the TGN/EEs but not to the Golgi apparatus or the prevacuolar compartment.

**AtECA1:sGFP Relocates to the Growing Cell Plate during Cytokinesis**

Varying levels of AtECA1:sGFP accumulation on cell plates suggested that the distribution of AtECA1:sGFP changes as the cell plate grows (Fig. 5A), as is the case for other proteins that relocate to the developing cell plate (Lauber et al., 1997; Chow et al., 2008). Therefore, the distribution of AtECA1:sGFP in root meristematic cells was monitored in conjunction with DNA visualization using 4′,6-diamino-phenylindole (DAPI), because stages of mitosis and cytokinesis could be differentiated by the varying morphological features of DAPI-stained nuclear DNA. AtECA1:sGFP was primarily localized to the plasma membrane until anaphase, with a minor portion localized to the endosomes. Accumulation of AtECA1:sGFP at the division plane was first observed during early telophase, when the cell plate starts to form, and GFP fluorescence remained associated with the cell plate until the end of cytokinesis (Fig. 5B). However, the fluorescence intensity at the cell plate was reduced in the later stages of cytokinesis, and it appeared to relocate to the plasma membranes of the daughter cells as the cell plate matured (Fig. 5B, g and h).

The dynamic colocalization of AtECA1:sGFP and FM4-64 was examined by time-lapse imaging (Fig. 5C). FM4-64 is targeted to young cell plates in plant cells undergoing cytokinesis (Dhonukshe et al., 2006). At 0 min, both FM4-64 and AtECA1:sGFP yielded strong fluorescence at the plasma membrane. At 3 min, FM4-64 was observed as a small line at the center of the cell (Fig. 5C, arrows), indicating that FM4-64 localizes to the nascent cell plate. However, at this time point, AtECA1:sGFP was barely detectable at the cell plate. At 6 min, the growing cell plate had expanded laterally and was labeled by both FM4-64 and AtECA1:sGFP. At subsequent time points, the two fluorescence signals overlapped in the growing cell plate. These results suggest that AtECA1 is involved in the expansion and maturation of the cell plate rather than in the early stage of formation, when Golgi-derived vesicles fuse with each other.

**The Localization Pattern of AtECA1:sGFP to the Growing Cell Plate Differs from Those of KNOLLE and DRP1C But Is Similar to That of CLC:mOrange**

To gain further insight into the role of AtECAs in cell plate formation, the localization of AtECA1:sGFP in the cell plate was compared with that of other cell plate proteins. KNOLLE, a cell plate-specific SNARE, is involved in the fusion of TGN-derived vesicles to generate cell plates (Lauber et al., 1997) and thus plays a crucial role in the initiation of cell plate formation. KNOLLE was visualized by indirect immunofluorescence microscopy in root cells expressing AtECA1:sGFP. Both KNOLLE and AtECA1:sGFP localized to the growing cell plate (Fig. 6, A and B), but their spatiotemporal distributions were slightly different. At the early stage of cell plate formation, both AtECA1:sGFP and KNOLLE closely overlapped at the growing cell plate (Fig. 6A). The KNOLLE-specific fluorescence was always more enriched at the periphery of the cell plate than AtECA1:sGFP fluorescence until the cell plate had grown out to the parental plasma membrane (Fig. 6, A and B, b and c, arrows). KNOLLE disappeared rapidly from the cell plate after it had connected with the plasma membrane, whereas AtECA1:sGFP remained in the maturing cell plate (Fig. 6, A and B, e). The differences in spatial localization patterns of AtECA1:sGFP and KNOLLE in relation to the growing cell plate were more clearly visible in a three-dimensional reconstruction of the cell plate (Fig. 6C).

Next, the spatiotemporal localization of AtECA1:sGFP was compared with that of DRP1C, a dynamin-
related protein known to play a role in membrane tubulation in the forming cell plate (Hong et al., 2003; Konopka et al., 2008; Konopka and Bednarek, 2008). It has been proposed that dynamin-related proteins are involved in two different steps during cytokinesis, formation of the cell plate and endocytosis from the cell plate (Kang et al., 2003; Konopka et al., 2008; Konopka and Bednarek, 2008; Mravec et al., 2011; Ito et al., 2012). To localize DRP1C to the forming cell plate, root tissues of AtECA1:sGFP plants were immunostained with an anti-DRP1C antibody. DRP1C localized to the cell plate (Fig. 7A), as observed previously with GFP-tagged DRP1C (Hong et al., 2003; Konopka et al., 2008). As was the case with KNOLLE, DRP1C and AtECA1:sGFP differed slightly in their temporal and spatial localization patterns. In the forming cell plate, DRP1C appeared earlier than AtECA1:sGFP (Fig. 7). DRP1C began to accumulate in the young cell plate at late anaphase, when AtECA1:sGFP was barely visible (Fig. 7, A and B, a). Soon after this stage, the fluorescence intensity of AtECA1:sGFP at the growing cell plate increased (Fig. 7, A and B, b–e). At subsequent stages in the central region of the growing cell plate, the fluorescence intensity of AtECA1:sGFP remained higher than that of DRP1C until the stage just before the cell plate reached the parental plasma membrane (Fig. 7, A and B, c and d). At the end of cytokinesis, when both ends of the growing cell plate reached the parental plasma membrane, the fluorescence intensity of AtECA1:sGFP was similar to that of DRP1C and their fluorescence intensity in the central region was lower than that at both ends of the cell plate (Fig. 7, A and B, e). Overall, DRP1C and AtECA1:sGFP exhibited similar but not identical spatial distribution along the cell plate, and their temporal distribution was also slightly different. These results suggest that

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**Figure 4.** AtECA1:sGFP-positive punctate stains represent localization in EFs. Transgenic plants expressing AtECA1:sGFP were crossed with transgenic plants expressing CLC:mOrange, mCherry:RabF2a/Rha1, or mRFP:SYP43. Double-transgenic plants expressing AtECA1:sGFP together with CLC:mOrange, mCherry:RabF2a/Rha1, or mRFP:SYP43 were obtained, and localization of these proteins was examined in root cells. Root tissues of AtECA1:sGFP plants were immunostained with anti-γ-COP or anti-VSR antibodies, and the localization of these proteins was examined. GFP fluorescence of AtECA1:sGFP was observed directly. In addition, cells were stained with DAPI (shown in blue in G and J). Bars = 10 μm.
the physiological role of AtECA1 in the biogenesis of the cell plate differs from that of DRP1C.

Next, the localization of AtECA1:sGFP in the cell plate was compared with that of CLC. CCVs play a role in endocytosis from the cell plate (Otegui et al., 2001; Jürgens, 2005). It has been shown that CLC localizes to the cell plate during cytokinesis (Konopka et al., 2008; Ito et al., 2012). To compare the distribution of AtECA1 and CLC in the cell plate, transgenic lines expressing both AtECA1:sGFP and CLC:mOrange were examined at different time points by time-lapse imaging. At the 0-min time point, when the cell plate was first detected by AtECA1:sGFP fluorescence, CLC:mOrange was not observed at the cell plate, indicating that AtECA1 arrives at the cell plate before CLC. At 5 and 20 min, their spatial localization patterns in relation to the cell plate overlap precisely (Fig. 8). These results suggest that AtECA1 and CLC work together in the cell plate for a certain period of the cell plate formation.

Figure 5. AtECA1:sGFP localizes to the growing cell plate in dividing cells. A, AtECA1:sGFP localization in root tissues. Root tip was stained with DAPI (blue), and localization of AtECA1:sGFP was examined. Bar = 20 μm. B, Temporal localization pattern of AtECA1:sGFP to the cell plate. Root tissues were stained with DAPI, and localization of AtECA1:sGFP was examined at various stages of cell division. The cell division stage was determined according to the DAPI staining pattern (blue) of chromosomes. Bars = 5 μm. C, Comparison of the cell plate localization of AtECA1:sGFP with FM4-64. Root tissues were stained with FM4-64, and localization to the cell plate was compared with the localization of AtECA1:sGFP during cell plate formation. Arrows indicate the cell plate. Bars = 5 μm.

**AtECA1 Interacts with Clathrin in Vitro**

The colocalization of AtECA1 and CLC in the growing cell plate suggested that AtECA1 might be
involved in CCV formation from the cell plate. To obtain additional evidence, an in vitro protein pull-down assay was performed to demonstrate an interaction between AtECA1 and clathrin. Recombinant AtECA1 was expressed in E. coli as a glutathione S-transferase (GST) fusion protein (GST:AtECA1) and was purified using the glutathione resin (Supplemental Fig. S4). Purified GST:AtECA1 was incubated with protein extracts from plant leaf tissues. Proteins bound to GST:AtECA1 were precipitated and analyzed by immunoblot analysis using an anti-clathrin H chain (CHC) antibody (Kim et al., 2001). CHC was detected in the precipitates (Fig. 9A), indicating that AtECA1 interacts with clathrin. As a negative control for the pull-down assay, the precipitates also were analyzed for α-adaptin, a subunit of a heterotetrameric adaptor protein complex-2 for CCVs (Robinson and Bonifacino, 2001; Supplemental Fig. S3B). α-Adaptin was not detected in the precipitates (Fig. 9B), confirming that the interaction between AtECA1 and clathrin is specific.

DISCUSSION

Three A/ENTH domain proteins, AtECA1, AtECA2, and AtECA4, which are characterized in this study, are most distantly related to the two A/ENTH domain-
containing proteins AtEpsinR1 and AtEpsinR2, which were characterized previously in plant cells (Song et al., 2006; Lee et al., 2007). The function of A/ENTH domain-containing proteins has been well characterized at the biochemical level. Together with other clathrin-binding proteins, A/ENTH domain-containing proteins are involved in CCV formation by acting as adaptors of clathrin (Legendre-Guillemin et al., 2004). In a similar mechanism as epsin-related proteins in animal and plant cells (Drake et al., 2000; Kalthoff et al., 2002; Song et al., 2006; Lee et al., 2007), AtECA1 interacts with clathrin via its C-terminal domain, which is consistent with its role as an adaptor for clathrin in CCV formation. Epsin-related proteins are involved in two different trafficking pathways: endocytosis and lysosomal/vacuolar trafficking. The majority of epsin-related proteins are involved in endocytosis (Chen et al., 1998; Rosenthal et al., 1999; Drake et al., 2000; Wendland, 2002), and only a few of them are involved in lysosomal/vacuolar trafficking (Kalthoff et al., 2002; Chidambaram et al., 2004). However, the physiological role of individual epsin-related proteins may not be easily predicted based on sequence homology. Plant AtEpsinR1 and AtEpsinR2 are thought to participate in vacuolar trafficking from the TGN (Song et al., 2006; Lee et al., 2007), but they are most closely related to animal Epsin1, which is involved in endocytosis. In vivo, AtECA1 localizes to the plasma membrane in nondividing cells and also localizes to the cell plate in dividing cells. Taken together with the biochemical properties of other epsin-related proteins, this localization pattern strongly suggests that AtECA1 plays a role in CCV formation at the plasma membrane and the growing cell plate in nondividing and dividing cells, respectively.

The growing cell plate is a dynamic organelle that undergoes extensive structural changes. Initially, vesicles derived primarily from the TGN fuse to generate the hourglass-shaped intermediates that are sequentially converted to a tubulovesicular network structure, then to a tubular network, and finally to a planar fenestrated sheet (Otegui et al., 2001; Jürgens, 2005). Thus, different proteins may be involved in different stages of biogenesis of the cell plate. In fact, many vesicle-trafficking proteins have been shown to play roles in different stages of cell plate formation. For example, KNOLLE is involved in the fusion of incoming vesicles at the division plane (Lauber et al., 1997). DRP1s are involved in anchoring incoming vesicles to microtubules at the phragmoplastin, the tubulation of hourglass-shaped intermediates, and the constriction of tubes (Otegui et al., 2001; Ito et al., 2012). Clathrin is involved in endocytosis from the forming cell plate (Jürgens, 2005; Ito et al., 2012). As an approach to understand the role of AtECA1 at the cell plate during cytokinesis, the spatial and temporal localization patterns of AtECA1:GFP were compared with those of three cell plate-localized proteins, KNOLLE, DRP1C, and CLC. Our results show that spatiotemporal localization of AtECA1:sGFP most
closely matches that of CLC, at the growing cell plate in particular, which suggests that AtECA1 may function together with CLC at the growing cell plate. Previous studies reported that endocytosis occurs at the growing cell plate (Otegui et al., 2001; Jürgens, 2005; Van Damme et al., 2008; Ito et al., 2012). Furthermore, many proteins involved in CCV-mediated endocytosis have been shown to accumulate in the cell plate during cell division (Otegui et al., 2001; Chow et al., 2008). Thus, the colocalization of AtECA1 and CLC at the growing cell plate suggests that AtECA1 may be part of the same complex with clathrin and may function in endocytosis at the cell plate by acting as an adaptor of clathrin, as has been observed with other epsin-related proteins in animals and plants (Rosenthal et al., 1999; Drake et al., 2000; Song et al., 2006; Lee et al., 2007). This notion is supported by in vitro experiments demonstrating the specific interaction of the C-terminal domain of AtECA1 with clathrin.

Large amounts of protein and lipid materials are necessary to produce the cell plate. These materials are transported to the nascent cell plate from the TGN (Mayer and Jürgens, 2004; Jürgens, 2005; Dhonukshe et al., 2006; Fendrych et al., 2010). Although the role of endocytosis mediated by AtECA1 at the cell plate during cell division is not clear, it is possible that the

**Figure 8.** AtECA1:sGFP and CLC:mOrange exhibit closely overlapping spatial localization patterns on the cell plate. A comparison of cell plate localization patterns of AtECA1:sGFP and CLC:mOrange is shown. A, Localization patterns of AtECA1:sGFP and CLC:mOrange were examined at different time points in root tissues of transgenic plants stably expressing both AtECA1:sGFP and CLC:mOrange. Bars = 5 μm. B, The signal intensity of AtECA1:sGFP and CLC:mOrange was determined along the cell plates indicated by horizontal lines in A at the indicated time points. The x axis denotes the dotted lines in A. au, Arbitrary units. Green traces indicate AtECA1:sGFP and red traces indicate CLC:mOrange.

**Figure 9.** AtECA1 interacts with clathrin using its C-terminal domain. The interaction of AtECA1 with clathrin is shown. Purified recombinant GST-fused AtECA1 proteins (GST-AtECA1, GST:AtECA1N, and GST:AtECA1C) were incubated with protein extracts from leaf tissues, and proteins were precipitated by glutathione agarose beads. AtECA1N contains the first 320 amino acids, representing the A/ENTH domain, and AtECA1C contains the rest of AtECA1. The precipitated proteins were analyzed by western blotting using anti-clathrin heavy chain and anti-GST antibodies. In addition, an identical blot was stained with Coomassie blue (A). Precipitated proteins were also detected using anti-α-adaptin antibody (B). Total (5%) represents 5% of input loaded on the gel as a positive control.
mediated endocytosis has not been identified. In animal cells, monoubiquitinated or polyubiquitinated proteins are recognized by epsin-related proteins (Shih et al., 2002; Hawryluk et al., 2006). However, AtECA1 does not contain any domain that is homologous to the ubiquitin-binding domain (Holstein and Oliviusson, 2005), raising the possibility that other domains in AtECA1 are involved in the recognition of cargo proteins. In fact, the ENTH domain has the ability to interact with SNAREs in the TGN to mediate endosome transport (Chidambaram et al., 2004).

Despite the strong possibility that AtECA1 is involved in CCV formation and cargo selection for endocytosis at the cell plate, we cannot exclude the possibility that AtECA1 plays a role in other processes in the biogenesis of the cell plate. For cell plate formation, vesicles arising from the TGN fuse to produce transient hourglass-shaped structures, which are further processed to planar fenestrated sheets through tubulovesicular networks and tubular networks (Mayer and Jürgens, 2004; Jürgens, 2005). This process requires proteins that modulate membrane structures. DRP1s have been implicated in this process due to their ability to tubulate membranes (Hong et al., 2003). Since the N-ANTH domain of AtECA1 has the ability to tubulate liposomes in vitro (Silkov et al., 2011), it is possible that AtECA1 plays a role in the modulation of membrane structures at the cell plate. Undoubtedly, further studies are necessary to fully define the physiological role of A/ENTH domain-containing proteins in cell plate formation in plants.

**MATERIALS AND METHODS**

**Growth of Plants**

Arabidopsis (Arabidopsis thaliana) plants were grown in a greenhouse or culture room at 22°C under a 16/8-h light/dark cycle. The transgenic plants were screened on B5 medium containing 2% Suc and antibiotics (20 mg L⁻¹ hygromycin and 30 mg L⁻¹ kanamycin) for 3 weeks after germination and then transferred to soil in a greenhouse to harvest seeds. To prepare protoplasts, Arabidopsis ecotype Columbia (Col-0) plants were cultured on B5 medium containing 2% Suc for 3 weeks. To observe fluorescent proteins from leaf or root tissues, seedlings were vertically grown on one-half-strength medium containing 2% Suc for 3 weeks. Transgenic plants were generated by the LAS3000 image-capture system (FujiFilm).

**Expression and Purification of Recombinant Proteins in E. coli**

GST fusion proteins were purified from Escherichia coli extracts. Proteins bound to beads were incubated with protein extracts from the Col-0 seedlings in protein pull-down buffer [20 mM HEPES, pH 7.5, 125 mM KCl, 2.5 mM Mg(OAc)₂, 1 mM EDTA, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitor cocktail (Roche)]. The beads were washed three times with the same volume of protein pull-down buffer and then boiled in sample buffer (12 mM Tris·HCl, pH 6.8, 5% glycerol, 0.4% SDS, and 2.9 mM β-mercaptoethanol). Proteins were separated using 10% SDS-polyacrylamide gels and analyzed by western blotting using anti-clathrin heavy chain (Kim et al., 2001), anti-GFP (Bio-Application), or anti-GST (Calbiochem) antibodies. The blots were developed using an ECL kit (Amersham Pharmacia Biotech), and images were obtained using the LAS1000 image-capture system (FujiFilm).

**Generation of Transgenic Plants**

To generate the transgenic plant expressing AIECA1::sGFP, the AIECA1::sGFP fragment was ligated to a binary vector, pBin121, with the CaMV 35S promoter, and the other AIECA1::sGFP fragments were ligated to the pCaVS1300 vector with the CaMV promoter. To express AIECA1::sGFP using the native promoter, the 2 kb promoter fragment (AIECA1p) was amplified by PCR using specific primers ECA1-promotor-F and ECA1-promotor-R, and this was used to replace the 35S promoter in the binary vector, pBin121. Subsequently, AIECA1::sGFP was ligated to the binary vector with AIECA1p to generate pBI-AIECA1p-AIECA1::sGFP. Transgenic plants were generated by the floral dipping method (Clough and Bent, 1998). Expression of the transgenes was examined by western-blot analysis using anti-GFP antibody (Clontech).

**In Vivo Imaging of Transiently or Stably Expressed AtECA1::sGFP**

For transient expression analysis, plasmid DNAs were purified using a DNA purification column and transformed into protoplasts derived from 3-week-old Arabidopsis leaf tissues (Col-0; Kim et al., 2005; Hyunjong et al., 2006). The primary roots and cotyledons of 6-d-old seedlings were used to perform in vivo imaging and immunohistochimistry as described previously (Park et al., 2005). To stain the cell plate with FM4-64, plant root tissues were treated with 12 μM FM4-64 for 1 min. The immunofluorescence samples were prepared as described previously (Lauber et al., 1997). The primary antibodies were anti-AIECA1 (1:500), anti-KNOLLE (1:500), anti-DRP1C (1:1000), anti-VDAC (1:1000), and anti-γ-COP antibodies. Antibody dilutions were 1/100 volume of preequilibrated glutathione agarose beads. The beads were washed three times with 10 μM DAPI for 5 min prior to mounting on a slide with Mowiol4-88 (Calbiochem). Images were captured using a Zeiss LSM 5 Meta laser scanning confocal microscope and processed using the LSM 5 image browser (Zeiss). The combinations of excitation wavelength/emission filter were 488 nm (argon ion laser)/505 to 530 bandpass for GFP and 543 nm/560 to 615 bandpass for mRFP, mOrange, and FM4-64 for mesophyll protoplasts and root tissues. In addition, images were obtained using a cooled CCD camera and a Zeiss Axioplan fluorescence microscope. The filter sets used were XFI16 (exciter, 474AF20; dichroic, 500DRLP; emitter, 510AF23) and XFI3/E (exciter, 535DF35; dichroic, 570DRLP; emitter, 605DF50 [Omega]) for GFP, fluorescein isothiocyanate and RFP/tetramethyl rhodamine isothiocyanate, respectively. The data were processed using Adobe Photoshop software, and the images were rendered in pseudocolor.

**Protein Pull Down and Western-Blot Analysis**

GST fusion proteins were purified from Escherichia coli extracts. Proteins bound to beads were incubated with protein extracts from the Col-0 seedlings in protein pull-down buffer [20 mM HEPES, pH 7.5, 125 mM KCl, 2.5 mM Mg(OAc)₂, 1 mM EDTA, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitor cocktail (Roche)]. The beads were washed three times with the same volume of protein pull-down buffer and then boiled in sample buffer (12 mM Tris·HCl, pH 6.8, 5% glycerol, 0.4% SDS, and 2.9 mM β-mercaptoethanol). Proteins were separated using 10% SDS-polyacrylamide gels and analyzed by western blotting using anti-clathrin heavy chain (Kim et al., 2001), anti-GFP (Bio-Application), or anti-GST (Calbiochem) antibodies. The blots were developed using an ECL kit (Amersham Pharmacia Biotech), and images were obtained using the LAS1000 image-capture system (FujiFilm).
buffered saline buffer. Proteins were eluted with 15 mM reduced glutathione (20 mM Tris-HCl, pH 8.0) and quantified by Bradford assay.

Generation of Antibodies

The construct MBpa-adaptinC was described previously (Lee et al., 2007). To construct Hisx6:AIECA1, the full-length cDNA of AIECA1 was ligated to the E. coli expression vector pSETC. MBpa-adaptinC and Hisx6:AIECA1 recombinant proteins were affinity purified and used to immunize rabbits and rats, respectively. To confirm the specificity of anti-a-adaptin antibodies, western-blot analyses were performed with the anti-a-adaptin antibody using protein extracts from transformants transformed with Hisx6-a-adaptin (Lee et al. 2007). To confirm the specificity of the anti-AIECA1 antibody, protein extracts from transgenic plants of AIECA1:sGFP and atc1a mutant plants were used in western-blot analyses with anti-GFP (1:1,000 dilution; Clontech), anti-actin (1:1,000 dilution; MP Biomedicals) antibodies.

Sequence data from this article can be found in the Arabidopsis Genome Initiative databases under the following accession numbers: AIECA1 (At2g16000), ATECA2 (At1g03050), AIECA4 (At2g25430), KNOLE (At1g08560), DRPIC (At1g14830), CEC (At2g40060), α-adaptin (At5g22770), SYP43 (At3g05710), RabF2a (At1g39640), γ-COP (At5g34450), AVS1 (At5g52850), and H+-ATPase (At1g30190).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Phylogenetic tree of A/ENTH domain-containing proteins.

Supplemental Figure S2. Anti-AIECA1 antibody generation and specificity determination.

Supplemental Figure S3. Anti-alpha-adaptin antibody generation and specificity determination.

Supplemental Figure S4. Expression of AIECA1 recombinant proteins.

Supplemental Table S1. Sequence of primers used in this study.

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LITERATURE CITED


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