The Arabidopsis Cell Plate-Associated Dynamin-Like Protein, ADL1Ap, Is Required for Multiple Stages of Plant Growth and Development

Byung-Ho Kang, James S. Busse, Carrie Dickey, David M. Rancour, and Sebastian Y. Bednarek*

Department of Biochemistry, University of Wisconsin, 433 Babcock Drive, Madison, Wisconsin 53706

Dynamin and dynamin-like proteins are GTP-binding proteins involved in vesicle trafficking. In soybean, a 68-kD dynamin-like protein called phragmoplastin has been shown to be associated with the cell plate in dividing cells (Gu and Verma, 1996). Five ADL genes encoding dynamin-like proteins related to phragmoplastin have been identified in the completed Arabidopsis genome. Here we report that ADL1Ap is associated with punctate subcellular structures and with the cell plate in dividing cells. To assess the function of ADL1Ap we utilized a reverse genetic approach to isolate three separate Arabidopsis mutant lines containing T-DNA insertions in ADL1A. Homozygous adl1A seeds were shriveled and mutant seedlings arrested soon after germination, producing only two leaf primordia and severely stunted roots. Immunoblotting revealed that ADL1Ap expression was not detectable in the mutants. Despite the loss of ADL1Ap, the mutants did not display any defects in cytokinesis, and growth of the mutant seedlings could be rescued in tissue culture by the addition of sucrose. Although these sucrose-rescued plants displayed normal vegetative growth and flowered, they set very few seeds. Thus, ADL1Ap is critical for several stages of plant development, including embryogenesis, seedling development, and reproduction. We discuss the putative role of ADL1Ap in vesicular trafficking, cytokinesis, and other aspects of plant growth.

Dynamin and dynamin-related proteins are a family of structurally related, but functionally diverse high Mr GTP-binding proteins (van der Bliek, 1999; McNiven et al., 2000a). All members of this family contain a conserved amino-terminal GTP-binding (GTase) domain and a carboxyl-terminal assembly/GTPase effector domain (GED). The dynamin family is further subdivided on the basis of other structural features. The brain-specific dynamin I, the defining member of this protein family, contains in addition to the conserved GTase and GED domains, pleckstrin-homology (PH) and C-terminal Pro-rich domains. Dynamin I and other dynamin isoforms, including the Drosophila melanogaster shibire gene product, have been demonstrated to function in clathrin-dependent endocytosis, trans-Golgi network (TGN) vesicle budding (Jones et al., 1998), and in the internalization of caveloae (Henley et al., 1998; Oh et al., 1998). Considerable biochemical, genetic, and morphological evidence (van der Bliek and Meyerowitz, 1991; Hinchshaw and Schmid, 1995; Takei et al., 1995) suggests that dynamin oligomerizes into multimeric rings around the neck of invaginating clathrin-coated vesicles and functions as a mechanoenzyme, releasing the nascent vesicles from the plasma membrane upon GTP hydrolysis. Dynamin is also thought to function as a regulatory molecule (Sever et al., 1999), recruiting various binding partners, including other components of the endocytic machinery, lipids, signaling molecules, and cytoskeletal proteins through its many domains (McNiven et al., 2000a). In addition to its role in vesicular trafficking, dynamin function has also been linked to actin cytoskeleton dynamics (McNiven et al., 2000b; Ochoa et al., 2000) and signal transduction (Fish et al., 2000).

Dynamin-like proteins, which generally lack the PH and Pro-rich domains found in dynamin, have been identified in a variety of organisms, including yeast, mammals, and plants. Many of these proteins also carry out processes related to membrane dynamics and vesicular trafficking. The yeast dynamin-like protein Vps1p functions in vesicular trafficking between the TGN and endosomes. However, unlike dynamin, it does not play a role in endocytosis (Rothman et al., 1990; Wilsbach and Payne, 1993; Nothwehr et al., 1995). Another yeast dynamin-related protein, Dnm1p, controls mitochondrial morphology by regulating the fission of outer mitochondrial membrane tubules (Bleazard et al., 1999; Sesaki and Jensen, 1999).

In plants, three dynamin-related protein subfamilies have been identified. One family consists of the soybean and Arabidopsis 68-kD dynamin-like pro-
teins, phragmoplastin and ADL1p, respectively, and another is defined by the 84-kD dynamin-like protein, ADL2p. ADL2p contains a chloroplast transit sequence and is localized to plastids (Kang et al., 1998), suggesting that ADL2p may play a role in chloroplast/plastid biogenesis and maintenance. A third subfamily consists of the ADL3 gene product. Similar to the prototypical dynamins, ADL3 encodes a large 100-kD GTPase-containing a PH domain (Mikami et al., 2000); however, its function has not been defined.

Dynamin-related proteins are thought to play a major role in plant cell division since the construction of the cytokinetic organelle, known as the cell plate, is highly dependent upon the formation, targeting, and fusion of secretory vesicles carrying membrane and cell wall material (for review, see (Staehelin and Hepler, 1996; Verma and Gu, 1996). Consistent with this idea is the recent observation that phragmoplastin is associated with the cell plate in dividing soybean and tobacco cells (Gu and Verma, 1996, 1997). There is, however, conflicting data regarding the localization of the ADL1 protein. One group (Lauber et al., 1997) localized ADL1p to the cell plate in embryonic cells, whereas another (Park et al., 1998) has suggested that ADL1p is associated with chloroplasts and is required for thylakoid membrane biogenesis. It interesting that although these two studies reached different conclusions, they both used the same ADL1p antibody preparation generated against the highly conserved dynamin GTPase domain consensus sequence of ADL1 (Park et al., 1997). However, this GTPase domain has been identified in several other genes in the Arabidopsis genome (see Fig. 1A) that are predicted to encode 68-kD dynamin-like proteins. This calls into question the specificity of the antibodies used in these studies.

In this study, we have re-examined the localization of ADL1Ap, one member of the ADL1 protein family encoded by aG68/ADL1 (Dombrowski and Raikhel, 1995; Park et al., 1997), and addressed its function in Arabidopsis using a combination of antibodies specific for the GTPase domain or an ADL1Ap-specific peptide. By subcellular fractionation and immunolocalization using the ADL1Ap-specific antibody we have established that ADL1Ap is targeted to the cell plate during cytokinesis and is not associated with chloroplasts. To understand the function of ADL1Ap we have taken a reverse genetic approach. Here we report on the initial characterization of Arabidopsis T-DNA insertion mutants in which the expression of ADL1A has been disrupted. Phenotypic analysis of these loss-of-function mutant plants suggests that other members of the ADL1 gene family have functions that partially compensate for the loss of ADL1Ap function. However, based upon our results, the ADL1A gene product must play a critical role in embryogenesis, seedling development, and reproduction.

RESULTS

The Arabidopsis 68-kD Dynamin-Like Protein, ADL1 Gene Family

Using the cDNA aG68/ADL1 (Dombrowski and Raikhel, 1995; Park et al., 1997) sequence we searched the Arabidopsis genome database and identified a gene family comprised of five highly related members, including the genomic copy of aG68/ADL1. The deduced amino acid sequences of these five gene products (named sequentially ADL1A through ADL1E; Fig. 1A), share significant (approximately 65%–84%) amino acid sequence identity to the soybean 68-kD dynamin-like protein, phragmoplastin (Gu and Verma, 1996). The genomic sequence corresponding to aG68/ADL1, which we have designated ADL1A, was found on the P1 clone, MJC20 (GenBank accession no. AB017067) (Kaneko et al., 1999), assigned to chromosome V (approximately 90 cM).

Characterization of an ADL1Ap-Specific Antibody

Previous ADL1p localization studies have relied on antisera generated against the highly conserved dynamin GTPase domain encoded by the aG68/ADL1 cDNA (Lauber et al., 1997; Park et al., 1997). Because of the high degree of sequence identity shared between the GTPase domains of each of the Arabidopsis approximately 68-kD dynamin-like protein family members (Fig. 1A), these antibodies are likely to cross-react with all ADL1p family members, calling into question previous localization studies. As is shown in Figure 2A, affinity-purified anti-ADL1p GTPase domain-specific antisera generated against amino acid residues 85 through 253 of aG68/ADL1 (Fig. 1A) recognized two distinct polypeptides, 68 and 70 kD, in protein extracts prepared from Arabidopsis suspension-cultured cells (Fig. 2A, lane 1) and seedlings (Fig. 2A, lanes 1 and 2). To determine which of these two polypeptides corresponds to ADL1Ap we generated ADL1Ap-specific antibodies against the peptide DVEKGNPTHSIFDRC (amino acids 498–512), indicated with stars (Fig. 1A). This peptide was chosen as an antigen because it corresponds to a highly variable, hydrophilic amino acid segment that connects the GTPase and effector domains of each of the five predicted ADL1p family members (Fig. 1A). Rabbits were immunized with the peptide coupled to keyhole limpet hemocyanin and affinity purified against an Escherichia coli-expressed GST-ADL1Ap (amino acids 232–523). As shown in Figures 2A and 7A, these antibodies are specific for ADL1Ap. On immunoblots of total protein extracts from suspension-cultured cells and seedlings, the affinity-purified ADL1Ap peptide antibodies detected a single protein of approximately 68 kD. The specificity of the ADL1Ap-specific antibodies and the identity of the 68-kD polypeptide as ADL1Ap were confirmed by demonstrating that homozygous ad1A
Figure 1. Arabidopsis 68-kD dynamin-like protein open reading frames. A, Alignment of the deduced amino acid sequences of the protein encoded by ADL1A, ADL1Ap (GenBank accession no. G2129608), and four other Arabidopsis approximately 68-kD dynamin-like protein open reading frames (GenBank accession nos. G6850867 [B], G8778229 [C], G3341679 [D], and G7076772 [E]). The alignment was generated using the Multiple Alignment Program (Smith et al., 1996). Identical and conserved amino acid residues are outlined in black and gray, respectively. Dashes depict gaps included to maximize sequence similarity. The 15-amino acid non-conserved hydrophilic region of ADL1Ap that was used to generate ADL1Ap-specific antibodies is overlined with asterisks. The 168-amino acid segment used to generate the general anti-ADL1p GTPase domain-specific antibody is overlined with bold dashes. The arrowheads indicate the positions at which the adl1A mutant proteins would likely be truncated. B, Schematic representation of ADL1A gene disruptions. The exon/intron structure of ADL1A is shown to scale, with black boxes representing exons. The positions of the translation initiation and termination codons are signified by ATG and TGA, respectively. The positions and orientations of T-DNA inserts with left border sequences (T₀; not drawn to scale) are indicated. Kan, T-DNA neomycin phosphotransferase selectable gene marker.
mutant seedlings (see below) lack the 68-kD protein (Fig. 5A, lanes 3–5). We have similarly confirmed, using protein extracts prepared from homozygous adl1E mutant seedlings, that the 70-kD polypeptide detected with ADL1p GTPase domain-specific antibodies (Fig. 2, lane 1) is encoded by ADL1E (B.H. Kang and S.Y. Bednarek, unpublished data), another member of the ADL1 gene family.

**Subcellular Fractionation**

To investigate the intracellular distribution of ADL1Ap, Arabidopsis protoplasts were gently disrupted by mechanical shearing and microsomal membranes and membrane-free cytosol were prepared. The relative amounts of ADL1Ap, as well as marker proteins for the cytosol and various subcellular compartments, were determined by immunoblot analysis and scanning densitometry. As shown in Figure 2B, the majority of ADL1Ap was associated with the microsomal membrane pellet generated by centrifugation of a post-nuclear supernatant at 150,000g (P150). Under the fractionation and detection conditions used we did not observe ADL1Ap in the 150,000g soluble fraction (S150), even with long immunoblot exposure times. At least 80% of the ADL1Ap was membrane-associated as determined by Suc gradient flotation fractionation and immunoblot/densitometric analysis of an Arabidopsis suspension-cultured P150 fraction (data not shown). ADL1Ap remained tightly membrane-associated even after treatments with 3 M urea or 1% (v/v) Triton X-100, conditions generally sufficient to solubilize most peripheral and integral membrane pro-
teins, respectively. Similar observations have been reported for the soybean cell plate-associated dynamin-like protein phragmoplastin (Gu and Verma, 1996). Treatment with 8 M urea was required to release ADL1Ap from the membrane fraction (data not shown), indicating that ADL1Ap is a tightly-associated peripheral membrane protein.

We also examined the association of ADL1Ap with chloroplasts since ADL1p has been suggested previously to function in thylakoid membrane biogenesis (Park et al., 1998). To determine if ADL1Ap is targeted to chloroplasts we prepared intact chloroplasts from bright-green Arabidopsis suspension-cultured protoplasts by Percoll gradient centrifugation. The two isoforms of phosphoglycerokinase, a 42-kD cytosolic form and a 43-kD mature chloroplast form, were used as marker proteins. Based upon the distribution of chloroplast phosphoglycerokinase (PGK), the isolated chloroplast fraction is significantly enriched (approximately 25-fold) for the PGK marker, whereas ADL1Ap was depleted (5- to 10-fold) from the chloroplast fraction when compared with the crude homogenate (Fig. 2B). The highly abundant membrane marker proteins AtSec12p (endoplasmic reticulum [ER]), AHA2 (plasma membrane), and Knolle (cell plate) were similarly depleted (5- to 10-fold) from the chloroplast fraction relative to the crude homogenate. We did not observe any significant association of other ADL1 gene family members with the chloroplast in extracts probed with anti-ADL1p GTPase domain antibodies (data not shown). These results, together with the fact that the deduced amino acid sequences of the members of the ADL1 gene family (Fig. 1A) lack the necessary N-terminal transit peptide (Emanuelsson et al., 1999), required for entry into the chloroplast, are inconsistent with the proposal that ADL1Ap or other ADL1p family members function in thylakoid biogenesis (Park et al., 1998).

**Immunolocalization Revisited**

We used immunofluorescence microscopy to further assess the subcellular localization of ADL1Ap. In brief, protoplasts from actively dividing Arabidopsis suspension-cultured cells were fixed and permeabilized under conditions that have previously been shown to preserve the plant cytoskeleton, including the phragmoplast (Goodbody and Lloyd, 1994), and were processed for indirect double-immunolabeling. In cells undergoing cytokinesis (Fig. 3, columns A–D), we observed a strong band of anti-ADL1Ap immunostaining across the expected plane of division. This band of immunofluorescence corresponded precisely to the middle of the phragmoplast, as determined by double-immunolabeling with anti-β-tubulin (Fig. 3, B and D), suggesting that ADL1Ap is targeted to the cell plate in dividing plant cells. No labeling of the cell plate or other intracellular membranes was observed in cells treated with anti-ADL1Ap preimmune sera (Fig. 3, columns E–H). In interphase cells (Fig. 3, columns I–L) and in dividing cells we also observed significant anti-ADL1Ap immunolabeling of punctate structures. These cytosolic structures did not correspond to chloroplasts (data not shown). Similar vesicular structures have been visualized previously using green fluorescence protein-tagged Golgi-resident proteins (Boeving et al., 1998; Nebeinfs et al., 1999). However, because no Golgi-resident marker protein antibodies suitable for indirect immunofluorescence microscopy are available, we could not confirm that the ADL1Ap-positive subcellular structures corresponded to Golgi stacks in these cells.

**Isolation of adl1A T-DNA Insertion Mutants**

ADL1Ap is highly concentrated at the cell plate in dividing Arabidopsis cells (Fig. 3). Therefore, we hypothesized that ADL1Ap function is required for cell plate development. To better understand the cellular role(s) of ADL1Ap, we sought to identify Arabidopsis mutants in which the expression of ADL1Ap was eliminated. Using a PCR-based approach (McKinney et al., 1995; Krysan et al., 1996, 1999) we identified three independent T-DNA-tagged adl1A alleles (Fig. 1B) that displayed similar embryogenesis and seedling development defects (see below). The first T-DNA insertion mutant, adl1A-1, was identified in a pool of 14,000 T-DNA-transformed Arabidopsis lines, as described in Krysan et al. (1996). We were unable to complement the defects associated with the adl1A-1 line using a genomic copy of ADL1A, suggesting that this line may contain an additional T-DNA-independent mutation(s), as has previously been reported for other T-DNA-tagged insertion lines (Krysan et al., 1999). To ensure that the mutant phenotypes we observed were specific to ADL1A, we isolated two additional alleles, adl1A-2 and adl1A-3, through the T-DNA lines available through the University of Wisconsin Biotechnology Center Arabidopsis Knockout facility (Krysan et al., 1999) that were “rescued” using a genomic copy of ADL1A as described below.

The T-DNA insertion site in each of the adl1A alleles was determined by DNA sequence analysis. Comparison of the cDNA and the genomic sequences shows that ADL1A is composed of 16 exons (Fig. 1B). The T-DNAs in adl1A-1 and adl1A-3 were inserted in the 13th intron and 14th exons, respectively, of ADL1A, which are upstream of the sequences encoding the critical dynamin GED of ADL1Ap. In the case of adl1A-2, the T-DNA was integrated within the first exon of ADL1A and is, therefore, most likely a null mutation. All three original adl1A lines were heterozygous for the T-DNA insertion and approximately 25% of the seeds from self-fertilized heterozygous plants were shriveled.
Figure 3. Immunolocalization of ADL1Ap in Arabidopsis suspension-cultured cells. Protoplasts from a 3-d culture of Arabidopsis cells were double immunolabeled with antibodies directed against β-tubulin (β-Tub) to visualize cortical and phragmoplast microtubules (B, F, and J) and either affinity-purified ADL1Ap-specific (C and K) or preimmune sera (G). Localization of the nuclear material was revealed by staining with 4',6′-diamidino-2-phenylindole (DAPI; A, E, and I). Electronically merged images of cells in cytokinesis (A–C) and (E–G) as demonstrated by the presence of two nuclei and interphase (I–K) are shown in D, H, and L, respectively. Bar = 50 μm, P, Phragmoplast; CP, cell plate. Arrows indicate ADL1Ap-positive subcellular structures.
Phenotypic Analysis of adl1A Mutants

To examine the growth and development of adl1A seedlings, wild-type and shriveled seeds from heterozygous adl1A plants were grown on germination media containing kanamycin (kan). Seedlings derived from each of the three independent heterozygous adl1A T-DNA-tagged lines fell into three phenotypic classes: wild-type kan-sensitive (kan\(^{-}\)), wild-type kan-resistant (kan\(^{+}\)), and mutant kan\(^{-}\) plants that segregated in an approximately 1:2:1 ratio. The mutant class of seedlings arose from the shriveled seeds and all three adl1A alleles displayed identical phenotypes. As shown in Figures 4A and 5, growth of mutant adl1A-2 seedlings has arrested after 5 d. PCR-based genotype analysis confirmed that the arrested seedlings from adl1A-2 (Fig. 4B), adl1A-3, and adl1A-1 (data not shown) were homozygous for the T-DNA insertion in ADL1A, indicating the mutant phenotype was associated with the insertion.

ADL1Ap Expression Is Abolished in adl1A Seedlings

Deletion of the N-terminal GTPase domain of the yeast dynamin-like protein Vps1p interferes with

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**Figure 4.** Genotype analysis of adl1A-2 seedlings. A, Five-day old wild-type kan\(^{+}\) (1), wild-type kan\(^{-}\) (2), and mutant seedlings (3) grown on germination media in continuous light (bar = 5.0 mm). B, Total DNA was prepared from individual 5-d-old seedlings and was analyzed by PCR using a mixture of three primers specific to ADL1A and the left T-DNA border (\(T_{L}\); see “Materials and Methods”). PCR amplification of DNA from plants homozygous for the wild-type ADL1A gene yielded only a single approximately 0.5-kb product (lane 2), whereas genomic DNA from the heterozygous adl1A::T-DNA-tagged plant (lane 1) yielded the wild-type 0.5-kb product and the approximately 0.3-kb T-DNA-tagged product. Homozygous adl1A-2 seedlings (lane 3) yielded only a single approximately 0.3-kb PCR product.
vacuolar protein sorting in a dominant-negative manner, whereas mutant forms of Vps1p lacking the carboxyl-terminal one-half of the protein and thus lacking the GED domain are recessive (Vater et al., 1992). By analogy we anticipated that the T-DNA element insertions in the three recessive adl1A alleles (Fig. 1B) would block the expression of ADL1Ap or result in the production of truncated mutant proteins lacking the critical carboxyl-terminal GED. To analyze the expression of ADL1Ap in the mutant seedlings, total protein was isolated from 5-d-old wild-type and adl1A seedlings and was analyzed by immunoblotting with antibodies directed against the GTPase domain or against the ADL1Ap-specific peptide. As shown in Figure 6A, the 68-kD polypeptide corresponding to ADL1Ap was not detected in the growth-arrested mutant seedlings, confirming the specificity of the ADL1Ap-specific antisera used for the subcellular fractionation and immunolocalization studies described above. The 70-kD dynamin-related protein recognized by the anti-ADL1p GTPase domain-specific antibody was still present in the mutant seedlings. The presence of the 70-kD dynamin-related protein and the loading control, AtCdc48p, in the adl1A seedling protein extracts demonstrates that the loss of ADL1Ap in the mutants is not simply a general consequence of the growth arrest phenotype. Reverse transcriptase- (RT) PCR analysis confirmed that there was no detectable expression of ADL1A mRNA expression in any of the three homozygous adl1A T-DNA-tagged mutant lines (data not shown). These results indicate that the T-DNA insertions in adl1A-1, adl1A-2, and adl1A-3 lines affect the proper transcription and/or stability of ADL1A mRNA and are most likely “loss-of-function” (i.e. null mutations).

Developmental Arrest of adl1A Seedlings

To more thoroughly characterize the stage at which adl1A seedlings arrest we examined the apical shoot and root meristem structure by scanning electron microscopy (SEM) and by brightfield and transmission electron microscopy (TEM). Mutant seedlings were similar to wild type in that they underwent typical photomorphogenesis with greening (Fig. 4A), hypocotyl and cotyledon expansion, as well hypocotyl hook unfolding (Fig. 5, A and B). However, seedling development in adl1A homozygous seedlings arrested after 5 d and elaboration of the plant body ceased. Root growth ceased in the mutant seedlings, although the dermal, ground, and vascular tissue differentiation appeared similar to wild type when examined with brightfield and TEM (data not shown). At the shoot apex, wild-type seedlings typically have initiated three leaf primordia by 5 d (Fig. 5C). In contrast, no more than two leaf primordia were initiated in homozygous adl1A seedlings (Fig. 5D).

The developmental arrest of the shoot cannot be attributed to a disrupted apical organization, since we found that in the mutant, as in the wild type, the shoot apical meristem has a typical tunica corpus organization (Fig. 7, A and B). In ADL1A and adl1A seedlings, two layers of tunica with anticlinal walls (L1 and L2) were found above the corpus. Further-
more, the organization and development of the leaf primordia was the same in *adl1A* as in the wild-type *ADL1A* with the exception that we did not observe trichome initiation in the mutants. Procambia for the midveins of the first two rosette leaf primordia was found in continuity with the vascular system, and no abnormalities were noted in the ground meristem or protoderm of the primordia when viewed with brightfield microscopy or TEM. Likewise, no difference could be found in the apical organization or

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**Figure 6.** Embryonic and seedling phenotype of *adl1A* mutants. Scanning electron micrographs of Arabidopsis 3-d-old wild-type seedling (A) and 5-d-old *adl1A-2/adl1A-2* seedlings (B). C, Wild-type shoot apex with three visible leaf primordia, labeled 1 through 3, above the cotyledons; D, *adl1A-2/adl1A-2* shoot apex; one cotyledon has been removed to visualize the apex. Two rosette leaf primordia, labeled 1 and 2, are visible on the flanks of the mutant shoot apical meristem. E, Wild-type mature embryo curved such that the hypocotyl-root-axis is parallel to the two cotyledons; F, *adl1A-2/adl1A-2* mature embryo with two cotyledons wrapped around the hypocotyl. A twist occurs in the lower portion of the mutant hypocotyl. A and B, Bars = 1 mm. C through F, Bars = 50 μm. C, Cotyledon; H, hypocotyl; R, radicle.
tissue differentiation of the wild-type and \textit{adl1A} mutant roots (data not shown).

Embryonic hypocotyl and cotyledon cells of \textit{ADL1A} and \textit{adl1A} are nearly completely packed with lipid and storage protein bodies when viewed with TEM. After 5 d these storage reserves have been nearly completely mobilized in wild-type and homozygous \textit{adl1A} seedlings (data not shown). TEM analysis of cotyledon chloroplasts also revealed no differences in the biogenesis of \textit{adl1A} thylakoid membranes (Fig. 7, C and D). Grana and stroma lamellae appeared morphologically normal. Accumulation of starch grains in mutant and wild type were similar. From this analysis we can conclude that the developmental arrest of the shoot apex cannot be attributed to a disrupted apical organization, storage reserve mobilization, or abnormal chloroplast biogenesis.

\textbf{ADL1A Is Involved in Embryo Development}

As described above, dry seeds containing \textit{adl1A} homozygous mutant embryos are shriveled, suggesting that ADL1Ap functions not only during seedling development, but in embryogenesis as well. Mutant seeds were generally shorter and narrower than their wild type counterparts; (wild type, 461 ± 6.5 × 263 ± 3.2 \(\mu\)m; \textit{adl1A}-1, 380 ± 8.7 × 223 ± 8.7 \(\mu\)m; \textit{adl1A}-2, 420 ± 14.8 × 273 ± 13.8 \(\mu\)m; and \textit{adl1A}-3; 420 ± 12.4 × 252 ± 4.7 \(\mu\)m). To determine at what stage in seed development homozygous \textit{adl1A} mutants were affected, immature siliques from self-fertilized heterozygous plants were split open and examined under a dissecting microscope. The number of seeds in heterozygous \textit{adl1A} siliques was identical to wild type, and mutant developing seeds were indistin-

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\caption{Shoot apical meristem structure of \textit{adl1A} mutants. Light micrographs of median longitudinal sections through shoot apices and first two rosette leaf primordia of wild-type (A) and mutant \textit{adl1A}-2/\textit{adl1A}-2 (B) seedlings. The tunica corpus apical organization of the mutant (B) is the same as that of the wild-type (A). Transmission electron micrographs of chloroplasts from upper palisade tissue of wild-type (C) and \textit{adl1A}-2/\textit{adl1A}-2 (D) cotyledons. A and B, Bars = 25 \(\mu\)m. C and D, Bars = 0.5 \(\mu\)m.}
\end{figure}
guishable from wild-type siblings at very early stages of development. During the organ expansion and maturation stage of embryo development (Goldberg et al., 1994), the mutant seeds became readily apparent; approximately 25% of the developing seeds in a silique from a heterozygous adl1A plant were pale green relative to wild type (Fig. 8A). The mutant seeds were pale because development of the mutant embryos was delayed with respect to wild type. To determine if the delay in mutant embryo development was due to a defect in cytokinesis we analyzed, by light microscopy, developing wild-type and mutant seeds that contained numerous actively dividing cells. No multinucleated cells were observed and all cell walls in the developing embryo and endosperm appeared normal (data not shown), indicating that proper cell division occurred in the adl1A mutant seeds. In addition, no defects in chloroplast/thylakoid development, which is initiated at or about the heart stage of embryogenesis (Mansfield and Briarty, 1991), were observed in the mutant embryos by TEM (data not shown). PCR analysis of embryos isolated from the pale-green seeds confirmed that the embryos were homozygous for the adl1A-1::T-DNA mutation (Fig. 8B). Consistent with its role in late embryo development we have shown by RT-PCR that ADL1A mRNA is expressed during late embryogenesis in wild-type embryos (Fig. 8C).

Wild-type embryos dissected from the seed are invariably curved with the hypocotyl-root axis parallel to the two cotyledons (Fig. 5E). Although smaller than wild type, homozygous adl1A embryos have a hypocotyl-root axis and two cotyledons (Fig. 5F). The majority (>50%) of the dissected adl1A embryos have a twist in the hypocotyl-root axis and/or the cotyledons, imparting a kink to the appearance of the mutant embryos. It is unclear whether the twisting of the mutant embryos occurs during development or upon seed desiccation.

The yeast dynamin-like protein Vps1p (Rothman et al., 1990) is required for vacuole protein targeting. Therefore, we wanted to know whether the defects in embryogenesis and seedling development we observed in the adl1A mutants were due to a defect in storage protein deposition, a process that is intimately dependent on secretory protein trafficking (Matsuoka and Bednarek, 1998). The structural organization of the mature Arabidopsis embryo, as well as the distribution of storage products, has been described extensively elsewhere (Mansfield and Briarty, 1992; Busse and Evert, 1999). Examination of the adl1A embryos by TEM revealed no differences in the structural organization or storage product distribution between mature wild-type embryos and homozygous adl1A embryos. Deposition of the 12S stor-
age protein, cruciferin, was also analyzed by SDS-PAGE and Coomassie staining, as described (Heath et al., 1986). The cruciferin subunit polypeptide profile of the mutants was identical to that of wild type. These results suggest that ADL1Ap does not function directly in storage product formation or deposition during embryogenesis.

Suc and Glc Stimulate Growth and Development of Arrested adl1A Seedlings

When 5-d-old adl1A seedlings were transferred to soil, none of the plants survived. However, the mutant seedlings remained viable for several weeks when cultured on germination media in continuous light, as indicated by their dark-green appearance and the lack of necrotic tissue. After 21 d we observed that 1% to 2% of 200 homozygous adl1A seedlings showed a slight degree of additional growth and had larger cotyledons and longer roots (approximately 0.2 cm). When these seedlings were transferred to soil they slowly continued to grow and eventually flowered.

Recovery of homozygous mutant plants in tissue culture was greatly facilitated by transferring growth-arrested homozygous adl1A-2 and adl1A-3 seedlings to media containing 88 mM Suc or Glc. After 10 d on sugar-containing germination media, all of the homozygous mutant seedlings had developed five to six rosette leaves and 2- to 3-cm roots. When transferred to soil these “rescued” plants grew and flowered (Fig. 9B). In contrast, growth of 5-d-old arrested adl1A-2 and adl1A-3 seedlings did not resume on media containing 88 mM sorbitol, indicating that the resumption of seedling development was dependent on the presence of metabolizable sugars and was not related to osmotic stress. Growth of arrested adl1A-2 and adl1A-3 seedlings could not be restored on germination media containing cytokinin (1.0 mg L⁻¹ 6-benzylaminopurine) and auxin (0.1 mg L⁻¹ 1-naphthaleneacetic acid) in the absence of sugar.

Analysis of Protein Expression in Rescued adl1A Plants

The conditional seeder arrest phenotype of adl1A plants prompted us to examine whether the expression of other ADL1 gene family members is induced in the “Suc-rescued” mutant plants. Arabidopsis cells have the potential of expressing five ADL1p isoforms (Fig. 1A) that are predicted to range in size between 68 to 70 kD. The induction of expression of one or more of these other isoforms might compensate for the loss of the ADL1Ap in the mutant seedlings and permit normal development. To examine this possibility we prepared total protein extracts from the leaves of homozygous adl1A plants and analyzed them by immunoblotting with the GTPase domain-specific antisera, which is expected to cross-react with all ADL1 protein family members (Fig. 1A). Similar to the adl1A seedlings (Fig. 6A), the leaves of rescued adl1A plants continue to express the 70-kD ADL1p (Fig. 6B). However, we were unable to detect the expression of ADL1Ap or other members of the approximately 68-kD ADL1 protein family in expanding leaves of homozygous adl1A-2 plants (Fig. 6B) and in 10-d-old “Suc-rescued” seedlings that were able to survive on soil (data not shown). Thus, the 70-kD ADL1 protein may be sufficient under certain growth conditions (i.e. in the presence of 88 mM Suc) to maintain growth and development of adl1A seedlings until they have established photosynthetically active leaves.

ADL1Ap Function Is Required for Reproduction

Flowers from homozygous adl1A-2 and adl1A-3 Suc-rescued plants are morphologically normal. However, self-pollinated homozygous adl1A-2 and adl1A-3 plants displayed severely reduced fertility, resulting in abnormally short siliques. Fully expanded siliques from homozygous adl1A-2 plants were characteristic of much shorter than wild type (5.2 ± 0.2 mm versus 18.3 ± 0.4 mm; Fig. 9D) and contained only one or two developing seeds. These seeds when mature were shriveled and gave rise to the characteristic adl1A arrested seedlings when grown in the absence of sugar.

Given that heterozygous adl1A mutants exhibited Mendelian, approximately 3:1 (wt:mutant), segregation for the mutant phenotype, typical of a defect in a sporophytically expressed gene (i.e. one not required for gametogenesis; Drews et al., 1998), we were surprised at the dramatic reduction in the fertility of the homozygous plants. To examine this issue we performed reciprocal crosses between mutant and wild-type Wassilewskija (WS) ecotype plants. As expected, mutant pollen was able to successfully fertilize wild-type plants and generate heterozygous ADL1A/adl1A seeds. In contrast, homozygous adl1A plants displayed poor seed set when pollinated with wild-type pollen, suggesting a maternal sporophytic defect in the mutant flowers.

The adl1A Phenotype Is Due to a Specific Disruption of ADL1A

Conclusive evidence that the phenotype observed in adl1A seedlings is due to disruption of ADL1A was obtained by transformation of homozygous adl1A “Suc-rescued” plants with a genomic copy of the gene. A 9-kb DNA fragment covering the entire protein-coding sequence of ADL1A plus 4.9 and 0.9 kB of the flanking 5′ and 3′ regions, respectively, was introduced into homozygous adl1A-2 and adl1A-3 plants. No other open reading frames have been identified in this genomic fragment. Ammonium glufoisinate- (BASTA) resistant homozygous adl1A-2
and adl1A-3 transformants were screened for normal growth on germination media in the absence of Suc. Complementation was confirmed by PCR analysis (Fig. 10) and by the normal appearance and development of siliques and seeds in flowering transgenic adl1A-2 (Fig. 9, C and D) and adl1A-3 plants (data not shown).

**DISCUSSION**

Our results reveal that ADL1Ap function is critical for plant growth and development. Mutants lacking this dynamin-like protein display defects in embryo maturation, seedling growth, and female fertility. It was previously proposed that ADL1Ap is targeted to...
and functions in the biogenesis of thylakoid membranes (Park et al., 1998). Evidence for such a role was based primarily on localization studies and on the analysis of dominant-interfering ADL1 constructs. The findings presented here and elsewhere (Lauber et al., 1997) are not consistent with this interpretation. First, we have demonstrated by immunofluorescence microscopy and quantitative subcellular fractionation that ADL1Ap as well as other members of the ADL1 protein family were not chloroplast-associated. Rather, ADL1Ap displayed a localization pattern identical to phragmoplastin (Gu and Verma, 1996). As shown in Figure 3, ADL1Ap is associated with punctate cytoplasmic structures and with the cell plate in dividing cells. Second, the thylakoid membranes of adl1A mutant chloroplasts appeared morphologically normal relative to wild type, and mutant seedlings did not display the yellow leaf phenotype observed by Hwang and colleagues (Park et al., 1998) in transgenic plants expressing various ADL1 deletion mutants. Given that dynamins self-assemble into oligomers (Hinshaw and Schmid, 1995; Shin et al., 1999; Zhang et al., 2000), one possible explanation for this apparent difference in phenotype is that overexpression of dominant-negative forms of ADL1p interfered with the proper localization and function of ADL1Ap and the chloroplast dynamin-like protein, ADL2p. In an alternate manner, the truncated forms of ADL1 mRNA encoded by the deletion mutants affected the expression of other ADL1 family members through post-transcriptional gene silencing (Meins, 2000), resulting in general loss of viability.

Mutant Phenotype

The adl1A mutant embryos are smaller and twisted relative to wild-type embryos, suggesting that ADL1Ap function is required during embryogenesis. Consistent with this observation, we have demonstrated that ADL1A mRNA is expressed during late embryogenesis. Analysis of the ADL1A promoter region using the Plant Cis-acting Regulatory DNA Elements Database (PLACE; Higo et al., 1998, 1999) has revealed several cis-acting elements found in the promoters of embryo-specific storage proteins (Baumlein et al., 1992; Ellerstrom et al., 1996) that are expressed highly during late embryogenesis. Based upon the cell plate localization of ADL1Ap we hypothesized that adl1A mutants would display defects in cytokinesis. Other Arabidopsis cytokinesis-defective mutants that exhibit defects in cell plate membrane formation and consolidation such as knolle (Lukowitz et al., 1996) and keule (Assaad et al., 1996; Waizenegger et al., 2000) display gross abnormalities during embryogenesis and seedling development. In a similar manner, adl1A mutants are seedling defective and arrest shortly after germination. To our surprise, however, homozygous loss-of-function adl1A mutants were not defective in cytokinesis and

Figure 10. Molecular analysis of homozygous and complemented adl1A-2 plants. PCR analysis of homozygous adl1A-2 transformed with the binary transformation vector pBK02B. A, Two oligonucleotide primer pairs, I = (SB7 + SB60) and II = (SB7 + SB59), were used for PCR to distinguish wild-type, heterozygous, and homozygous adl1A-2::pBK02B transgenic plants. Oligonucleotides SB7, SB60, and SB59 are specific to the 5’ end of ADL1A, pBK02B, and to the 3’ genomic DNA sequence flanking ADL1A, which was not included in the pBK02B, respectively. Primer pair I is specific to pBK02B and II is specific to the endogenous wild-type copy of ADL1A. B, PCR amplification of genomic DNA from untransformed wild-type ADL1A/ADL1A plants (lanes 1 and 2), transgenic ADL1A/adl1A-2::pBK02B plants (lanes 3 and 4), transgenic homozygous adl1A-2::pBK02B plants (lanes 5 and 6), and purified pBK02B plasmid DNA (lanes 7 and 8). PCR analysis of DNA from homozygous adl1A-2::pBK02B plants confirms the presence of the complementing ADL1A genomic copy and confirms that the plants are homozygous for the T-DNA insertion in ADL1A. Cross-hatched box, pBK02B vector DNA; specckled box, P1 MJC20 DNA flanking the 15-kb Xhol/KpnI restriction fragment containing ADL1A; gray triangle, ADL1A upstream regulatory sequences; gray square, ADL1A 3’ untranslated sequence.
showed normal patterns of cell division during embryogenesis and seedling germination.

Models for the Role of ADL1Ap

Given that adl1A mutants are not defective in cytokinesis, what, if any, role do members of the ADL1p/phragmoplastin dynamin-subfamily play in the assembly of the cell plate? By analogy with other dynamins we postulate that there are several potential, but not mutually exclusive, roles for ADL1Ap and other ADL1 isoforms in vesicular trafficking and cell plate formation. ADL1 proteins may function in a manner analogous to dynamin in the formation of TGN-derived exocytic vesicles (Jones et al., 1998). By virtue of its association with these TGN-derived vesicles, ADL1Ap would be transported and concentrated at the site of cell plate formation during cytokinesis. The following observations lend support to this model: green fluorescence protein-tagged dynamin has been shown to remain associated with mammalian endocytic vesicles after they bud from the plasma membrane (M. McNiven, personal communication), and phragmoplastin is predominantly associated with the leading edge of the cell plate (Gu and Verma, 1997; i.e. the area of the cell plate to which the cell plate vesicles are targeted). We have observed, by confocal microscopy, that ADL1Ap is associated with the leading edge of the cell plate in dividing Arabidopsis cells (C. Dickey and S.Y. Bednarek, unpublished data). Based on this model, ADL1Ap may function in vesicular trafficking from the Golgi apparatus.

Multiple parallel vesicular routes that transport distinct sets of cargo from the Golgi to the plasma membrane have been demonstrated in yeast and mammalian cells (Govindan et al., 1995; Harsay and Bretscher, 1995; Ikonen et al., 1995; Yoshimori et al., 1995). In a similar manner, analysis of glycoprotein and complex polysaccharide trafficking has hinted at the possibility of multiple distinct transport routes to the plasma membrane in plant cells, as well (Moore and Staehelin, 1988). During cell plate formation, these multiple exocytic pathways likely become polarized toward the plane of division, as they appear to do during cell division in Saccharomyces cerevisiae. If ADL1 protein family members are required for the formation of one or more of these TGN-to-plasma membrane vesicle classes, then loss of a single branch of the exocytic pathway in the adl1A mutants would not be expected to have a dramatic effect on the formation of the cell plate membrane. In this regard it is interesting that disruption of GNOM/EMB30, an ADP ribosylation factor-guanine nucleotide exchange factor required for the proper trafficking and localization of an auxin efflux carrier to the plasma membrane, does not disrupt cell plate formation (Steinmann et al., 1999). To examine whether ADL1Ap and other ADL1 isoforms cooperate during cell plate formation we are generating multiple mutant lines through genetic crosses with plants that contain T-DNA disruptions in different ADL1 genes.

A second potential role for the ADL1 protein family is in recycling of membranes and proteins from the forming cell plate. Consistent with this idea is the observation that expansion of the cell plate is accompanied by the appearance of clathrin-coated vesicles that appear to bud from the surface of this compartment and the appearance of endocytic multivesicular bodies adjacent to the cell plate (Samuels et al., 1995).

A third hypothesis, originally proposed by Samuels and Staehelin (Samuels et al., 1995) and later expanded upon by Verma and colleagues (Verma and Gu, 1996; Zhang et al., 2000), is that phragmoplastin/ADL1 proteins play a role in the formation of novel membrane tubules that have been observed to extend from individual cell plate vesicles as they begin to fuse (Samuels et al., 1995). These tubules appear to be protein coated and are about the same diameter (20–25 nm) as dynamin-encircled, clathrin-coated vesicle bud necks that form at the plasma membrane of neuronal cells and around artificial liposomes under conditions that prevent GTP hydrolysis (Takei et al., 1995; Sweitzer and Hinshaw, 1998). The tips of the narrow cell plate vesicle tubules are likely to be highly fusogenic because of their high radius of curvature (Chernomordik and Zimmerman, 1995). Formation and/or stabilization of these cell plate structures by the polymerization of ADL1Ap (or another ADL1 family member) around them may therefore drive the fusion of cell plate vesicles and membranes. This model would require the presence of additional cellular factors to regulate the GTPase activity of ADL1 proteins. A fourth possibility is that members of the ADL1 dynamin protein subfamily play a regulatory role (e.g. signal transduction), instead of an enzymatic function in membrane trafficking and fusion (Fish et al., 2000).

Supplemental Suc Stimulates Normal Development of adl1A Mutant Seedlings

Growth of adl1A seedlings is limited primarily to cell expansion, including those cells that give rise to first two leaf primordia (Fig. 5), which are established during embryogenesis (Conway and Poethig, 1997; J. Long and M.K. Barton, personal communication). Homozygous adl1A mutant seedlings arrest at about the time (3–5 d after germination) when wild-type seedlings have depleted most of their lipid and protein storage reserves and have become dependent on photosynthesis for energy production (Mansfield and Briarty, 1996). In this regard it is interesting that the metabolizable sugars Suc and Glc stimulate the growth of arrested adl1A seedlings. Suc and its derivatives may act indirectly or directly to stimulate the growth of adl1A arrested seedlings. Suc, the major transport form of photosynthetically assimilated car-
bon in plants, provides intermediates that enter the metabolic cycle of the cell and furnish the energy and substrates required for viability and growth. In addition, Suc availability modulates cell division rates by regulating the expression of the CycD cyclins, which regulate the G1-to-S phase cell cycle transition (Riou-Khamlichi et al., 2000).

The ADL1 Protein Family

The Arabidopsis genome encodes four other approximately 68-kD dynamin-like proteins that share a high degree of sequence similarity with ADL1Ap. The deduced amino acid sequences of ADL1Ap and ADL1Bp are most closely related to the soybean dynamin-related cell plate protein, phragmoplastin (approximately 84% sequence identity), than to ADL1 Cp, ADL1Dp, or ADL1Ep (approximately 65% identity). The significance, if any, of this remains to be understood. As discussed above, one possibility is that different members of the ADL1p dynamin subfamily are targeted to different branches of the secretory pathway to perform a similar function (e.g. vesicle formation), as has been observed for mammalian dynamin isoforms (Cao et al., 1998). In an alternate manner, the ADL1p isoforms may be functionally redundant and are differentially expressed in response to developmental or environmental signals. It is likely that another ADL1 isoform(s) can compensate for the loss of ADL1Ap upon the reinitiation of growth of arrested adl1A seedlings.

One isoform that may conditionally compensate for the loss of ADL1Ap during seedling growth is the 70-kD dynamin-like protein encoded by ADL1E (B.H. Kang and S.Y. Bednarek, unpublished data). ADL1Ep appeared to be constitutively synthesized in arrested, as well as Suc-rescued adl1A plants, whereas we were unable to detect the expression of other ADL1 family members in adl1A seedlings and leaves (Fig. 6). This raises the question of why adl1A mutant seedlings arrest if ADL1Ep is functionally redundant with ADL1Ap. One possibility is that the expression of ADL1-interacting protein(s) may be blocked in the Suc-starved seedlings. In an alternate manner, the activity of ADL1Ep may be regulated by post-translational modification. Previous studies have demonstrated that membrane binding of dynamin and dynamin-related proteins is regulated by post-translational modifications, including dephosphorylation (Liu et al., 1994a, 1994b; Park et al., 1997). It is, therefore, conceivable that the pool of ADL1Ep in the arrested seedlings is inactive, but is activated in response to Suc. In this regard it is interesting that induction of G1 cyclin expression in response to Suc is mediated through the activation of type 2A protein Ser-threonine phosphatases (Riou-Khamlichi et al., 2000). Experiments are in progress to determine if ADL1Ep is post-translationally modified in response to sugar treatment in the arrested adl1A seedlings.

A Role for ADL1Ap in Reproduction

In addition to being essential for embryo maturation and seedling growth, the conditional seedling lethal phenotype of adl1A mutants has permitted us to identify one additional stage in development that requires ADL1Ap function—reproduction. Genetic analysis of the highly infertile adl1A plants suggests that the mutation is a maternal sporophytic defect. One possibility is that pollen germination and/or guidance through the maternal adl1A tissue are affected in the mutants due to general defects related to protein trafficking (e.g. failure to secrete chemotropic guidance signals that direct pollen tube growth to the ovule). In an alternate manner, adl1A may be a sporophytic maternal mutation that disrupts normal megagametogenesis. In this case, heterozygous adl1A mutants may not exhibit a 50% defective seed phenotype observed in other classes of female gametophyte mutants (Drews et al., 1998) because of the presence of a maternally inherited pool of ADL1Ap. Further characterization of reproductive processes in the adl1A mutants, including megagametogenesis, pollen germination, and guidance will greatly aid in the elucidation of the function of the ADL1 family of dynamin-like proteins.

MATERIALS AND METHODS

General Reagents

Enzymes were purchased from New England Biolabs (Beverly, MA) or Pharmacia Biotech (Piscataway, NJ) unless indicated. All other reagents, unless specified, were from Sigma Chemical (St. Louis).

Plant Material and Culture Conditions

For subcellular fractionation and localization studies we utilized the Arabidopsis ecotype Columbia T87 suspension-cultured cell line (Axelos et al., 1992) provided by Dr. Michele Axelos (Institut National de la Recherche Agronomique, France). T87 cells were maintained axenically in liquid Murashige-Skoog 0.2 medium (Murashige and Skoog, 1962) supplemented with 0.2 mg L−1 2,4-dichlorophenoxyacetic acid and 1.32 mM KH2PO4 at 28°C at 125 rpm on a gyratory shaker. Cells were subcultured weekly. All other experiments were performed using Arabidopsis ecotype WS. Surface-sterilized seeds were germinated and grown on germination media (1.3 g L−1 Peters 15-5-15 [Grace Sierra, Milpitas, CA], 3 mM MES [2-(N-morpholino)-ethanesulfonic acid], and 0.6% [w/v] phytoagar [Gibco-BRL, Rockville, MD]) in the presence or absence of 40 μg mL−1 kan at 22°C under continuous light. Kan-resistant plants were planted on soil (Germination Mix, Conrad Farafar, Agawam, MA) and grown at 22°C under continuous light or under long-day conditions (16 h of light/8 h of dark). To examine the effects of various growth media, 5-d-old wild-type and mutant seedlings were transferred to agar-solidified Murashige-Skoog me-
dia containing 1% to 3% (w/v) Suc. After 10 d, “rescued” homozygous adl1A mutants were transferred to soil.

**GST-ADL1A Expression Plasmid Construction and Protein Purification**

An 870-bp DraI-BamHI fragment of aG68 (Dombrowski and Raikhel, 1995) was subcloned into pGEX-5A-2 (Pharmacia Biotech). This plasmid pGST-ADL1A(232–523) resulted in a translational fusion between glutathione-S-transferase and amino acids 232 to 523 of ADL1Ap. For protein expression, a 1.5-L culture of E. coli expressing GST fusion proteins as described (C. Dickey, D.M. Rancour, and S.Y. Bednarek, unpublished data). Antibodies against AHA2p (DeWitt and Sussman, 1995), AtSec12p (Bar-Peled and Raikhel, 1997), and PGK were kindly provided by M. Sussman (University of Wisconsin, Madison), N. Raikhel (MSU-DOE Plant Research Labs), and J. Thorner (University of California, Berkeley), respectively. Various commercially available antibodies were used, including donkey anti-rabbit IgG-horseradish peroxidase (Amersham Life Science, Arlington Heights, IL), monoclonal rat anti-tubulin (MAS078p; Harlan Sera-Lab, Loughborough, UK), rabbit anti-chicken IgY-horseradish peroxidase, Cy3-conjugated goat anti-rabbit IgG and fluorescein isothiocyanate (FITC) conjugated goat anti-rat IgG (Jackson ImmunoResearch Laboratories, West Grove, PA).

**Preparation of ADL1A-Specific Peptide Antibodies**

To generate ADL1Ap-specific antibodies, the peptide NH₂-DVEKGNNPHTSSDPRC-COOH was synthesized by the University of Wisconsin-Madison Biotechnology Center Peptide Synthesis Facility, coupled via the C-terminal Cys residue to activated keyhole limpet hemocyanin (Pierce Chemicals, Rockford, IL), and used to immunize a rabbit. For affinity purification, approximately 2 mg of GST-ADL1A(232–523) was dialyzed against 50 mm NaHCO₃, pH 9.0, 0.15% (v/v) SDS. Protein concentrations were quantified by SDS-PAGE followed by Coomassie Blue staining and scanning densitometry using bovine serum albumin (BSA) as a known standard.

**Other Antisera Used in This Study**

Affinity-purified anti-ADL1 GTPase domain-specific antisera generated against amino acid residues 1 through 253 of the protein encoded by aG68 (Dombrowski and Raikhel, 1995) was kindly provided by W. Lukowitz and C. Somerville (Carnegie Institution of Washington, Stanford, CA). Affinity-purified polyclonal antibodies to Knolle and AtCdc48p were generated against E. coli-expressed GST fusion proteins as described (C. Dickey, D.M. Rancour, and S.Y. Bednarek, unpublished data). Antibodies against AHA2p (DeWitt and Sussman, 1995), AtSec12p (Bar-Peled and Raikhel, 1997), and PGK were kindly provided by M. Sussman (University of Wisconsin, Madison), N. Raikhel (MSU-DOE Plant Research Labs), and J. Thorner (University of California, Berkeley), respectively. Various commercially available antibodies were used, including donkey anti-rabbit IgG-horseradish peroxidase (Amersham Life Science, Arlington Heights, IL), monoclonal rat anti-tubulin (MAS078p; Harlan Sera-Lab, Loughborough, UK), rabbit anti-chicken IgY-horseradish peroxidase, Cy3-conjugated goat anti-rabbit IgG, and fluorescein isothiocyanate (FITC) conjugated goat anti-rat IgG (Jackson ImmunoResearch Laboratories, West Grove, PA).

**Protoplast Isolation**

The procedure for isolation and purification of protoplasts from T87 cells was modified from that described in Bednarek et al. (1990) and Bar-Peled and Raikhel (1997). T87 cells from two 3- to 5-d-old 50-mL cultures were collected by filtration through a 94-μm steel mesh screen (Cellctor, Belco Glass, Vineland, NJ) and were washed with 50 mL of T87 protoplast wash buffer (PWB; 0.4 mm betaine, 3 mm MES-KOH, pH 5.7, and 10 mm CaCl₂). The cells were digested in 15 mL of PWB containing 1.0% (w/v) betaine, 3 m KOAc, and 2 m bitol, 50 m a-acetate, and 50 m KOAc, and were washed extensively with 50 mL of PWB containing 1.0% (w/v) mesopectolyase Y23 (Karlan Research Products, Santa Rosa, CA), 0.5% (w/v) macerozyme (Karlan Research Products), and 0.1% (w/v) pectolyase Y23 (Karlan Research Products) at 28°C with gentle shaking on a gyratory shaker at 75 rpm for 1 to 2 h. Protoplasts were separated from undigested cell clumps by filtration through a 94-μm steel mesh screen and were collected by centrifugation at 50g for 5 min at room temperature. The protoplasts were gently resuspended and washed three more times with 50 mL of PWB. Protoplast yields were quantified using a hemacytometer and the viability was determined by fluorescein diacetate staining (Widholm, 1972).

**Chloroplast Isolation**

Protoplasts were isolated from 3-d-old T87 cells and resuspended at 5 × 10⁶ cells mL⁻¹ in 10 mL of chloroplast isolation buffer (CIB; 50 mm Hepes [4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid]-KOH, pH 7.0, 250 mm Sorbitol, 50 mm KOAc, and 2 mm EDTA) containing 1 mm dithiothreitol plus a protease inhibitor cocktail (1 mm phenylmethylsulfonyl fluoride, 5 μg mL⁻¹ pepstatin A, 1 μg mL⁻¹ chymostatin, 1 μm p-aminobenzamidine, 1 μm e-aminocaproic acid, 5 μg mL⁻¹ aprotinin, 1 μg mL⁻¹.
leupeptin, and 1 μg mL⁻¹ E64). The protoplasts in CIB were gently homogenized (12 strokes in a 10-mL Dounce homogenizer, B pestle) and the lysate was clarified by centrifugation at 100,000 g for 10 min at 4°C to remove unbroken material and large debris. The resulting post-nuclear supernatant (S0.1) was subsequently recentrifuged at 2,500,000 g for 10 min and the supernatant (S2.5) was loaded onto an 8-mL Percoll (Pharmacia) step gradient (3 mL 20% Percoll, 5 mL 40% [v/v] Percoll, in CIB) in a 15-mL Corex tube and was centrifuged at 7,500 g for 10 min at 4°C to remove large aggregates. The resulting post-nuclear supernatant was designated as the total crude homogenate (S0.1). Total microsomal membranes (P150) and membrane-free cytosol (S150) were prepared by centrifugation of the 100,000 g supernatant at 150,000 g for 15 min at 4°C, for 10 min at 4°C to remove unbroken material the lysate was centrifuged twice at 100,000 g for 10 min at 4°C and the supernatant was designated as the total crude homogenate (S0.1). Total microsomal membranes (P150) and membrane-free cytosol (S150) were prepared by centrifugation of the 100,000 g supernatant at 150,000 g in a JA20 rotor (Beckman Instruments, Fullerton, CA). The loose pellet was diluted with at least 10 volumes CIB and centrifuged at 2,500,000 g for 10 min at 4°C, gently resuspended in 200 μL of CIB, quick frozen in liquid nitrogen, and stored at −80°C.

**Preparation of Subcellular Fractionations**

For the preparation and analysis of soluble and membrane fractions, protoplasts in PWB were diluted 1:10 in chilled membrane isolation buffer (20 mM HEPES-KOH, pH 7.0, 50 mM KOAc, 1 mM Mg(OAc)$_2$, and 250 mM Sorbitol) supplemented with 1 mM dithiothreitol and protease inhibitor cocktail and lysed by six passages through a 25-gauge needle. To remove unbroken material the lysate was centrifuged twice at 100,000 g for 10 min at 4°C and the supernatant was designated as the total crude homogenate (S0.1). Total microsomal membranes (P150) and membrane-free cytosol (S150) were prepared by centrifugation of the 100,000 g supernatant at 150,000 g in a TLA100.3 rotor (Beckman Instruments) for 30 min at 4°C.

**Analysis of Subcellular Fractions**

The protein concentration of isolated subcellular fractions was measured using the DC Protein Assay Reagent (Bio-Rad, Richmond, CA) and BSA as a known standard. Subcellular fractions were further characterized by measuring the content of various marker proteins by SDS-PAGE and immunoblotting. For immunoblotting, samples were separated on 12.5% and 7.5% (w/v) SDS-polyacrylamide minigels, and were electroblotted to nitrocellulose in 25 mM Tris, pH 8.3, 192 mM Gly, 20% (v/v) methanol, and 0.005% (w/v) SDS at 300 mA for 1.5 h using a minigel tank blotter (Hoefer, San Francisco). Immunodetection was performed using the enhanced chemiluminescence western blotting detection system (Amersham Life Science) according to the manufacturer’s instructions. For quantitative immunoblotting, band intensities were determined by scanning densitometry using several different film exposures to verify linearity of the film response.

For preparation of total protein, extracts from wild-type and mutant seedling and plants, five 5-d-old seedlings or leaf tissues (approximately 50 mm$^2$) were homogenized in 100 μL of SDS-PAGE sample buffer and were incubated at 95°C for 5 min. The samples were cleared of insoluble debris by centrifugation for 5 min at 17,000 g and 10 μL of the supernatant was resolved on a 7.5% (w/v) SDS-polyacrylamide minigel and was analyzed by immunoblotting.

**Immunofluorescence Microscopy**

Arabidopsis protoplasts were resuspended and fixed in 5 mL of microtubule stabilizing buffer (MTSB; Goodbody and Lloyd, 1994; 50 mM PIPES [1,4-piperazinediethanesulfonic acid]-KOH, pH 6.9, 5 mM MgSO$_4$, and 5 mM EGTA) containing 0.4 μM Sorbitol and 4% (w/v) paraformaldehyde at room temperature for 30 min.

The fixed protoplasts were collected by centrifugation at 50g for 5 min at room temperature and were washed twice with MTSB and 0.4 μM Sorbitol. The suspension was plated onto eight-well poly-L-lys-coated slides or ProbeOn Plus slides (Fisher Scientific, Pittsburgh). Wells were hand-drawn onto slides with a PAP pen slide marker (Research Products International, Mount Prospect, IL). Protoplasts were allowed to settle for 10 min at room temperature, and were then air dried and stored overnight at 4°C.

For immunolabeling, the slides were warmed to room temperature and then immersed in −20°C methanol for 10 min. All subsequent steps were carried out in a moist chamber at room temperature. The cells were permeabilized in MTSB, 3% (v/v) Triton X-100, and 10% (v/v) dimethyl sulfoxide for 15 min and the slides were blocked with 3% (w/v) BSA in MTSB (BSA/MTSB) for 1 h. Primary antibodies and preimmune sera were diluted in BSA/MTSB and were layered over the cells for 1 h. Unbound antibodies were removed with five washes for 5 min each with MTSB. The washed cells were incubated in the appropriate secondary antibody solution for 1 h. The secondary antibodies, Cy3-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories) and FITC-conjugated goat anti-rat IgG (Jackson ImmunoResearch Laboratories), were diluted to 7.5 μg mL⁻¹ and 15 μg mL⁻¹ in BSA/MTSB, respectively. After washing the slides five times in MTSB, the protoplasts were stained with 0.5 μg mL⁻¹ DAPI for 10 min. The cells were rinsed in MTSB and mounted in Vectashield antifade mounting medium (Vector Laboratories, Burlingame, CA).

Epifluorescence microscopy was performed on an Axioskop (Carl Zeiss, Thornwood, NY) equipped with a cooled charge-coupled device digital camera containing a 1,317 × 1,035 pixel array (MicroMax, Princeton Instruments, Trenton, NJ). All images were acquired using a 63× (numerical aperture 1.4) PlanAPO Chromat oil immersion objective lens. For fluorescence microscopy, emission/excitation filters for DAPI, FITC, and Cy3 were used. Image acquisition and processing were carried out on a Macintosh computer (PowerPC 8500, Apple Computer, Cupertino, CA) using IPLab Spectrum, version 3.2 (Signal Analytics, Vienna, VA) and Adobe Photoshop 5.0 (Adobe Systems, San Jose, CA) imaging software. Image acquisition times ranged from 25 to 200 ms. A sample with no primary antibody was always included to control for background generated by the secondary antibodies. For double labeling experiments, control localization studies were performed with each primary antibody separately to examine the amount of “bleed through” signal in each fluorescence emission channel.
Isolation of adl1A T-DNA Insertion Mutants

PCR-based identification of T-DNA insertions in ADL1A was performed as described (Krysan et al., 1996). Two separate Arabidopsis T-DNA insertion libraries containing 14,000 and 60,480 plants (available through the Arabidopsis Knock-Out Facility, Biotech Center, University of Wisconsin, Madison) were screened for adl1A::T-DNA using oligonucleotide primers JL202, SB7, and SB8. Three independent lines were identified and the positions of the T-DNA inserts in ADL1A were determined by sequencing the PCR-amplified product and by DNA gel-blot analysis. Heterozygous adl1A plants were backcrossed to wild-type WS ecotype plants and lines that segregated for a single T-DNA insert were selected. The genotype of segregating plants was confirmed by PCR using allele-specific primer pairs.

Oligonucleotides

All oligonucleotides used in this study were synthesized by Integrated DNA Technologies (Coralville, IA; Table I).

Complementation

A 15-Kb Xhol-KpnI fragment containing ADL1A was isolated from the P1 clone MJC20 (Kaneko et al., 1999) and was subcloned into the modified binary transformation vector pTZP221-B (Hadjukiewicz et al., 1994) containing the glufosinate (BASTA)-resistance gene to generate pBK02B. The plasmid pBK02B was introduced into homozygous adl1A-2 plants using the Agrobacterium-mediated floral dip transformation method (Clough and Bent, 1998). Transgenic plants were selected for growth on germination medium containing 20 μg mL⁻¹ ammonium glufosinate (Crescent Chemicals, Hauppauge, NY).

Analysis of ADL1A mRNA Expression

Total RNA was isolated from wild-type and adl11 seedlings using TRI reagent according to the manufacturer’s instructions (Sigma Chemical), and 2 μg of total RNA was treated with RQ DNase (Promega, Madison, WI) to eliminate genomic DNA. After heat inactivating RQ DNase, 1 μg of total RNA was transcribed by Moloney Murine Leukemia Virus RT (Promega) using oligo(dT) to produce the first-strand cDNA in a 20-μL reaction mixture. A 1-μL aliquot of a 25-fold dilution of the reverse transcribed DNA was subsequently PCR-amplified with the oligonucleotide primer pair SB86 and SB87.

Table I. Oligonucleotides used in this study

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5’ → 3’ )</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>SB7</td>
<td>CAGCTACTGTATTTTGGGCTTCTCATTG</td>
<td>Mutant isolation, ADL1A 5’ primer</td>
</tr>
<tr>
<td>SB8</td>
<td>CATGGTACCGAGCTCCCAGGAAAAGTGT</td>
<td>Mutant isolation, ADL1A 3’ primer</td>
</tr>
<tr>
<td>SB59</td>
<td>GTCAACGAAATTTGATACGTGAGAAAG</td>
<td>ADL1A-specific RT-PCR 5’ primer</td>
</tr>
<tr>
<td>SB60</td>
<td>GCGGATAACAATTTTTCACACAGGAACCG</td>
<td>ADL1A-specific RT-PCR 3’ primer</td>
</tr>
<tr>
<td>SB86</td>
<td>GGTGTTAACCCCTCACACTCCATTTC</td>
<td>Genotyping adl1A-2</td>
</tr>
<tr>
<td>SB87</td>
<td>AACGATACGTAACATATCGGGTGATGGAC</td>
<td>ADL1A-specific RT-PCR 5’ primer</td>
</tr>
<tr>
<td>SB106</td>
<td>AAGTACAGAAACCCCTCTCCACAACATC</td>
<td>Complementation verification</td>
</tr>
<tr>
<td>SB106</td>
<td>CAGCTACTGTATTTTGGGCTTCTCATTG</td>
<td>Mutant isolation, ADL1A 5’ primer</td>
</tr>
<tr>
<td>SB106</td>
<td>GCGGATAACAATTTTTCACACAGGAACCG</td>
<td>ADL1A-specific RT-PCR 3’ primer</td>
</tr>
<tr>
<td>JL202</td>
<td>CATTITATAAAACGGCCTGGACATCTACA</td>
<td>Mutant isolation, T-DNA left border</td>
</tr>
</tbody>
</table>

SEM

Embryos and seedlings were fixed for 4 h in 2.5%/2.5% (w/v) paraformaldehyde/glutaraldehyde in 0.05 M sodium cacodylate, pH 7.0, with a change of fresh fixative after the first 2 h. Following fixation, material was rinsed with buffer and embryos were dissected using 28-gauge needles. All tissues were rinsed with buffer, dehydrated with ethanol, and critical-point dried. Samples were coated with 480Å gold. The material was viewed with a scanning electron microscope (S-570, Hitachi, Rolling Meadows, IL) at 5 or 10 kV and was photographed with positive/negative 4 × 5 instant sheet film (Polaroid 55, Eastman-Kodak, Rochester, NY). The number of seeds examined were 70 wild type, 44 adl1A-1/adl1A-1, 23 adl1A-2/adl1A-2, and 19 adl1A-3/adl1A-3. The number of mature embryos examined were 38 wild type, 70 adl1A-1/adl1A-1, 36 adl1A-2/adl1A-2, and 44 adl1A-3/adl1A-3. The number of 5-d-old seedlings examined were 24 wild type, 17 adl1A-1/adl1A-1, 17 adl1A-2/adl1A-2, and 23 adl1A-3/adl1A-3.

TEM

Initial seedling fixation and buffer washing were performed as described for SEM. All tissues were post-fixed overnight at 4°C with 2% (w/v) osmium tetroxide in 0.05 M sodium cacodylate followed by buffer rinsing and dehydration through a graded acetone series. Tissue was then transferred to propylene oxide, embedded in Spurr’s resin, and polymerized at 70°C (Spurr, 1969). Gold sections were cut with a Sorvall Porter-Blum MT-2 ultramicrotome, mounted on 0.5% (w/v) pioloform-coated 75-mesh or uncoated 300-mesh copper grids, stained with 3% (w/v) uranyl acetate in 30% (v/v) ethanol, and post-stained in Reynold’s lead citrate. Sections were viewed at 80 kV with a JEOL 100CX or JEM-1200EX transmission electron microscope electron scope and were photographed with electron microscope (S-570, Hitachi, Rolling Meadows, IL).

Brightfield Microscopy

Material prepared for TEM was sectioned at 2 μm, affixed to glass slides with heat, and stained with toluidine blue-O. Sections were examined with an ultra-phot (Zeiss) and were photographed using T-MAX 100 film (Kodak).

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LITERATURE CITED


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Wilsbach K, Payne GS (1993) Vps1p, a member of the dynamin GTPase family, is necessary for Golgi membrane protein retention in Saccharomyces cerevisiae. EMBO J 12: 3049–3059
