A dynamin-like protein in *Arabidopsis thaliana* is involved in biogenesis of thylakoid membranes

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Dynamin, a GTP-binding protein found in rat brain, plays a role in endocytosis. Suborganellar fractionation studies of *Arabidopsis* leaf tissue revealed that a dynamin-like protein, ADL1, is localized in the thylakoid membranes of chloroplasts. This notion was supported further by *in vivo* targeting experiments using an ADL1–green fluorescent fusion protein and immunogold labeling with the anti-ADL1 antibody. Transgenic plants harboring various deletion mutant genes of *ADL1* had a yellow leaf phenotype where the cells had very few chloroplasts. In addition, the remaining chloroplasts appeared morphologically not fully developed. The detailed structure of the chloroplasts revealed by electron microscopy showed a greatly reduced amount of thylakoid membranes. Also, the level of thylakoid membrane proteins such as the light-harvesting complex II and CP29 was greatly reduced in these transgenic plants. When we examined the expression of the *ADL1* deletion mutant genes, these genes were highly expressed at the transcriptional level. However, the mutant ADL1s were not detectable at the protein level by Western blot analysis. Moreover, the endogenous ADL1 protein level was greatly reduced in these transgenic plants, probably due to a post-transcriptional silencing effect of the transgenes. We propose, therefore, that ADL1 is involved in the biogenesis of thylakoid membranes.

Keywords: *Arabidopsis* dynamin-like 1/chloroplast/thylakoid membrane biogenesis/transgenic plants

Introduction

Dynamin is a high molecular weight GTP-binding protein with an intrinsic GTPase activity. Recently many genes belonging to the dynamin family have been isolated from a variety of eukaryotic systems ranging from yeast to human (Shpetner and Vallee, 1989; Horisberger *et al.*, 1990; Obar *et al.*, 1990; Rothman *et al.*, 1990; Chen *et al.*, 1991; van der Bliek and Meyerowitz, 1991; Nakata *et al.*, 1993; Dombrowski and Raikhel, 1995; Gu and Verma, 1996). As a result, it appears that these family members are quite diverse with regard to the degree of sequence

similarity among them as well as the biological processes in which these proteins are thought to be involved. The primary structure of these proteins can be divided into two regions. The N-terminal region containing the GTP-binding motifs is highly conserved among these proteins, whereas the C-terminal half of the molecules shows varying degrees of sequence conservation. Thus, the members of the dynamin family can be divided into several subfamilies based on their sequence similarity. One of them is the dynamin subfamily consisting of mammalian dynamin isoforms, Dyn1, Dyn2, Dyn3 and *Drosophila* shibire (Obar *et al.*, 1990; Chen *et al.*, 1991; Nakata *et al.*, 1993; Cook *et al.*, 1994). Another subfamily is the Vps1p family which is composed of the yeast homologs Vps1p and Dnm1p (Rothman *et al.*, 1990; Gammie *et al.*, 1995). Two plant homologs, SDL of soybean and aG68 of *Arabidopsis*, can also be grouped into a separate subfamily (Dombrowski and Raikhel, 1995; Gu and Verma, 1996). In addition, there are two subfamilies, Mx (Aebi *et al.*, 1989; Horisberger *et al.*, 1990) and Mgm1p (Jones and Fangman, 1992), which are most distantly related to the dynamin family based on amino acid sequence similarity as well as proposed biological functions.

Among all these proteins, dynamin 1, the brain-specific isoform, has been characterized most thoroughly (Robinson *et al.*, 1993; Miki *et al.*, 1994; Seedorf *et al.*, 1994; Valee and Okamoto, 1995). Many studies in animal cells indicate that dynamin 1 is involved in endocytosis for neurotransmitter recycling in the brain, especially at the step of vesicle formation from invaginated plasma membranes (van der Bliek and Meyerowitz, 1991; Herskovits *et al.*, 1993a; Robinson *et al.*, 1993; Damke *et al.*, 1994). Thus it has been postulated that dynamin 1 may function as a molecular switch in regulating endocytosis, because the GTPase activity of dynamin 1 can be modulated in various ways, such as by interaction with proteins containing SH3 domains, by a phosphorylation/dephosphorylation cycle, and by interaction with membranes (Gout *et al.*, 1993; Herskovits *et al.*, 1993b; Robinson *et al.*, 1993; Miki *et al.*, 1994; Seedorf *et al.*, 1994). In yeast, the dynamin homolog Vps1p plays a different role. Here it has been shown to be involved in vacuole protein sorting (Rothman *et al.*, 1990; Vater *et al.*, 1992; Wilsbach and Payne, 1993). Another protein, Dnm1p, also a member of the Vps1p subfamily, is thought to be involved in pheromone-mediated endocytosis in yeast (Gammie *et al.*, 1995). The plant homolog SDL is thought to be involved in yet another biological process (Gu and Verma, 1996, 1997). SDL is localized specifically at the cell division plate during the early stage of cell plate formation in dividing soybean root tip cells or tobacco suspension culture cells, as observed by *in situ* cytohistochemistry or *in vivo* targeting experiments,

respectively. Based on this observation, Gu and Verma (1996, 1997) suggested that SDL may play a role in transporting materials needed for cell plate formation.

As described above, the members of the dynamin family appear to be involved in very diverse biological processes. However, there is a common feature that is shared among most of these proteins; it is the involvement in trafficking of materials. As a means to investigate intracellular trafficking in plants, we decided to clone cDNAs encoding dynamin-like proteins in *Arabidopsis*. We isolated a cDNA clone, *ADL1*, that encodes a dynamin-like protein in *Arabidopsis* and found that the nucleotide sequence of this clone was identical to that of a previously published cDNA, *aG68*. We used the cloned cDNA to look into the biological role of the encoded protein, ADL1, in *Arabidopsis*.

Here we present evidence that the *Arabidopsis* dynamin-like 1 (ADL1) protein encoded in the *aG68* cDNA is localized in the thylakoid membranes in leaf tissue and that it possibly is involved in thylakoid membrane biogenesis during chloroplast development.

Results

Presence of ADL1 in chloroplasts

In order to study intracellular trafficking in plants, we first attempted to isolate a cDNA encoding a dynamin homolog from *Arabidopsis*. Degenerate oligonucleotides corresponding to the conserved GTP-binding motif were used as probes for library screening (Hall and Brown, 1985; Ma *et al.*, 1990). The cDNA clone, *ADL1*, isolated from an *Arabidopsis* leaf λ gt11 cDNA library had exactly the same sequence as the *Arabidopsis aG68* cDNA (Dombrowski and Raikhel, 1995). Using this cDNA, we investigated the biological role of the encoded protein, ADL1, in *Arabidopsis*. We first employed Western blot analysis using a polyclonal antibody raised against the N-terminal region (~300 amino acid residues starting from the initiation codon) of ADL1. The Western blot analysis of cell extracts from leaf, root, flower and silique tissue showed that ADL1 was present in all tissues examined (data not shown). This result is in a good agreement with the Northern data of Dombrowski and Raikhel (1995) which showed that the *aG68* gene was transcribed at high levels in leaf, root, flower and silique tissue. To characterize the protein further, we then examined the cellular localization of ADL1 in leaf tissue by Western blot analysis. The leaf tissue was homogenized gently and fractionated on a Percoll gradient. The top fraction of the Percoll gradient was fractionated further into supernatant and pellet fractions by ultracentrifugation. As shown in Figure 1A, the majority of ADL1 was co-purified with the chloroplasts (either intact or broken). Only a minor portion was present in the top fraction of the Percoll gradient. When the top fraction was fractionated again by ultracentrifugation, ADL1 stayed with the membranes in the pellet fraction. However, upon longer exposure, a weak band appeared consistently in the supernatant of the ultracentrifugation step, indicating that a very small amount of ADL1 may be present in a soluble form.

Since the chloroplast is composed of multiple suborganelles, we pursued the question of the localization of ADL1 within the chloroplast even further. The intact

Fig. 1. Subcellular and suborganellar localization of ADL1 in leaf tissue. (A) Total leaf homogenate was fractionated into top, broken chloroplast and intact chloroplast fractions on a Percoll step gradient. The top fraction was fractionated further into supernatant and pellet fraction by ultracentrifugation. These fractions were examined for the presence of ADL1 by Western blot analysis using the anti-ADL1 antibody. Total, Top, Br. Chl. and Intact Chl. indicate total homogenate, top fraction of the Percoll gradient, broken chloroplasts and intact chloroplasts, respectively. (B and C) ADL1 in the thylakoid membranes was examined after additional fractionation of the intact chloroplasts into supernatant (stroma and envelope membranes) and pellet (thylakoids). Proteins were resolved by SDS-PAGE and stained with Coomassie brilliant blue (B) or subjected to immunostaining with the anti-ADL1 antibody (C). An identical blot was probed with the anti-OEP34 antibody as an outer envelope membrane marker.

chloroplasts were lysed and subsequently fractionated into supernatant and pellet by low speed centrifugation. After this fractionation, the pellet contains thylakoid membranes and the supernatant contains chloroplast envelope membranes as well as soluble stromal proteins. The proteins in the supernatant and pellet were separated by SDS-PAGE and probed with the anti-ADL1 antibody. As shown in Figure 1C, the majority of ADL1 was present in the pellet containing the thylakoid membranes, and only a minor portion remained in the supernatant. When the supernatant was fractionated further into a soluble and a pellet fraction by ultracentrifugation, most of the ADL1 co-purified with the membranes in the pellet fraction (data not shown), indicating that the small amount of ADL1 in the supernatant of the low speed centrifugation is very likely attached to the membranes of the chloroplast envelope. As a control for the suborganellar fractionation of the chloroplast, an identical blot was probed with a polyclonal antibody raised against OEP34, a subunit of the chloroplast protein import complex localized on the outer membrane of the chloroplast (Seedorf *et al.*, 1995). The antibody recognized a protein species in the super-

Fig. 2. Association of ADL1 with thylakoid membranes. (A) The thylakoid membrane fraction was treated with 1.0 M NaCl or 0.1 M Na₂CO₃ on ice for 30 min. Solubilized proteins were removed after centrifugation in a microcentrifuge. The pellet was washed with the buffer once and then reconstituted to the original volume. Proteins in equal volumes of samples were subjected to Western blot analysis. (B) The thylakoid fraction was incubated with thermolysin (100 µg/ml) in the presence or absence of Triton X-100. The remaining proteins were resolved by SDS-PAGE and probed with the anti-ADL1 antibody.

natant as expected. Thus the control experiment confirms that there is very little, if any, contamination of chloroplast membranes in the thylakoid fraction. These results clearly establish that the majority of ADL1 in chloroplasts is present in the thylakoid membranes, although a minor portion may be present in the membranes of the chloroplast envelope.

In order to understand better the association of ADL1 with the thylakoid membranes, we treated the thylakoid membrane fraction with high salt (1.0 M NaCl) or high pH (0.1 M Na₂CO₃, pH 11.5) and subsequently precipitated the thylakoid membranes by low speed centrifugation. The amount of ADL1 remaining in the pellet was then examined by Western blot analysis. As shown in Figure 2A, a large portion of ADL1 was washed away at high pH, indicating that ADL1 is a peripheral membrane protein. In another experiment, the thylakoid membrane fraction was digested with thermolysin in the presence or absence of Triton X-100 and then examined for the presence of ADL1 by Western blot analysis. As shown in Figure 2B, ADL1 was digested away under both conditions, suggesting that ADL1 is most likely located on the outward-facing side of the thylakoid membranes.

Targeting of ADL1 into the chloroplast

As an alternative approach to show the presence of ADL1 in the chloroplasts, we employed an *in vivo* targeting experiment using green fluorescent protein (GFP) tagging on ADL1 (Sheen *et al.*, 1995; Davis and Viestra, 1996). We generated a fusion protein between ADL1 and smGFP (ADL1-smGFP). We also fused the transit peptide of the chlorophyll *a/b*-binding protein (Cab) to smGFP (Cab-smGFP) in order to use it as a control protein for chloroplast targeting (Karlin-Neumann and Tobin, 1986; Smeekens *et al.*, 1986) (Figure 3A). DNA constructs encoding the two fusion proteins and smGFP were introduced into soybean suspension culture cells (soybean

Fig. 3. Targeting of the ADL1-smGFP fusion protein to chloroplasts. (A) Schematic presentation of fusion constructs. a, b and c indicate ADL1-smGFP, Cab-smGFP and smGFP, respectively. (B) The soybean suspension cells were transformed with either ADL1-smGFP (panels A and B), Cab-smGFP (panels C and D) or smGFP (panels E and F). The left and right panels are fluorescent and light microscopic images of the cells, respectively. Bars indicate 12 µm.

photoautotrophic cell, SB-P) by the particle bombardment method (Takeuchi *et al.*, 1992). Expression of the introduced genes was examined under a fluorescence microscope. As shown in Figure 3B, the majority of the ADL1-smGFP fusion protein was targeted to the chloroplast as indicated by the green fluorescent staining of the chloroplasts. The staining pattern was nearly identical to that of Cab-smGFP. In contrast to ADL1-smGFP and Cab-smGFP, smGFP remained in the cytosol as expected. In addition, there was a weak staining at the

Fig. 4. Immunogold labeling of ADL1 in the thylakoid membranes. The sections prepared from leaf tissue were labeled with immunogold coated with the anti-ADL1 antibody (A, B and C). The control sample was treated with a polyclonal antibody against ARSK1 which is a root-specific protein (D). S indicates starch granules. The bars represent 1.2 μ m (A), 0.15 μ m (B) and 0.2 μ m (C and D).

boundary of the cells transformed with the *ADL1-smGFP* fusion gene, suggesting that a minor portion of ADL1-smGFP might be targeted to the plasma membrane.

Immunogold labeling of ADL1 in thylakoid membranes

To obtain direct evidence for the presence of ADL1 in the thylakoid membranes, we employed the immunogold labeling method using the polyclonal anti-ADL1 antibody. Four-week-old leaf tissue was fixed and processed for immunogold labeling. As shown in Figure 4, the immunogold label concentrated in the chloroplasts. The majority of the label was located in the thylakoid membranes, with only minor labeling of the membranes of the chloroplast envelope. Thus, the results of the immunogold labeling experiment are in agreement with those of the Western blot analysis described in Figure 1.

Yellow leaf phenotype of the transgenic plants harboring deletion mutant genes

To gain insight into the biological role of ADL1 in the chloroplasts, we decided to generate dominant-negative

mutants by expressing mutant forms of the *ADL1* cDNA in transgenic plants. The deletion mutant genes shown in Figure 5A were constructed, and transgenic plants were generated by the *Agrobacterium*-mediated transformation method (Valvekens *et al.*, 1988). The deletion constructs were under the control of the 35S cauliflower mosaic virus (CaMV) promoter to obtain strong expression in leaf tissue. The T_1 generation of the transformants was rescreened for kanamycin resistance. We then examined the transgenic plants visually for phenotypic changes. As shown in Figure 5B, one obvious phenotypic effect was manifested in the yellow leaves of the transgenic plants. The yellow leaf phenotype was reproducible in the T_2 generation. The transgenic plants retained the phenotype when grown in soil, but at a somewhat reduced frequency. This phenotype was observed in many independent lines of transgenic plants derived from the same deletion mutant gene and from different deletion mutant genes, albeit that it differed in frequency and severity as shown in Table I. The results strongly suggest that the yellow leaf phenotype is caused by the introduction of the mutant gene. The variation in the frequency of the phenotype between the

Fig. 5. Phenotype of transgenic plants. **(A)** Schematic diagrams of the deletion mutant genes of *ADL1* cDNA. The three elements of the consensus GTP-binding motif are indicated by thick lines. **(B)** Phenotype of the T1 progeny of transgenic *Arabidopsis* plants. Transgenic plants containing pBI121 (a control transformation vector) (a) and *ADL1del2* (b).

transgenic lines and between the plants with various deletion mutant genes is probably due to the randomness in their integration into the genome.

In search for the mechanism underlying the yellow leaf phenotype, we further characterized the transgenic plants. First, we examined the expression of the introduced deletion mutant genes by Northern blot analysis using total RNA obtained from the respective transgenic plants. The transgenic plants showed a high level of expression of the deletion mutant genes as expected. A typical result of Northern blot analysis is shown in Figure 6A. We also carried out Western blot analysis to measure the accumulation of the truncated proteins encoded by the deletion mutant genes. Surprisingly, we found no accumulation of any truncated form of ADL1, regardless of which deletion mutant gene had been introduced. An example of Western blot analysis is shown in Figure 6B. In order to rule out the possibility that a frameshift mutation occurred during the construction of the deletion mutant genes, we translated the deletion constructs *in vitro* using wheat germ extract and examined the translation products

Fig. 6. Northern and Western analysis of transgenic plants transformed with the *ADL1del2* construct. **(A)** High level transcription of the *ADL1del2* deletion mutant gene. Total RNA was isolated from transgenic plants containing the *ADL1del2* deletion mutant gene and analyzed by Northern blot hybridization using the *ADL1* cDNA as a probe. **(B)** Suppression of endogenous ADL1 by the introduced deletion mutant genes. Protein extracts were prepared from transgenic plants of the T1 generation. The level of endogenous ADL1 and deletion mutant *ADL1del2* was examined by Western blot analysis with the anti-ADL1 antibody. The numbers denote independent lines of transgenic plants harboring *ADL1del2*. pBI121 indicates transgenic plants harboring a transformation vector, pBI121.

Table I. Phenotype analysis of transgenic plants

Construct	Line	Total No. (T1)	Phenotype		
			Km ^r Green	Yellow	Km ^s
<i>ADL1del1</i>	7	18	5	8	5
	8	16	5	4	7
	10	12	3	7	2
	15	14	11	0	3
<i>ADL1del2</i>	2	15	5	5	5
	12	18	4	10	4
	25	16	10	1	5
	28	19	7	6	6
<i>ADL1del4</i>	1	14	3	6	5
	13	13	7	1	5
	14	15	2	9	4
	15	11	5	3	3
<i>ADL1ΔN</i>	3	13	2	6	5
	12	11	2	7	2
	21	15	5	9	1
	24	18	6	8	4
<i>ADL1ΔC</i>	1	12	6	4	2
	2	13	3	4	6
	5	8	4	2	2
	19	17	5	7	5

Approximately 20 seeds from the T1 generation of each line indicated were sterilized and planted on MS plates containing kanamycin (50 μg/ml). Plants were scored for kanamycin resistance and for the phenotype 3 weeks after planting.

by Western blot analysis with the anti-ADL1 antibody. We observed that the deletion constructs not only yielded the expected truncated forms of ADL1, but also that the *in vitro* translated products were recognized by the anti-

ADL1 antibody (data not shown). In contrast to the absence of accumulated truncated ADL1s, the transgenic plants showed various levels of endogenous ADL1 ranging from the control level to >10-fold reduced levels. The Western blot results thus stand in contrast to the Northern blot results. A possible explanation for this may be a post-transcriptional silencing effect by the introduced deletion mutant gene on the endogenous gene in the transgenic plants, in spite of our initial attempt to create a dominant-negative effect when introducing the deletion mutant genes. It has been shown before that gene silencing can occur either at the transcriptional level or at the post-transcriptional level (de Carvalho *et al.*, 1992; Seo *et al.*, 1995; Elmayan and Vaucheret, 1996).

Thylakoid membrane and thylakoid membrane protein reduction in the transgenic plants

To address the phenotypic changes at the cellular level, sections of leaf tissue were examined under a microscope. In the leaves of the transgenic plants, the overall size of individual cells was slightly enlarged, while the number of cells was reduced. However, the most prominent changes in the transgenic plants occurred in the number and the morphology of the chloroplasts. The cells in the leaf tissue contained almost no or only very few chloroplasts. Furthermore, the remaining chloroplasts in the transgenic plants appeared to be not fully developed. We examined these chloroplasts with an electron microscope. As shown in Figure 7A and C, they show a marked reduction in the amount of thylakoid membranes without grana stacks. Instead the chloroplasts in the leaf cells of the transgenic plants had more lipid granules (plastoglobules, Pg) and were filled with small particulates. Usually the chloroplasts of a mature leaf cell exhibit thylakoid membranes that are organized into grana stacks at a high density, as shown in Figure 7B and D (Apel *et al.*, 1983; Melis, 1984; Anderson and Beardall, 1991). We also addressed the changes in the level of thylakoid membrane polypeptides in the transgenic plants. Total cell protein was extracted from transgenic plants harboring pBI121 (a transformation vector) and the deletion mutant genes, and probed with polyclonal antibodies raised against maize proteins CP29, and the light-harvesting complex II (Bergantino *et al.*, 1995). As shown in Figure 8, the amount of thylakoid membrane proteins such as CP29 and the LHCII complex was greatly reduced in the transgenic plants harboring the deletion mutant genes (*ADL1del1* and *ADL1del2*) as compared with the control transgenic plants (pBI121) when equal amount of total protein was separated by SDS-PAGE. The level of these polypeptides correlated well with that of ADL1 in the transgenic plants.

Therefore, our results strongly suggest that the cells in the leaf tissue of transgenic plants are defective with regard to the accumulation of thylakoid membranes as well as thylakoid membrane proteins in their chloroplasts. In addition, there were other changes, such as a reduction in the volume of the cytosol as well as in the number of mitochondria; however, it is not clear whether these changes were the direct effect of the reduced ADL1 level or the indirect consequence of poor chloroplast development.

Discussion

In this study, we investigated the biological role of ADL1 in *Arabidopsis*. By subcellular localization studies of ADL1 using *Arabidopsis* leaf tissue, we found that a large portion of the ADL1 pool is present in chloroplasts. Subsequent suborganellar fractionation experiments revealed that the majority of the chloroplast ADL1 is associated with the thylakoid membranes. This finding was supported by two additional independent approaches. An ADL1-smGFP fusion protein was targeted to the chloroplasts when expressed transiently in soybean suspension cells, SB-P. The immunogold labeling studies, using the anti-ADL1 antibody, unequivocally demonstrated that the majority of the ADL1 in the chloroplasts is localized at the thylakoid membrane.

As a means to elucidate the biological role of ADL1, we initially tried to generate dominant-negative mutants by expressing various deletion mutant genes in transgenic plants, since the protein seems to be composed of multiple domains and also exists in high molecular weight complexes *in vivo* (Park *et al.*, 1997). The transgenic plants containing these deletion constructs had yellow leaves. Microscopic inspection of the yellow leaf phenotype at the cellular level showed that cells in the leaf tissue contain almost no or very few chloroplasts, which then appear not fully developed. Moreover, the chloroplasts as seen by electron microscopy contained a greatly reduced amount of thylakoid membranes. On the other hand, there was an increased number of lipid granules and an accumulation of small particulates. Also, the amount of thylakoid membrane polypeptides was greatly reduced in the transgenic plants showing the yellow leaf phenotype. It has been proposed that dynamin 1 plays a role in severing the neck of the invaginated membrane in the vesicle formation process. Vps1p, the yeast homolog, is also thought to be involved in the formation of vesicles during the process of vacuolar protein sorting. It is possible, therefore, that ADL1 may play a role in a similar biological process in the chloroplast. The presence of vesicles in chloroplasts has been shown in a variety of plants, especially when leaves were incubated at low temperature (Morré *et al.*, 1991). Recently a plastid protein, Pftf, which shares a high degree of amino acid sequence similarity with NSF of yeast and animals and FtsH of bacteria, has been isolated from hot pepper and implicated in the vesicle fusion process (Hugueney *et al.*, 1995). The observation suggests that a vesicle fusion process operates in chloroplasts that is similar to the processes observed in the cytosol. Therefore, one possible role for ADL1 in the chloroplast could be an involvement in vesicle formation as in the case of dynamin.

In the leaf cell, the young proplastid goes through many developmental stages before becoming a mature chloroplast (Engelbrecht and Weier, 1967; Sundqvist *et al.*, 1980; Meier and Lichtenthaler, 1981; Apel *et al.*, 1983; Melis, 1984; Anderson and Beardall, 1991). During this developmental process, thylakoid membranes proliferate and form grana stacks. The thylakoid membranes have a lipid composition similar to that of the chloroplast inner membranes (Carde *et al.*, 1982; Douce and Joyard, 1984; Morré *et al.*, 1991). Based on this observation, it has been postulated that the thylakoid membranes are derived from

Fig. 7. Comparison of chloroplast development in transgenic and wild-type plants. Leaf sections of transgenic and wild-type plants were examined by transmission electron microscopy. A transgenic plant with the yellow leaf phenotype (harboring *ADL1del2*) (A and C) and a wild-type plant (B and D). S, g and Pg indicate starch, grana stacks and plastoglobules (lipid granules). Bars represent 2.0 μm (A and B) and 0.1 μm (C and D).

Fig. 8. Accumulation of thylakoid membrane polypeptides in the transgenic plants. Total cell extracts were prepared from transgenic plants harboring the *ADL1del1* (del1-7, del1-10) and *ADL1del2* deletion mutant (del2-12, del2-25) genes as well as transgenic plants harboring a transformation vector, pBI121 (pBI121). Proteins (10 μg) were separated by 10% SDS-PAGE and stained with Coomassie brilliant blue to confirm equal loading (data not shown). For Western blot analysis, identical gels were blotted onto PVDF membranes. The blots were probed with antibodies raised against ADL1, CP29 (maize) and the light-harvesting complex II (maize, LHCII).

the inner membrane of the chloroplast envelope. A form of vesicles has been suggested as a possible mechanism for the transfer (Carde *et al.*, 1982; Douce and Joyard,

1984). Without ADL1 or with a reduced amount of ADL1, as in the case of the transgenic plants, the vesicle formation at the inner membrane could be hampered, thereby precluding the transfer of lipid membranes to the thylakoids. The reduced amount of the thylakoid membranes or the lack of the grana stacks, in turn, may result in the inhibition of chloroplast development and chloroplast proliferation. Also, the observed increase in lipid granules may reflect the excess amount of lipids that are synthesized for thylakoid membrane biogenesis but cannot be organized into thylakoid membranes because of a defect in the ADL1 activity. However, we cannot exclude the possibility of a different function for ADL1 in the biogenesis of the thylakoid membranes or grana stacks in the chloroplasts.

In this study, we focused on the biological role of ADL1 during chloroplast development. Further studies will be necessary before we can fully understand the biological function of ADL1 in *Arabidopsis*, because there is evidence that ADL1 may be involved in other biological processes. The *aG68/ADL1* gene is expressed in cells that have no chloroplasts (Dombroski and Raikhel, 1995). Furthermore, the ADL1-smGFP targeting experiment has shown that the fusion protein was also targeted to the plasma membranes, although to a lesser extent. Finally, it is noteworthy that ADL1 is highly homologous to SDL

which seems to be involved in the transport of materials to the cell division plate during cytokinesis.

Materials and methods

Expression of recombinant ADL1 in *E.coli* and protein purification

To prepare a specific antibody, the N-terminal region containing ~300 amino acid residues (ADL1-N) was expressed in *E.coli* using pRSET B (Invitrogen, Inc.). The construct was then transformed into BL21 (DE3) pLysS cells and the encoded protein was induced according to the manufacturer's protocol (Invitrogen, Inc.). Since most of the expressed protein was found in the inclusion body fraction, the protein was purified by Ni²⁺-NTA affinity chromatography under denaturing conditions following the manufacturer's instructions (QIAGEN). The protein was purified further by SDS-PAGE and injected into a rabbit to raise antibody (Harlow and Lane, 1988). The antibody was affinity-purified using the purified ADL1-N protein (Harlow and Lane, 1988).

Northern blot analysis

Total RNA was prepared from transgenic plants and used for Northern blot analysis using the *ADL1* cDNA as a hybridization probe (Ausubel *et al.*, 1989).

Isolation and suborganelle fractionation of the chloroplasts

Plants were grown on MS plates at 20°C for 2 weeks. Homogenates were prepared and separated into top, broken chloroplast and intact chloroplast fractions on a Percoll step gradient (Cline *et al.*, 1985; Robinson and Barnett, 1988). The intact chloroplasts were lysed and fractionated further into a supernatant and a pellet fraction (Li *et al.*, 1991). To investigate the nature of the ADL1 association with the thylakoids, the thylakoid fraction was treated with 1.0 M NaCl or 0.1 M Na₂CO₃ (pH 11.5) on ice for 30 min and then centrifuged at 5000 g for 10 min. The pellet was resuspended to the original volume in 10 mM Tris-HCl, pH 7.5, and 2 mM EDTA and analyzed via Western blot analysis. For protease treatment of ADL1 in the thylakoid membranes, the thylakoid fraction was treated with thermolysin in the presence or absence of 1% Triton X-100 at room temperature for 10 min. Western blot analysis was carried out according to a published protocol using the anti-ADL1 antibody as the primary antibody and the horseradish peroxidase-conjugated anti-rabbit IgG antibody as the secondary antibody (Harlow and Lane, 1988). Blots were developed using the ECL detection system for Western blots (Amersham).

Construction and transient expression of fusion genes

The termination codon of the *ADL1* cDNA was removed after PCR using two PCR primers, ADL 5' (5'-GTCAAGCTTGGACCAAGCAACAGC-3') and the T7 primer of pBluescript (5'-AATACGACTCACTATAG-3'). The resulting molecule was in-frame fused to smGFP (Davis and Viegas, 1996). To construct the *Cab-smGFP* fusion gene, a DNA fragment of the *cab* cDNA (accession No. X56062) containing the first 89 amino acid residues was ligated in-frame to the smGFP coding region. The fusion constructs were introduced into soybean suspension culture cells (soybean photoautotrophic cells, SB-P) by the particle bombardment method (Takeuchi *et al.*, 1992).

Construction of deletion mutants and generation of transgenic plants

The deletion mutant genes were generated utilizing various restriction sites within the *ADL1* cDNA. The *ADL1del1* construct had a deletion from the *Ecl136II* site to the *XmaI* site, the *ADL1del2* construct from *SmaI* to *SalI*, the *ADL1del4* construct from the *XbaI* site to the *BamHI* site, the *ADL1ΔN* from the second codon to *SmaI*, and the construct *ADL1ΔC* from 521 to the C-terminus. The deletion mutant genes were introduced into transgenic plants using the *Agrobacterium*-mediated transformation method (Valvekens *et al.*, 1988).

Electron microscopy and immunogold labeling

Leaves from wild-type and transgenic plants were processed for transmission electron microscopy as described previously (Hawes, 1994). Ultra-thin sections were made, stained with uranium and lead, and examined by electron microscopy as described previously (Roth *et al.*, 1981). Grids containing ultra-thin sections were blocked with 3% bovine serum albumin in Tris-buffered saline (TBS), reacted with the affinity-purified anti-ADL1 antibody at a dilution of 1:50 or 1:100 in TBS, and

washed in TBS. They were then reacted with 20 nm gold-conjugated goat anti-rabbit IgG (Zymed, diluted 1:10 or 1:30) and washed with TBS and distilled water. Sections were post-stained with standard solutions of uranyl acetate and lead citrate and examined with a Hitachi H-600 electron microscope.

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