

# Regulation of ADL6 activity by its associated molecular network

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## Summary

Plant dynamin-like proteins consist of a group of high molecular weight GTPase with diverse structural arrangements and cellular localizations. In addition, unlike animal dynamins, there was no evidence for the involvement of any plant dynamin-like protein in clathrin-mediated vesicle trafficking. In this study we demonstrate that ADL6 (*Arabidopsis* dynamin-like protein 6), due to its domain arrangement, behaves similarly to the animal dynamins. The association of ADL6 with clathrin-coated vesicles was demonstrated by co-fractionation and immunocytochemical studies. ADL6 also interacted via its C-terminus with  $\gamma$ -adaptin, an adaptor protein of clathrin-coated vesicles. Our results suggest that ADL6 participates in clathrin-mediated vesicle trafficking originating from the Golgi. In addition, our studies demonstrate that ADL6 intrinsic GTPase activity is regulated by its association with acidic phospholipids and an SH3 (Src homology 3)-containing protein.

**Keywords:** clathrin, dynamin, GTPase, pleckstrin homology (PH) domain, proline-rich domain, Src homology 3 (SH3) domain.

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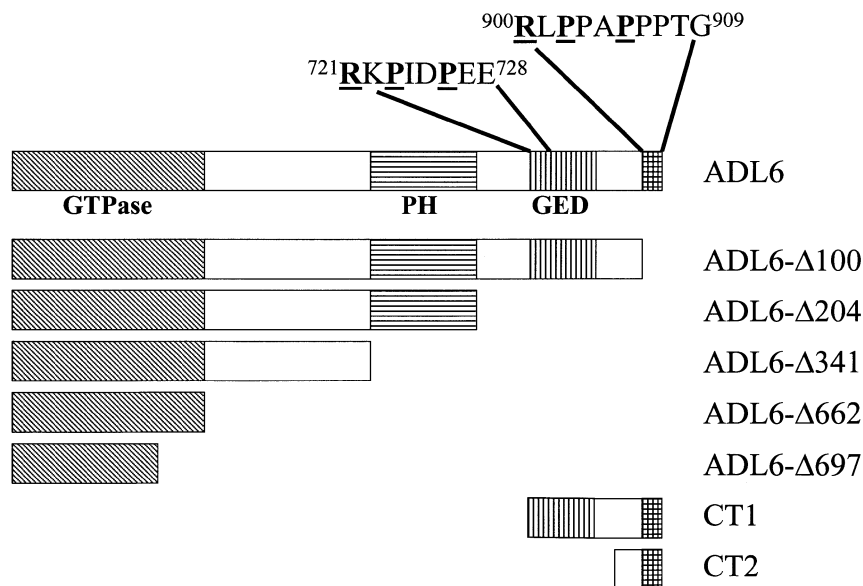
## Introduction

Clathrin-mediated vesicle trafficking is a multi-step process that includes the formation of a clathrin-coated pit, the invagination of the pit, the fission of the coated vesicle and the uncoating of the vesicle in the cytoplasm. In mammalian and yeast cells each of these steps is regulated by an accessory protein complex that consists of both enzymatic and non-enzymatic proteins (reviewed by Kirchhausen, 2000). One of the major accessory proteins is the GTPase dynamin. Originally identified through the *shibire* mutation (van der Bliek and Meyerowitz, 1991; Chen *et al.*, 1991) in *Drosophila melanogaster*, dynamin is the mechanoenzyme driving membrane fission at the plasma membrane and at the Golgi (McNiven *et al.*, 2000).

Apart from the N-terminus GTPase catalytic domain, the mammalian dynamin has a lipid-binding pleckstrin homology domain (PH domain), a GTPase effector domain (GED), and a C-terminus proline-rich domain (PRD) containing several PXXP motifs (P, proline; X, any amino acids), which are the targets of several Src homology 3 (SH3) domain-containing accessory proteins (McNiven *et al.*, 2000). Protein–lipid and/or protein–protein interactions through these functional domains are critical in

the regulation of the dynamin GTPase activity (Barylko *et al.*, 1998). Moreover, the interaction of the PXXP motifs with scaffolding proteins such as amphiphysin is important for the recruitment of dynamin to the site of clathrin-coated vesicle formation (Shupliakov *et al.*, 1997).

Several plant dynamin-like proteins have been identified recently. These include phragmoplastin in soybean (Gu and Verma, 1996), and various *Arabidopsis thaliana* dynamin-like (ADL) proteins: ADL1 (Park *et al.*, 1998); ADL2 (Kang *et al.*, 1998); ADL3 (Mikami *et al.*, 2000); and ADL6 (Jin *et al.*, 2001). Other than ADL6, all these dynamin-like proteins have domain arrangements that differ from those in animal dynamins. Most plant dynamin-like proteins consist of the GTPase and GED domains, while the presence of the PH domain and the PRD is not ubiquitous to all of them. Moreover, their subcellular localization is drastically different from that of the mammalian dynamins. Phragmoplastin was found to be associated with the forming cell plate (Gu and Verma, 1996). While ADL1 was originally shown to associate with the thylakoid membrane (Park *et al.*, 1998), a more recent immunolocalization study using an ADL1-specific antibody



**Figure 1.** Domain organization of ADL6.

Domain organization of ADL6 as predicted by SMART (Schultz *et al.*, 1998). ADL6 (914 amino acids) contained a GTPase domain (diagonal hatching); a pleckstrin-homology (PH) domain (horizontal hatching); a GTPase effector domain (GED, vertical hatching); and two proline-rich domains (PRDs, crossed hatching). The sequence of PRDs is shown with the potential SH3-binding PXXP motif in bold and underlined. CT1 and CT2 represented the C-terminus fragments of ADL6 used in subsequent expression studies and yeast two-hybrid studies. ADL6- $\Delta$ 100, ADL6- $\Delta$ 204, ADL6- $\Delta$ 341, ADL6- $\Delta$ 662 and ADL6- $\Delta$ 697 represent the C-terminus truncation mutants of ADL6 used in the GTPase activity assays.

by Kang *et al.* (2001) suggested that ADL1 is associated with the cell plate. ADL2 was localized to the plastids (Kang *et al.*, 1998). ADL6 was shown to associate with the Golgi apparatus (Jin *et al.*, 2001). The cellular compartment to which ADL3 associates is currently unknown.

Apart from difference in subcellular localization, the cellular functions of dynamin-like proteins revealed so far are very different from those of the mammalian dynamins. In animals, dynamins are major mechanoenzymes that are involved in both clathrin-dependent and clathrin-independent receptor-mediated endocytosis. Caveolae internalization and fluid-phase pinocytosis are also regulated by different isoforms of dynamin. Participation of dynamins in trafficking to and from the Golgi was also observed (McNiven *et al.*, 2000). In plants, there was no direct observation of the involvement of dynamin-like proteins in clathrin-mediated vesicle trafficking in the endosomes. However, expression of a dominant negative mutant of ADL6 caused an accumulation of lytic vacuole-targeted cargo within the *trans*-Golgi network, suggesting the importance of ADL6 for trafficking from Golgi to vacuole (Jin *et al.*, 2001). On the other hand, the association of phragmoplastin, ADL1 and ADL2 to compartments unique to plants suggested that plant dynamins could have more diverse functions in vesicle trafficking. At the physiological level, a recent study using T-DNA insertional mutant lines of *ADL1* suggested that ADL1 could be involved in various

stages of plant development such as embryogenesis and seed development (Kang *et al.*, 2001).

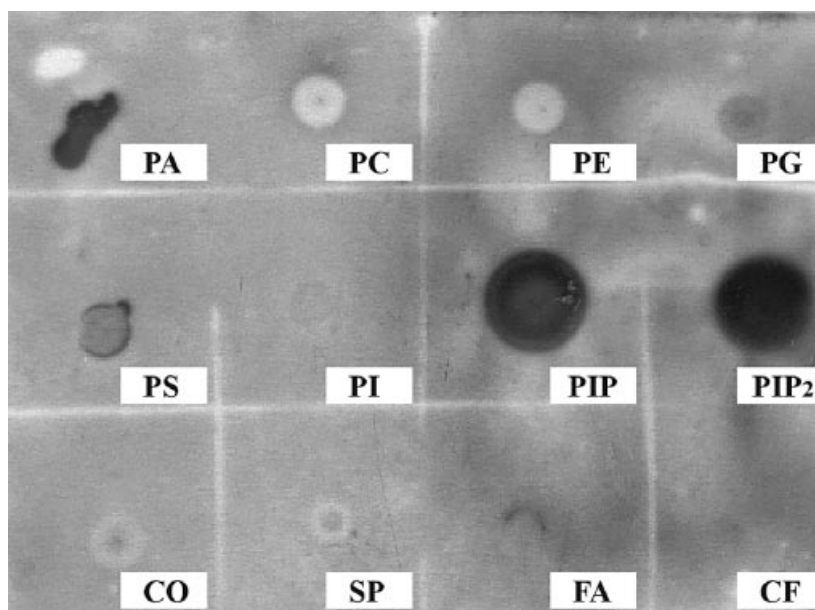
Reports on plant dynamin-like proteins at the biochemical level in general, and on the regulation of their GTPase activity in particular, are limited. In this report we identify several molecules that bind to different regions of ADL6 and provide results suggesting the role of these interactions in the regulation of GTPase activity in the ADL6-protein complex.

## Results

### Domain prediction

Functional domains of ADL6 were predicted by submitting the amino acid sequence of ADL6 (Jin *et al.*, 2001) to SMART (Schultz *et al.*, 1998). ADL6 consists of an N-terminus GTPase domain (residues 4–250); a pleckstrin-homology (PH) domain (residues 573–698); and a C-terminus GTPase effector domain (GED, residues 725–811) (Figure 1). Within the coding sequence there are two potential SH3-binding PXXP motifs: PRD1 (<sup>721</sup>RKPIDPEE<sup>728</sup>) within the GED domain; and PRD2 (<sup>900</sup>RLPPAPPPTG<sup>909</sup>) at the C-terminus of ADL6 (Figure 1). Notably, both motifs contain the consensus RXPXXP motif that represents a reversed version of the mammalian class II (PXXPXR) SH3-binding motifs (Mayer and Eck, 1995).

**Figure 2.** ADL6 binds acidic phospholipids. Various lipids (5 µg) were spotted onto nitrocellulose membrane in PH domain-binding assays as described in Experimental procedures. The lipids used were phosphatidic acid (PA); phosphatidylcholine (PC); phosphatidylethanolamine (PE); phosphatidylglycerol (PG); phosphatidylserine (PS); phosphatidylinositol (PI); phosphatidylinositol-4-phosphate (PIP); phosphatidylinositol-4,5-phosphate (PIP<sub>2</sub>); cholesterol (CO); sphingomyelin (SP); fatty acid-CoA (FA). Chloroform (CF) was used as a negative control. The figure represents three independent experiments.



**Table 1.** Interaction between ADL6 and AtSH3P3

AtSH3P3 bait	ADL6 prey		
	pJG4-5	pJG-CT1	pJG-CT2
pEG-P3	1.48	25.50	2.41
pEG-NT	0	0.60	0.12
pEG-SH3	0	5.60	0

β-galactosidase activity was calculated in Miller units. Results are the average of three independent experiments with two replicates per plasmid combination.

#### Each PRD of ADL6 binds different targets

In animals, the C-terminus PRD of dynamin contained binding sites to numerous SH3-containing proteins. With a domain arrangement similar to that of animal dynamins, ADL6 could be involved in an SH3-PRD-dependent protein–protein interaction. Our previous work (Lam *et al.*, 2001) showed that there were only a limited number of *A. thaliana* SH3-containing proteins, as predicted by sequence homology. Hence, instead of performing a full screen for potential protein interactor(s) to the PRDs of ADL6, we used yeast two-hybrid assays to determine whether one or both of the ADL6 PRDs interact with the SH3 domains of the recently identified *A. thaliana* AtSH3P protein family (Lam *et al.*, 2001). CT1 and CT2, containing both PRDs and only PRD1 of ADL6, respectively (Figure 1), were generated and fused to the activation domain of LexA as the prey fusions. On the other hand, the SH3 domain of

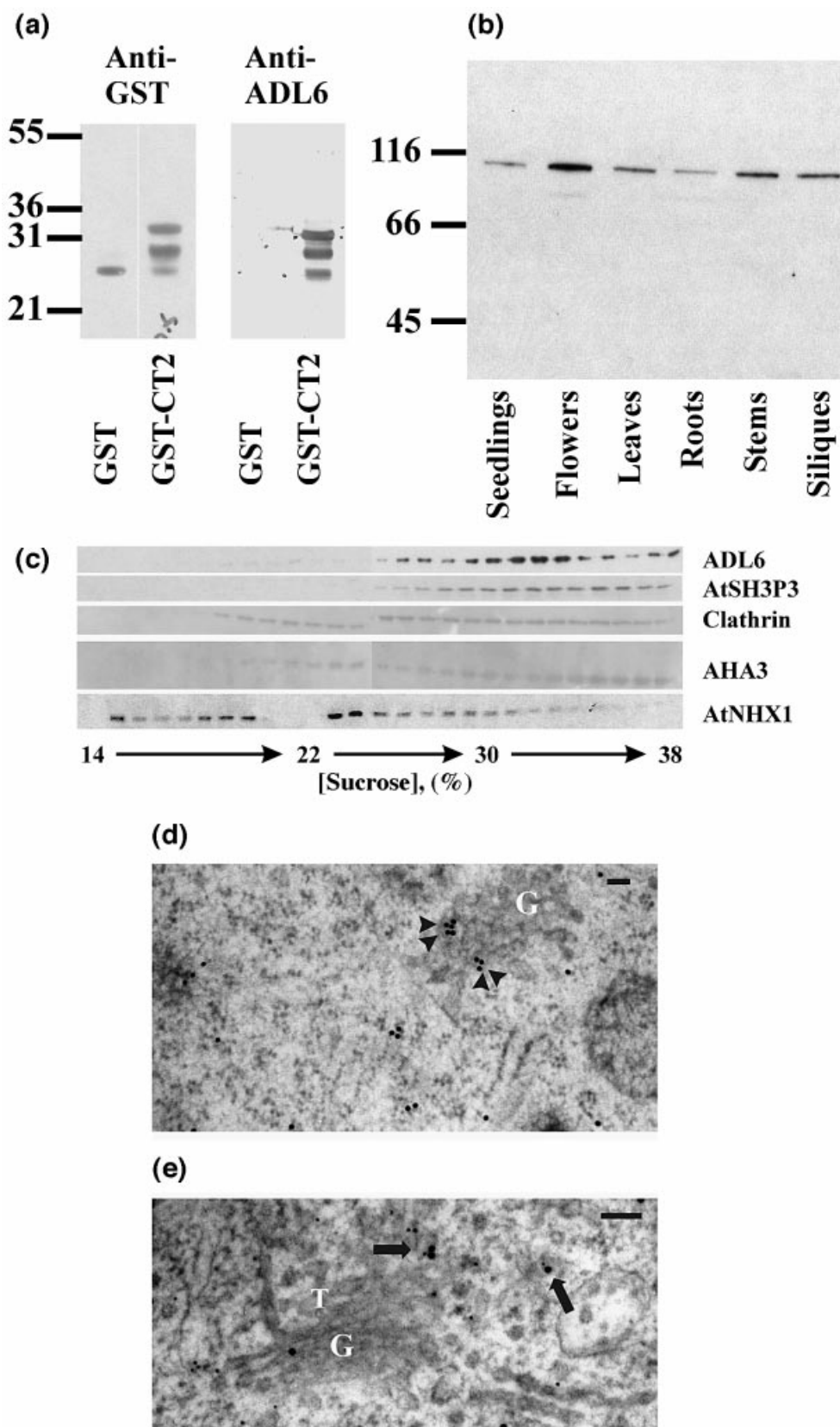
**Table 2.** Interaction between ADL6 and an *Arabidopsis thaliana* γ-adaptin

ADL6 bait	Prey	
	pJG4-5	pJG-γ-adaptin
pEG202	0	0
pEG-CT1	0	32.4
pEG-CT2	0	49.6

β-galactosidase activity was calculated in Miller units. Results are the average of three independent experiments with two replicates per plasmid combination.

each of AtSH3P1, AtSH3P2 and AtSH3P3 was fused to the DNA-binding domain of LexA as the bait fusions. After transformation of plasmids containing the bait and prey fusions into yeast strain EGY48, we found that the SH3 domains of AtSH3P1 and AtSH3P2 did not interact with either CT1 or CT2 of ADL6 (results not shown). On the other hand, the SH3 domain of AtSH3P3 interacted with CT1 (Table 1). However, the SH3 domain of AtSH3P3 did not bind CT2, indicating that the interaction was mediated through PRD1, but not PRD2. In addition, the full-length AtSH3P3 interacted strongly with CT1, while AtSH3P3 lacking the SH3 domain lost its CT1 affinity, suggesting that the SH3 domain was critical in this interaction (Table 1).

In order to identify interacting partners of PRD2, CT2 was then used as the bait to screen an *A. thaliana* cDNA library. Consistent with the results discussed above, we were unable to identify any SH3 domain-containing protein as a potential interactor of CT2. One clone that was consistently



obtained encoded a partial sequence of an *A. thaliana*  $\gamma$ -adaptin-like protein (GenBank accession no. AAD28247). Retransforming EGY48 with the adaptin plus either CT1 or CT2 bait activated the reporters (Table 2) while adaptin by itself did not generate any  $\beta$ -galactosidase activity.

#### The PH domain of ADL6 binds phospholipids

A GST-fusion of the predicted PH domain of ADL6 was generated and used as the probe for *in vitro* lipid-binding assays (Figure 2). The PH domain bound strongly to phosphatidic acid (PA), phosphatidylinositol-4-phosphate (PIP) and phosphatidylinositol-4,5-bisphosphate (PIP<sub>2</sub>). It also bound, although weakly, to phosphatidylserine (PS) and phosphatidylglycerol (PG). It did not bind to phosphatidylcholine (PC) and phosphatidylethanolamine (PE), the major components of plant lamellar membrane.

#### ADL6 exists as a 100 kDa polypeptide in plants and co-fractionated with clathrin and AtSH3P3

Rat antibodies were generated specifically against the C-terminus 94 amino acid unique region of ADL6 (Figure 3a). When the antibodies were used in protein-gel blots with total extracts of various *A. thaliana* tissues, a single polypeptide band of 100 kDa was detected. Cross-reactivity of the anti-ADL6 antibodies was the strongest in flowers, although signals were detected in all tissues examined (Figure 3b).

Microsomal membranes of *A. thaliana* were isolated and subjected to continuous sucrose density gradient fractionation according to Lam *et al.* (2001). Protein-gel blots were performed using antibodies raised against the full-length AtSH3P3, the C-terminus 94 amino acids of ADL6, clathrin (Blackbourn and Jackson, 1996), the *A. thaliana* plasma membrane H<sup>+</sup>-ATPase (AHA3), and the *A. thaliana* vacuolar Na<sup>+</sup>/H<sup>+</sup> antiport (AtNHX1) (Figure 3c). ADL6 and AtSH3P3 co-localized to fractions representing sucrose concentration of 25–38%. On the other hand, the plasma membrane AHA3 was abundant in fractions of  $\approx$ 33–38%. Clathrin was more widely distributed in fractions from 20 to 38%, probably due to its presence throughout the endomembrane system which includes plasma membrane, Golgi and some prevacuolar compartments. In

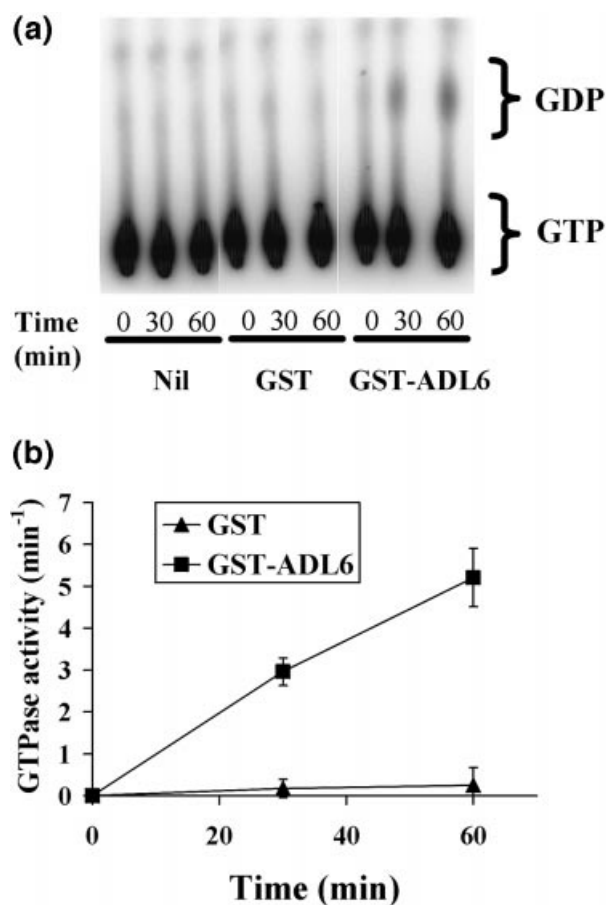
contrast, only the vacuolar AtNHX1 was localized to the fractions of 14–22%. This suggested that both AtSH3P3 and ADL6 were localized in vesicle fractions originating from both plasma membrane and the Golgi/ER system.

Immunocytochemical studies demonstrated that the ADL6 protein localized to the Golgi and associated vesicles (Figure 3d). Notably, ADL6 co-localized with clathrin at these cellular locations, suggesting the association of ADL6 with clathrin-coated vesicles originating from the Golgi (Figure 3e). ADL6 also localized to the partially coated reticulum and plasma membrane (results not shown). One-way ANOVA indicated that localization events of ADL6 at the Golgi, partially coated reticulum and plasma membrane were not statistically different from one another ( $df = 2$ ,  $H = 2.841$ ,  $P = 0.069$ ). However, localization events at these endomembrane compartments were significantly different ( $df = 6$ ,  $H = 0.44$ ,  $P < 0.001$ ) from localization at other cellular compartments of the cytoplasm to include the endoplasmic reticulum, mitochondria, and plastids. Mean percentage ADL6 localization to the Golgi, partially coated reticulum and plasma membrane was 28.63 (5.13), 21.73 (5.07) and 27.43 (3.53), respectively, where values in parentheses represent the 95% confidence interval. Mean percentage ADL6 localization to all other cellular compartments was  $<4$ , except for free vesicles which represented 9.81 (3.3) of the localization events.

#### Recombinant ADL6 contained intrinsic GTPase activity

Recombinant GST-fusion of the full-length ADL6 was generated, purified and used to measure [ $\alpha$ -<sup>32</sup>P]GTP hydrolysis using a thin-layer chromatography-based method (Figure 4a). With no protein added to the reaction or at time 0 min, the majority of the signals remained close to the origin (bottom, Figure 4a) and represented non-hydrolysed GTP. After 30 min initiation of the reaction by adding GST-ADL6, there was an increase in the amount of [ $\alpha$ -<sup>32</sup>P]GDP (top, Figure 4a), indicating GTP hydrolysis. The amount of [ $\alpha$ -<sup>32</sup>P]GDP was doubled at 60 min, indicating a time-dependent GTP hydrolysis (Figure 4b). On the other hand, GST, a negative control protein purified in identical fashion to GST-ADL6 isolation, did not have any detectable time-dependent GTP hydrolysis (Figure 4a,b). This sug-

**Figure 3.** ADL6 and AtSH3P3 co-localized with vesicles enriched in clathrin. (a) Purity verification of anti-ADL6. Purified GST (lane 1) and GST-CT2 (lane 2, used for antibody generation) were probed by anti-GST antibodies (left) and anti-ADL6 antibodies (right) in a protein-gel blot. (b) 15  $\mu$ g total extract from *A. thaliana* 7-day seedlings, flowers, leaves, roots, stems and siliques were subjected to protein gel blot using anti-ADL6 antibodies. (c) One-tenth of 1 ml fractions of a continuous sucrose density gradient of *A. thaliana* were resolved on SDS-PAGE and probed with antibodies raised against ADL6, AtSH3P3, plant clathrin, vacuolar AtNHX1 and plasma membrane AHA3. The corresponding sucrose concentration of each fraction is also presented. Figures are representative of two independent experiments. (d) Immunolocalization of ADL6 to budding vesicles from the Golgi network in pollen grains of *A. thaliana* (arrowheads). (e) Co-localization of ADL6 with clathrin in the Golgi and associated vesicles (arrows). Bars = 100 nm. ADL6, 18 nm gold particles; clathrin, 10 nm gold particles. Abbreviations: G, Golgi; T, *trans*-region of Golgi.

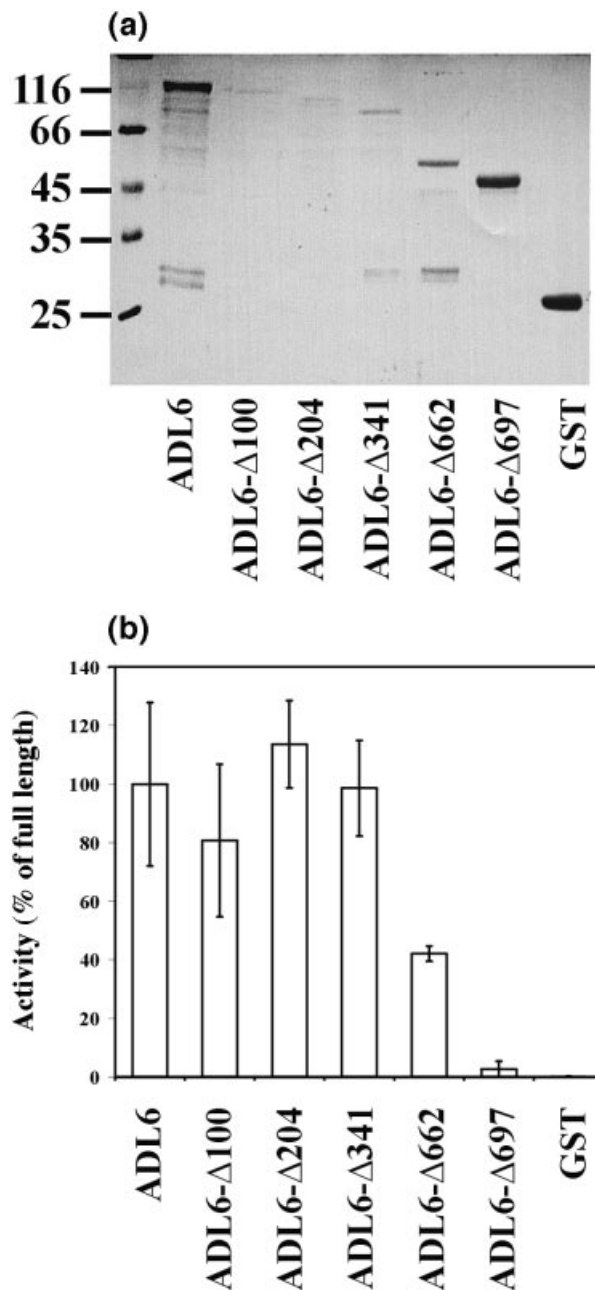


**Figure 4.** Time-dependent activity of ADL6. [ $\alpha$ -<sup>32</sup>P]GTP hydrolysis by GST-ADL6 or GST was performed according to Experimental procedures. (a) Representation of the GTPase assay. Reactions were resolved on cellulose polyethyleneimine TLC plates and the relative intensity of [ $\alpha$ -<sup>32</sup>P]GTP or [ $\alpha$ -<sup>32</sup>P]GDP was measured. Concurrent reactions with no protein added (Nil) were performed as a negative control. (b) Graphical representation of the GTPase activity of GST-ADL6 (■) and GST (▲). GTPase activity was calculated as the amount of [ $\alpha$ -<sup>32</sup>P]GDP generated as a percentage of total [ $\alpha$ -<sup>32</sup>P]GTP used (sum of [ $\alpha$ -<sup>32</sup>P]GTP and [ $\alpha$ -<sup>32</sup>P]GDP of each lane) at the time indicated. Values are mean  $\pm$  SD ( $n = 5$ ).

gested that the GTPase activity detected in GST-ADL6 was due specifically to the presence of ADL6.

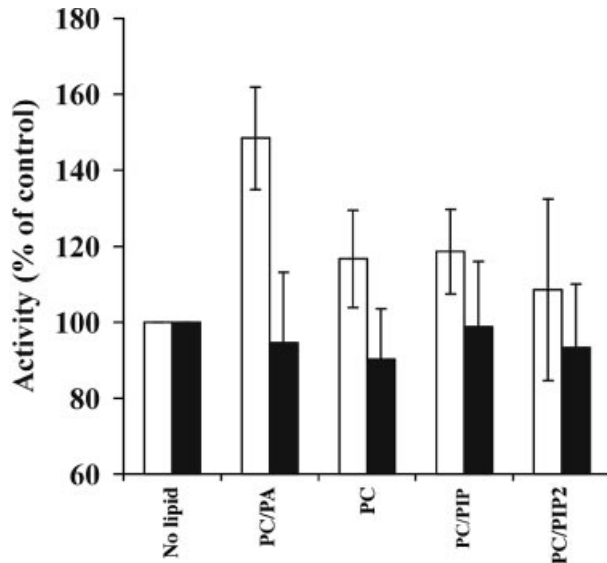
*Intrinsic GTPase activity of recombinant ADL6 does not require the presence of PRD, GED or PH domain*

In order to identify the minimal functional unit of ADL6 for GTPase activity, as well as to verify whether the various non-catalytic regions exert regulatory effects on GTPase, several C-terminus truncated versions (described in Figure 1) of GST-ADL6 were generated. Figure 5a shows a Coomassie blue-stained SDS-PAGE gel resolving the purified recombinant proteins from a prokaryotic expres-



**Figure 5.** Deletion mutants of ADL6 still possess GTPase activity. (a) GST-fusions of the various truncation mutants described in Figure 1 and GST were resolved on SDS-PAGE and visualized by Coomassie blue staining. (b) GST-fusions (0.5  $\mu$ M) of ADL6, ADL6- $\Delta$ 100, ADL6- $\Delta$ 204, ADL6- $\Delta$ 341, ADL6- $\Delta$ 662 and ADL6- $\Delta$ 697 were subjected to GTPase assay as described. Bars represent mean  $\pm$  SD ( $n = 5$ ).

sion system. Although some degradation products were observed for each of the truncated proteins, the original GST-fusions still represented the majority of proteins in each sample. Each recombinant protein was subjected to GTPase assays (Figure 5b). Notably, the activities of ADL6- $\Delta$ 100, ADL6- $\Delta$ 204 and ADL6- $\Delta$ 341 were not significantly



**Figure 6.** ADL6 GTPase activity was stimulated by phosphatidic acid. GST fusions of ADL6 (open bars) and ADL6-Δ341 (black bars) were preincubated with liposomes containing 100% PC; 90% PC with 10% PA; PIP; or PIP2 and tested for GTPase activity as described in Experimental procedures. GTPase activity was expressed as percentage of control reaction without any lipid. Bars represent mean  $\pm$  SD ( $n = 5$ ).

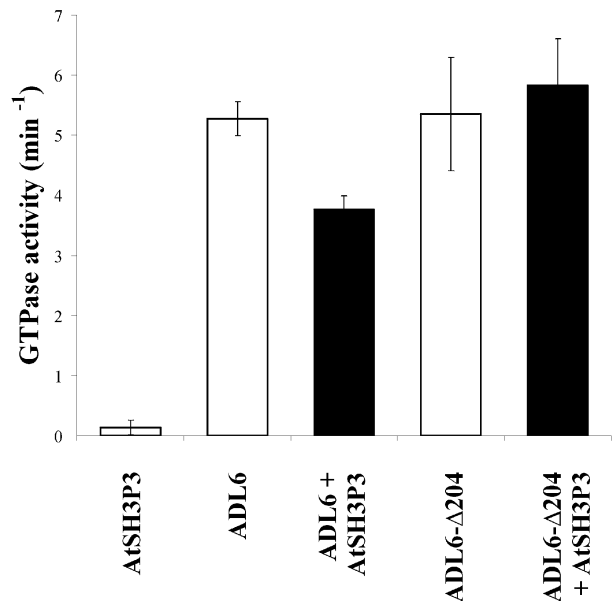
different from that of the full-length ADL6 (Student's *t*-test,  $P < 0.05$ ). This suggested that the elimination of PRD2, GED (including PRD1) and PH domain did not affect the GTPase activity of ADL6. On the other hand ADL6-Δ662, which contained only the predicted GTPase domain, had <50% of the full-length GTPase activity. As predicted, the elimination of 35 amino acids from the GTPase domain in ADL6-Δ697 almost completely abolished GTPase activity to the level of the negative control of GST (Figure 5b).

#### *ADL6 is activated by phosphatidic acid*

GST fusions of ADL6 and ADL6-Δ341 were incubated with liposomes made up of 100 or 90% PC with 10% PA, PIP or PIP2 prior to GTPase activity assays (Figure 6). PC, PC/PIP and PC/PIP2 slightly increased the full-length ADL6 GTPase activity by  $\approx$ 20% or less. On the other hand, in the presence of PC/PA the GTPase activity of ADL6 was stimulated by 48%. The activity of ADL6 in the presence of PC/PA was significantly greater than that in the presence of other phospholipid combinations (Student's *t*-test,  $P < 0.05$ ). In contrast, in the absence of the PH domain in ADL6-Δ341, the GTPase activity remained the same as control regardless of the type of liposome applied (Figure 6).

#### *GTPase activity of ADL6/AtSH3P3 complex*

In order to determine the physiological significance of the AtSH3P3-ADL6 interaction, we studied the effect of



**Figure 7.** GTPase activity of the ADL6/AtSH3P3 complex. 0.5  $\mu$ M of GST-ADL6 or GST-ADL6-Δ204 (lacking GED) was preincubated with (black bars) or without (open bars) 0.5  $\mu$ M AtSH3P3 and tested for GTPase activity as described in Experimental procedures. Bars represent mean  $\pm$  SD ( $n = 3$ ).

AtSH3P3 on ADL6 GTPase activity (Figure 7). Purified recombinant AtSH3P3 had a small but negligible GTPase hydrolysis activity (Figure 7) which may be due to the presence of a small quantity of co-purified bacterial GTPases. Notably, the incubation of ADL6 with AtSH3P3 rendered a rate of GTP hydrolysis that was significantly lower (Student's *t*-test,  $P < 0.05$ ) than GTPase activity of ADL6 alone (Figure 7). This change was obviously not due to merely adding two different proteins together, as adding another recombinant protein (GST or GST-CT2) did not alter ADL6 GTPase activity (results not shown). Moreover, the inhibition of GTPase activity on incubation of ADL6 with AtSH3P3 was drastically reduced when ADL6 was substituted with ADL6-Δ204 which lacked the AtSH3P3-binding GED/PRD2 (Figure 7).

#### **Discussion**

Dynamin plays an important role in vesicle trafficking in animals and yeast. It is one of the major mechanoenzymes that provides a driving force in membrane fission. In spite of their sequence similarity to other dynamins, dynamin-like plant proteins appear to have different cellular roles from the animal dynamins. Here we provide evidence supporting the notion that ADL6 is the plant dynamin-like protein that most resembles the animal counterpart. ADL6 co-fractionated with clathrin-enriched vesicles on a continuous sucrose density gradient; the C-terminus of ADL6 interacted with the clathrin-adaptor  $\gamma$ -adaptin; and the

GTPase activity of ADL6 could be regulated by phospholipids as well as an SH3 domain-containing protein.

*In vivo* targeting experiments using ADL6::GFP fusion by Jin *et al.* (2001) suggested the association of ADL6 with the Golgi. In addition, expressing a dominant negative mutant of ADL6 in *A. thaliana* blocked the trafficking from Golgi to vacuole (Jin *et al.*, 2001). The present immunolocalization study supports the above observations and the interaction between the C-terminus of ADL6 with  $\gamma$ -adaptin, a member of the adaptor complex of the clathrin-coated vesicle in the *trans*-Golgi network (Kirchhausen, 2000), provides additional evidence supporting the involvement of ADL6 in clathrin-mediated vesicle trafficking. The dynamin-dependent clathrin-coated vesicle transport from Golgi is still controversial in animals (Hinshaw, 2000), and it is not clear which dynamin-splice variants or their associated proteins are involved in this process. On the other hand, it was shown that clathrin-mediated vesicle trafficking from Golgi in yeast required Vps1p and Inp53p, which were similar to dynamin and synaptojanin, respectively (Bensen *et al.*, 2000). These results suggest the requirement of dynamin-like proteins for vesicular trafficking from the Golgi. The identification of other ADL6-binding partners may help elucidate the regulation of clathrin-coated vesicle formation from Golgi in plants. It is also intriguing that, even though endocytosis (Kubitscheck *et al.*, 2000) and clathrin-coated vesicles (Lam *et al.*, 2001) have been observed at the plasma membrane in plants, a dynamin-like protein associated with vesicle trafficking from plasma membrane to the endosome has not been found. Our fractionation studies and that of Jin *et al.* (2001) suggested that ADL6 co-migrated with the dense fractions (30–35% in Figure 3c) that also included the plasma membrane vesicles. In addition, we also detected the presence of ADL6 at the plasma membrane in our immunolocalization study. Whether ADL6 or another plant dynamin-like protein is responsible for vesicle trafficking from the plasma membrane is currently under investigation.

The identification of protein- and lipid-binding partners of ADL6 suggests, that similarly to animals, ADL6 participated in an accessory protein complex for vesicle trafficking. In fact, ADL6 is the only plant dynamin-like protein identified so far that has a domain organization identical to the animal dynamins – an N-terminus GTPase domain, a PH domain, a GED domain and a C-terminus PRD with putative SH3-binding sites. Also similarly to animal dynamins, ADL6 bound to acidic phospholipids via its PH domain. ADL6 also interacted with AtSH3P3, a protein with structural similarity (N-terminal coiled-coil domain and C-terminal SH3 domain) to the animal amphiphysin and endophilin, in an SH3-PRD-dependent manner. Similarly to the amphiphysin–dynamin interactions (McNiven *et al.*, 2000), AtSH3P3 may be involved in the recruitment of ADL6 to the accessory protein complex. Hence the pres-

ence of protein–protein or protein–lipid binding domains in ADL6 supports the notion that the accessory protein complex for vesicle trafficking is thoroughly co-ordinated via interactions that are mediated by several protein modules. This co-ordination allows for specific spatial and temporal associations between ADL6 and its binding partners and the control of the formation of a clathrin-coated vesicle.

The molecular network associated with other dynamin-like proteins was unknown until the recently published work on phragmoplastin (Hong *et al.*, 2001a; Hong *et al.*, 2001b). Instead of associating with vesicle trafficking proteins, phragmoplastin interacted with an enzymatic complex consisting of callose synthase (Hong *et al.*, 2001a) and UDP-glucose transferase (Hong *et al.*, 2001b). Together with its unique subcellular localization, phragmoplastin could be involved in the deposition of callose at the cell plate. The fact that phragmoplastin, ADL1 and ADL2 do not possess a similar domain arrangement to animal dynamins may correlate with functions other than vesicle trafficking. This suggests that plant dynamin-like proteins might be divided into two subclasses: PH-containing GTPases (ADL6) associated with endosomal vesicle trafficking; and non-PH-containing GTPases (phragmoplastin, ADL1 and ADL2) associated with trafficking in other compartments and/or other cellular processes. Studies of other PH-containing GTPases such as ADL3 could help to verify this hypothesis.

Lipid-binding assays suggested that the ADL6 PH domain had affinity to acidic phospholipids including PA, PIP and PIP<sub>2</sub>. Moreover, the GTPase activity of ADL6 was significantly increased in the presence of PA, and the activation was dependent on the presence of the PH domain. These results contrast with those reported in animal dynamins, where GTPase was activated by phosphoinositides to the greatest extent (Barylko *et al.*, 1998). Although no evidence suggested the regulation of animal dynamin GTP hydrolysis by PA, it was shown that the decrease in PA inhibited the formation of vesicles (Chen *et al.*, 1997). Notably, the presence of PA allowed for the maximum penetration of dynamin into lipid monolayers (Burger *et al.*, 2000). The affinity of ADL6 to PA may have two physiological consequences: (i) PA promotes a negative membrane curvature that results in severe membrane bending, thus providing a docking site for the PH domains and the recruitment of ADL6 to the membrane; (ii) the local increase in PA at the sites of invagination would also ensure that ADL6 assumes the highest GTPase activity at the correct subcellular location for membrane fission. Increasing evidence suggests that PA is an important second messenger of various signalling pathways in plants (reviewed by Munnik, 2001). Local variation of PA could be regulated by either the phospholipase D or the phospholipase C/DAG kinase pathway. Both pathways are



known to be responsive to various external signals (Munnik, 2001). Hence the regulation of ADL6 activity by PA may provide a link between signal transduction and vesicle trafficking. Although the activity of ADL6 was not affected by phosphoinositides, the *in vitro* binding assays suggested the affinity of the PH domain of ADL6 to these lipids. A similar study on the PH domain of ADL2 also suggested its binding to phosphoinositides (Kim *et al.*, 2001). Phosphoinositides are known to maintain the stability of the coat proteins of a vesicle (Martin, 1998), and fluctuation of these lipids may alter the degree of association of ADL6 to the coated vesicles.

The GED and PH domains of animal dynamins have been postulated to be critical for dynamin GTPase activity (Hinshaw, 2000). As discussed above, the binding of phospholipids to the PH domain could lead to the intramolecular stimulation of dynamin GTP hydrolysis. Whether the GED domain could regulate dynamin activity remains controversial. One view suggests that the GED could bind the GTPase domain (Muhlberg *et al.*, 1997), and the GED could act as a GTPase-activating protein (GAP; Barylko *et al.*, 2001). Different conclusions were reached in a more recent study using various mutated dynamins in similar assays (Marks *et al.*, 2001). In particular, it was shown that a GED-defective dynamin had similar GTPase activity to that of the wild type. Our own data support the latter view, as deleting the GED of ADL6 did not affect its activity. Under our assay conditions, none of the non-catalytic domains was required for full ADL6 activity. ADL6- $\Delta$ 662 (containing the predicted GTPase domain only) displayed a relatively lower GTPase activity, suggesting the presence of other regulatory elements responsible for full activity between the GTPase domain and the PH domain in ADL6. On the other hand, it is also possible that the minimal active unit of the GTPase domain is larger than that predicted by the program SMART.

However, the GED of ADL6 may still contribute to the regulation of GTPase activity of the ADL6 protein complex. This was suggested by our *in vitro* assays, where ADL6 GTP hydrolysis rate was significantly lower in the presence of AtSH3P3. On the other hand, AtSH3P3 did not affect the GTPase activity of a truncated form of ADL6 lacking the GED (Figure 7). A possible explanation for these observations is that, under normal conditions, GED binds to – or is in close proximity to – the GTPase domain (Muhlberg *et al.*, 1997), and this association does not affect GTPase activity. However, the binding of AtSH3P3 to GED may alter the GED/GTPase interaction and negatively regulate the GTPase activity. On the other hand, AtSH3P3 could be linked physically to the GTPase domain via GED and exert an inhibitory effect. The negative effect of AtSH3P3 on ADL6 activity contrasted with the results observed in the SH3-PRD interactions in the animal dynamins, where the SH3 domain of Grb2 (growth factor receptor-bound

protein 2) was shown to stimulate dynamin activity (Barylko *et al.*, 1998). The region of interaction between Grb2 and dynamin is distinct from the GED at the C-terminus region.

It has been suggested that the GED could bind to itself, resulting in the assembly of dynamins into tetramers or other higher-order structures (Okamoto *et al.*, 1999). Such helical structures may allow dynamin to wrap around the invaginating coated pits and provide the constriction needed for vesicle budding. On the other hand, Marks *et al.* (2001) suggested that the oligomerization of dynamin may not be important for the fission stage, as expression of dynamin with mutated GED did not alter endocytosis. More work is needed to establish the role of GED in dynamin activity and dynamin oligomerization in plants.

In conclusion, we have provided evidence suggesting mechanisms for the regulation of a plant dynamin-like protein, ADL6, by its interacting partners. The diversity of domains and/or structural arrangements of plant dynamin-like proteins suggest that the mode(s) of ADL6 control may not be applied to other dynamin-like proteins. The lack of PH and SH3-binding domains in many dynamin-like isoforms, and the limited number of SH3 domain-containing proteins identified in plants (Lam *et al.*, 2001), suggest that the regulation of plant dynamin-like proteins could be as diverse as their cellular functions.

## Experimental procedures

### Isolation of ADL6 cDNA clone

Searches were performed for potential C-terminus SH3-binding PXXP motifs in the sequence of ADL-related genes and putative ADLs deposited in GenBank. This led to the identification of ADL6, first deposited as GenBank accession no. AF180732 and subsequently published (Jin *et al.*, 2001) during the preparation of this manuscript. The primers ADL6-FLF (5'-CGGAATTCATGGAGCGATCGATGAGTTG-3') and ADL6-CTR (5'-CCGCTCGAGGTTCTAATACCTGTAAGCTGA-3') were used to amplify a 2.7 kb fragment from an *Arabidopsis thaliana* seedling cDNA library (Kieber *et al.*, 1993) and used as a probe to screen the cDNA library. A cDNA clone encoding an amino acid sequence identical to that of Jin *et al.* (2001) was obtained from the screen and used as the template for subsequent cloning.

### Primers and constructs

ADL6-CT1 (5'-GGAATCTTGCAAAGGTTATCCAGGCT-3') and ADL6-CTR were used to amplify CT1, a 675 bp fragment that encodes the C-terminus 224 amino acids of ADL6. ADL6-CT2 (5'-GGAATCCGCGCAGCAGCTGCTCAAGT-3') and ADL6-CTR were used to amplify CT2, a 285 bp fragment that encodes the C-terminus 94 amino acids of ADL6. Both of these fragments were cloned in-frame with LexA activation domain in pJG4-5 (Clontech, Palo Alto, CA) to give pJG-CT1 and pJG-CT2. Both CT1 and CT2 were also cloned in-frame with LexA DNA-binding domain in pEG202 (Clontech) to generate pEG-CT1 and pEG-CT2.

ADL6-FLF and ADL6-CTR were used to generate FL, the DNA fragment encoding the entire length of ADL6. Various C-terminus truncation mutants of ADL6 were generated by amplification of PCR fragments of ADL6- $\Delta$ 100, ADL6- $\Delta$ 204, ADL6- $\Delta$ 341, ADL6- $\Delta$ 662 and ADL6- $\Delta$ 697 using ADL6-FLF and the reverse primers  $\Delta$ 100 (5'-CCGCTCGAGCTGCCTCGTGAGTTAGA-3'), ADL6-PHR (5'-CCGCTCGAGACTCTGCCTCATGGAACACT-3'),  $\Delta$ 341 (5'-CCGCTCGAGCTCCCCCTCAGGTCCAGCA-3'),  $\Delta$ 662 (5'-CCGCTCGAGTCCAGACTGTGCTGACGCAA-3') and ADL6- $\Delta$ 697 (5'-CCGCTCGAGTGCAAGGGCTTTCGAGTTCTC-3'), respectively. PH, a 426 bp fragment encoding the PH domain of ADL6 was generated by ADL6-PHF (5'-CGGAATTCGACCTGAGGGGAGATAACA-3') and ADL6-PHR. FL, the PCR fragments corresponding to the various truncation mutants, CT2 and PHR were all cloned in-frame with GST into pGEX-5  $\times$  1 (Amersham Pharmacia, Quebec) to give pGST-FL, pGST-ADL6- $\Delta$ 100, pGST-ADL6- $\Delta$ 204, pGST-ADL6- $\Delta$ 341, pGST-ADL6- $\Delta$ 662, pGST-ADL6- $\Delta$ 697, pGST-CT2 and pGST-PH, respectively.

A PCR fragment encoding the SH3 domain of AtSH3P3 was generated using P3-SH3 (5'-GGAATTCTCATACTTTCTTGCTGAGTGA-3') and P3-CTR (5'-ACGCGTGCAGTAACTTCAGCAGCAAA-3'). P3-NT (5'-GGAATTCATGGATGCGTTTAGAAGACAA-3') and P3-CTR were used to generate the fragment containing the coding sequence of the full-length AtSH3P3. P3-NT and P3-deltaC (5'-CCGCTCGAGCGTTTCTCTGAGCCGT-3') were used to generate a PCR product encoding AtSH3P3 lacking the C-terminus SH3 domain. All three aforementioned products were cloned in-frame with the LexA DNA-binding domain in pEG202 to generate pEG-SH3, pEG-P3 and pEG-NT, respectively.

#### Yeast two-hybrid screening

To study the potential interaction between the SH3 domain of AtSH3P3 and the C-terminus PXXP motifs of ADL6, bait plasmids pEG-SH3, pEG-P3 or pEG-NT were co-transformed with prey plasmids pJG-CT1 or pJG-CT2 into yeast strain EGY48 consisting of both  $\beta$ -galactosidase and *Leu* reporter (Finley and Brent, 1994). pEG202 and pJG4-5 were used as negative controls. As the expression of prey fusions was under the control of the *GAL4* promoter, the galactose-responsive interactions (reporter activity in the presence of galactose minus reporter activity in the absence of galactose) were quantified using a liquid assay, as described (Ausubel *et al.*, 1994).

To identify putative interactors to the C-terminus 94 amino acids of ADL6, pEG-CT2 and an *A. thaliana* pJG4-5 cDNA library were co-transformed into EGY48. Co-transformants were screened for the galactose-dependent activation of both *Leu* and  $\beta$ -galactosidase reporters. Plasmids of putative interactors were rescued and co-transformed with either pEG-CT1 or pEG-CT2 back into EGY48 to confirm the interactions. The interactions were quantified as described above.

#### Recombinant proteins and antibody generation

pGST-FL, pGST-ADL6- $\Delta$ 100, pGST-ADL6- $\Delta$ 204, pGST-ADL6- $\Delta$ 341, pGST-ADL6- $\Delta$ 662, pGST-ADL6- $\Delta$ 697, pGST-CT2 and pGST-PH were all transformed into *Escherichia coli* BL21 (pLys) and GST-fusions were purified as described previously (Aharon *et al.*, 1998). GST was not cleaved from the recombinant proteins, as we observed significant proteolysis in this process. As shown in another study, GST-fusions appear to have no effect on GTPase activity (Kost *et al.*, 1999).

Due to the similarity of the GTPase domain of ADL6 to other proteins in the dynamin superfamily, as well as the fact that the PH domain and the GEDs are very similar between ADL3 and ADL6, we generated antibodies against CT2, the 94 amino acids of the C-terminus unique region of ADL6. Serum from two independent Wistar rats injected with the GST-CT2 fusion was obtained and affinity-purified as described previously (Lam *et al.*, 2001).

#### Protein-gel blots

To verify the purity of the anti-CT2 antibodies, 10  $\mu$ g of total lysates of *E. coli* BL21 (pLys) expressing either GST or GST-CT2 were resolved on SDS-PAGE and subjected to protein-gel blot assay as previously described (Lam *et al.*, 2001) using a 1/1000 dilution. Protein gel blots using anti-GST antibodies (1/2000 dilution, Amersham, Pharmacia) were performed to verify the presence of the GST-fusions.

Protein-gel blots using total extracts from various *A. thaliana* tissues were performed as described (Lam *et al.*, 2001) using 15  $\mu$ g extract per lane. The dilution of anti-CT2 used was 1/300.

Fractions of microsomal membranes of *A. thaliana* resolved on a continuous sucrose density gradient were obtained as described previously (Lam *et al.*, 2001). Protein-gel blots of the various membrane fractions were performed as described (Lam *et al.*, 2001) using anti-AtSH3P3 (1/500 dilution, Lam *et al.*, 2001); anti-ADL6-CT2 (1/500 dilution); and anti-plant clathrin (1/2000 dilution, Blackburn and Jackson, 1996). Antibodies against the vacuolar Na<sup>+</sup>/H<sup>+</sup> exchanger, AtNHX1 (1/2000, Apse *et al.*, 1999), and the plasma membrane H<sup>+</sup>-ATPase, AHA3 (1/5000, Pardo and Serrano, 1989) were used as markers for fractions enriched in tonoplast and plasma membranes, respectively.

#### Electron microscopy and immunolocalization

Developing pollen was prepared for immunolocalization at the level of the transmission electron microscope as described by Lam *et al.* (2001). Sections were probed with anti-ADL6 and/or anticlathrin antibodies using 1 : 50 dilutions. Controls omitted either the primary or secondary antibody. Also, specificity of antibody distribution was quantitatively and statistically assessed on six pollen grains from three separate flowers as described by Lam *et al.* (2001).

#### In vitro lipid-binding assays

The lipid-binding ability of the PH domain of ADL6 was investigated as described previously (Lam *et al.*, 2001) using purified GST-PH domain from *E. coli* BL21(pLys) transformed with pGST-PH. Binding was detected by incubating with anti-GST antibodies (1/2000 diluted, Pharmacia) followed by incubating with anti-goat IgG secondary antibodies conjugated with horseradish peroxidase and subsequently detected by chemiluminescence. Negative controls using GST-AtSH3P1-SH3 domain (Lam *et al.*, 2001) were performed concurrently (results not shown).

#### GTPase activity

GTPase activity assays using charcoal (Aharon *et al.*, 1998) or organic extraction (Barylko *et al.*, 2001) to separate radioactive inorganic phosphate from GTP or GDP yielded low sensitivity. Instead, we applied a thin-layer chromatography-based method

(Damke *et al.*, 2001) to measure the GTPase activity of ADL6. For the time-course assays, 0.5  $\mu\text{M}$  of various GST-ADL6 truncated recombinant proteins or GST were incubated in the reaction buffer (20 mM Hepes pH 7.5, 2 mM EGTA, 1 mM  $\text{MgCl}_2$ , 1 mM DTT) to a final volume of 17.5  $\mu\text{l}$  at room temperature. The reaction was started by the addition of 2.5  $\mu\text{l}$  labelled GTP from a stock of 4 mM GTP (buffered by 10 mM TrisCl pH 7.5) and [ $\alpha$ - $^{32}\text{P}$ ]GTP (Amersham Pharmacia). At 0, 30 and 60 min after reaction initiation, 2  $\mu\text{l}$  of each reaction were withdrawn and spotted onto flexible cellulose polyethyleneimine TLC plates (Selecto Scientific, Norcross, GA). The plates were air-dried and developed in 1 : 1 volume of 1 M LiCl and 2 M formic acid. The plates were then air-dried and subjected to quantitative autoradiography using a phosphorimager (Bio-Rad, Hercules, CA). To determine the relative positions of GDP and GTP, 1  $\mu\text{l}$  50 mM unlabelled GTP or GDP was spotted on separate lanes and developed together with the reactions. The developed plate was sprayed with 0.002% (w/v) fluorescein solution and inspected for the position of each nucleotide using UV illumination.

In order to study the effect of phospholipids on ADL6 activity, liposomes containing 100 or 90% PC with 10% PA, PIP or PIP2 were generated and resuspended in reaction buffer. GST-ADL6 or GST-ADL6- $\Delta$ 341 (no PH domain) was preincubated with liposomes equivalent to 1  $\mu\text{M}$  phospholipid content for 10 min at room temperature before initiation of the reaction as described above. Activity was measured 60 min after reaction initiation. The effect of AtSH3P3 on ADL6 activity was studied as follows: 0.5  $\mu\text{M}$  GST-ADL6 or GST-ADL6- $\Delta$ 204 (no GED), 0.5  $\mu\text{M}$  AtSH3P3 or combinations of above were preincubated for 10 min at room temperature before initiation of the reaction. As a negative control, 0.5  $\mu\text{M}$  GST or GST-CT2 was preincubated with either ADL6 or AtSH3P3. Activity was measured 60 min after reaction initiation.

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