Chilling Tolerance in Arabidopsis Involves ALA1, a Member of a New Family of Putative Aminophospholipid Translocases

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The lipid composition of membranes is a key determinant for cold tolerance, and enzymes that modify membrane structure seem to be important for low-temperature acclimation. We have characterized *ALA1* (for *aminophospholipid ATPase1*), a novel P-type ATPase in Arabidopsis that belongs to the gene family *ALA1* to *ALA11*. The deduced amino acid sequence of ALA1 is homologous with those of yeast DRS2 and bovine ATPase II, both of which are putative aminophospholipid translocases. *ALA1* complements the deficiency in phosphatidylserine internalization into intact cells that is exhibited by the *drs2* yeast mutant, and expression of *ALA1* results in increased translocation of aminophospholipids in reconstituted yeast membrane vesicles. These lines of evidence suggest that *ALA1* is involved in generating membrane lipid asymmetry and probably encodes an aminophospholipid translocase. *ALA1* complements the cold sensitivity of the *drs2* yeast mutant. Downregulation of ALA1 in Arabidopsis results in cold-affected plants that are much smaller than those of the wild type. These data suggest a link between regulation of transmembrane bilayer lipid asymmetry and the adaptation of plants to cold.

INTRODUCTION

Low temperature is one of the major environmental factors limiting plant growth. Cold-sensitive plants are injured and stunted in growth when temperatures are well below those for normal growth but still above the freezing point (reviewed in Nishida and Murata, 1996; Pearce, 1999; Thomashow, 1999). Many tropical or subtropical plant species, including a large number of crops such as tomato, rice, cotton, cucumber, and maize, are susceptible to chilling injury, and substantial losses in productivity result from the inability of such crops to withstand cold stress. Normal functioning of integral membrane proteins such as transporters and receptor proteins depends on the fluidity of the membrane, which is strongly influenced at a given temperature by its lipid composition (Squier et al., 1988; Gasser et al., 1990; reviewed in Hazel, 1995).

A major factor determining the fluidity of lipid membranes is the degree of unsaturation of membrane lipids. Thus, membranes with unsaturated acyl chains in phospholipids remain fluid at lower temperatures than do membranes with saturated lipids (Kates et al., 1984; Cevc, 1991; Cossins, 1994). One of the best-documented responses of plants to chilling stress is the increase in polyunsaturated acyl chains of membrane phospholipids, which allows membrane fluidity to be maintained (Hugly and Somerville, 1992; Nishida and Murata, 1996). This response indicates that after transfer to the cold, plants may increase the amount of unsaturated lipids by upregulating the activity of desaturase enzymes. Accordingly, expression in tobacco of a desaturase gene from a cyanobacterium results in increasing membrane lipid unsaturation in most membrane lipids concomitant with an increase in chilling tolerance (Ishizaki-Nishizawa et al., 1996).

The proportion of unsaturated fatty acids in the lipid acyl chains is particularly high in chloroplast membranes (Harwood, 1988). Several genetic loci (*FAD2*, *FAD3*, *FAD4*, *FAD5*, *FAD6*, *FAD7*, and *FAD8*) encoding enzymes involved in fatty acid desaturation of lipids in chloroplast or microsomal membranes have been identified (Browse and Somerville, 1991; Somerville and Browse, 1991). Although these mutants do not exhibit a visible phenotype when grown at 22°C, some of them, such as *fad5* (defective in chloroplast Δ12 desaturase) and *fad2* (defective in microsomal Δ12 desaturase), show diminished growth and partial chlorosis when grown at 5°C (Hugly and Somerville, 1992; Miquel et al., 1993). In addition, tobacco plants overexpressing the Arabidopsis *FAD7* gene (coding for the chloroplast ω-3 desaturase) have enhanced cold tolerance (Kodama et al., 1994). Extensive unsaturation of phosphatidylglycerol, an abundant

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Table 1. Overview of the *ALA* Gene Family in Arabidopsis

GenBank Accession No.	Swiss-Prot Accession No.	Suggested Gene Name	Chromosomal Location (Centimorgans)[a]	Mol. Wt.[b]	Length (Amino Acids)	pI
AB005245	P98204	*ALA1*	5.15	130,329	1158	5.9
AB005239	P98205	*ALA2*	5.94	124,836	1107	5.9
AC007258	Q9XIE6	*ALA3*	1.93	127,169	1123	7.7
AC022492	Q9LNQ4	*ALA4*	1.23	138,189	1216	7.5
AC010926	Q9SGG3	*ALA5*	1.115	139,341	1228	6.9
AC005287	Q9SLK6	*ALA6*	1.83	140,682	1244	7.1
AB019229	Q9LVK9	*ALA7*	3.20	140,928	1247	7.2
AP000371	Q9LK90	*ALA8*	3.48	135,309	1189	6.2
AC008075	Q9SX33	*ALA9*	1.104	136,045	1200	5.9
AP001313	Q9LI83	*ALA10*	3.38	136,279	1202	5.7
AC007357	Q9SAF5	*ALA11*	1.16	136,584	1203	6.5

[a] The position in centimorgans is the position of the nearest known genetic marker according to the Arabidopsis Information Resource (http://www.arabidopsis.org).

[b] Mol. Wt., calculated molecular weight.

phospholipid in thylakoid membranes (Harwood, 1988), correlates with improved chilling tolerance of tobacco (Murata et al., 1982; Murata, 1983). Furthermore, overexpression of glycerol-3-phosphate acyltransferases in tobacco, which leads to decreased concentrations of saturated species of phosphatidylglycerol, results in plants that are more tolerant to chilling (Murata et al., 1992; Wolter et al., 1992; Moon et al., 1995).

For animal, bacterial, and viral systems, various membranes are known to have a characteristic asymmetry of fluidity (e.g., Cogan and Schachter, 1981; Seigneuret et al., 1984; Foley et al., 1986; Dudeja et al., 1991; Kitagawa et al., 1991, 1998; Julien et al., 1993; Müller et al., 1994; Schroeder et al., 1995; Igbavboa et al., 1996). Variation of lipid fluidity between individual hemileaflets may therefore be a general feature of biological membranes. Asymmetry in lipid fluidity of the two leaflets appears to be associated with an asymmetric phospholipid headgroup composition (reviewed in Hazel, 1995). For example, an increased content of phosphatidylcholine (PC) in the outer leaflet and of anionic phospholipids in the inner leaflet has often been associated with distinct lipid fluidity of individual leaflets of biological membranes. Interestingly, in several poikilothermic organisms, asymmetric alterations in membrane phospholipid headgroup composition are associated with low-temperature adaptation (Hazel, 1995; Miranda and Hazel, 1996). In plants, asymmetric transbilayer distribution of phospholipids has been documented (Cheesebrough and Moore, 1980; Rawyler and Siegenthaler, 1981; Dorne et al., 1985; Tavernier and Pugin, 1995; O'Brien et al., 1997), but the physiological significance of the phenomenon is not known.

Although a thorough understanding of the mechanisms generating membrane lipid asymmetry has not emerged, some of the enzymes that may play a major role in these processes have been identified in nonplant systems (Dolis et al., 1997). Yeast protein DRS2 (Tang et al., 1996) and bovine ATPase II (Tang et al., 1996; Ding et al., 2000) are putative aminophospholipid translocases and may play a role in lipid flipping. Both are members of a distinct subgroup of P-type ATPases, type IV (P_4) ATPases (Axelsen and Palmgren, 1998), also referred to as third-type ATPases (Halleck et al., 1998). The *drs2* mutant displays less capacity for internalization of aminophospholipids into intact cells (Tang et al., 1996), impairment in the assembly of the 40S ribosomal subunit (Ripmaster et al., 1993), hypersensitivity to Zn^{2+}, Co^{2+}, Mn^{2+}, and Ni^{2+} but not to Ca^{2+} or Mg^{2+} (Siegmund et al., 1998), and inability to grow at temperatures colder than 23°C (Ripmaster et al., 1993). The latter phenotype suggests a role for DRS2 in cold tolerance of yeast.

Here, we have identified a gene family of P_4 ATPases in Arabidopsis (*ALA1* to *ALA11*; for aminophospholipid ATPase1 to 11). We provide genetic and biochemical evidence that *ALA1* encodes an aminophospholipid translocase and that this gene is involved in cold tolerance of Arabidopsis.

RESULTS

Cloning an Arabidopsis P₄-Type ATPase

Using database searches, we identified the presence of at least 11 P_4 ATPases in Arabidopsis (GenBank accession numbers AB005245, AB005239, AB007258, AC022492, AC010926, AC005287, AB019229, AP000371, AC008075, AP001313, and AC007357), distributed over three different chromosomes (Table 1). The derived amino acid sequences all include the typical conserved domains of the P-type ATPase superfamily, including the DKTGTLT aspartyl phos-

phorylation motif (Axelsen and Palmgren, 1998). Moreover, they also contain variants of the SPDEx(A/S)(F/L)(V/L) and GxT(A/G)(I/V)ED(K/R)LQ motifs, which are believed to be specific to P$_4$ ATPases (Axelsen and Palmgren, 1998; Halleck et al., 1998).

A full-length cDNA, *ALA1*, corresponding to a gene found on the genomic clone AB005245, was isolated (GenBank accession number AF175769) and found to encode a protein of 1158 amino acids with a predicted molecular mass of 130.3 kD. Hydrophobicity analysis of the ALA1 protein revealed a hydropathy pattern indicating 10 transmembrane helices (Figure 1A), a result very similar to those for the other
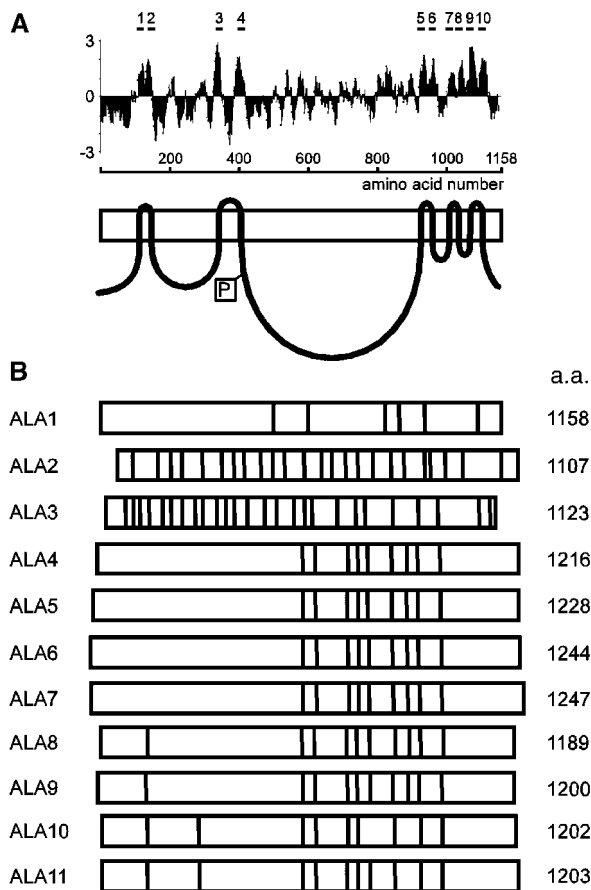

Figure 1. Sequence Analysis of Members of the *ALA* Gene Family.

(A) Prediction of putative membrane-spanning domains of ALA1. Hydropathy plots were performed with a window of 17 amino acids (Kyte and Doolittle, 1982). Predicted membrane-spanning domains were confirmed by referring to the P-type ATPase database (http://biobase.dk/~axe/Patbase.html) and are marked at top as bars designated 1 to 10. At bottom is shown a simplistic model of the protein. The boxed P denotes the phosphorylation site.
(B) Distribution of introns and exons in the *ALA* gene family. a.a., amino acids. The positions of introns are shown by a vertical line within a box representing the coding sequence.
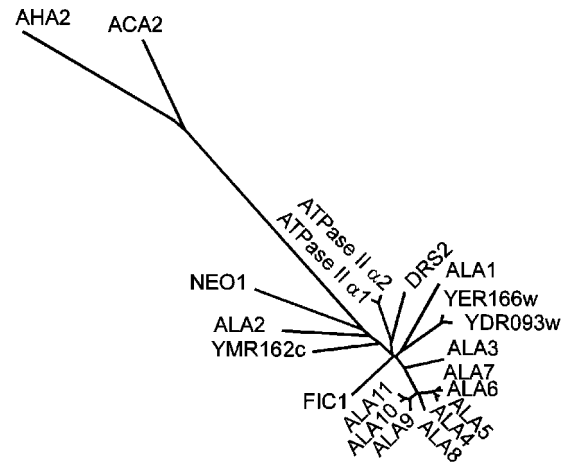

Figure 2. Phylogenetic Tree of Conserved Sequence Segments of Selected P-Type ATPases.

The sequences are as follows. Type P$_4$ ATPases: ALA1 to ALA11 (see Table 1 for accession numbers), *Arabidopsis thaliana*; YER166w (P32660), *Saccharomyces cerevisiae*; YDR093w (Q12675), *S. cerevisiae*; DRS2 (P39524), *S. cerevisiae*; ATPase II α1 (Q29449), *Bos taurus*; ATPase II α2 (Q9Y2Q0), *Homo sapiens*; FIC1 (O43520), *H. sapiens*; YMR162c (Q12674), *S. cerevisiae*; and NEO1 (P40527), *S. cerevisiae*. Type P$_{2B}$ ATPases: autoinhibited Ca^{2+}-ATPase ACA2 (O81108), *A. thaliana*. Type P$_{2A}$ ATPases: endoplasmic reticulum–type Ca^{2+}-ATPase (O04987), *A. thaliana.* Type P$_{3A}$ ATPases: plasma membrane H$^+$-ATPase AHA2 (P19456), *A. thaliana*. The tree is based on the Fitch–Margoliash and least-squares method (PHYLIP; Felsenstein, 1989). The conserved segments used in the analysis are defined in Axelsen and Palmgren (1998).

predicted ALA proteins (data not shown). The exon structures of the different *ALA* genes are highly variable, but the close resemblance between genes *ALA4* through *ALA11* indicates that those pumps form a subfamily (Figure 1B). A phylogenetic tree based on a comparison of the 11 ALA1-like amino acid sequences and other selected P-type ATPases is depicted in Figure 2. ALA1, ALA2, and ALA3 are the most divergent members of the ALA family. The closest relatives to ALA1 are yeast DRS2 and bovine ATPase II, whereas ALA4 through ALA11 cluster together and form a separate group in the phylogenetic tree.

Biochemical Properties of ALA1

Lipid internalization assays were performed to identify a biochemical function for ALA1. The outer leaflet of the yeast plasma membrane was labeled with the fluorescent phosphatidylserine (PS) and PC derivatives 2-(6-[7-nitrobenz-2-oxa-1,3-diazol-4-yl]-amino)hexanoyl (NBD)-PS and NBD-PC under conditions that block endocytosis, thus enabling measurements of the phospholipids being internalized by the cell (Tang et al., 1996). NBD-PS, but not NBD-PC, was
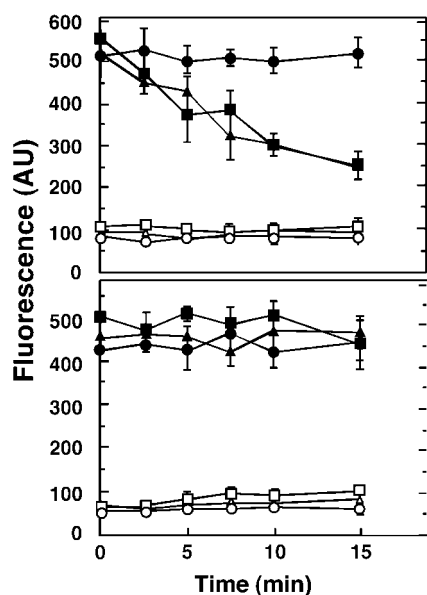
Figure 3. Influence of ALA1 Expression on Aminophospholipid Flipping in Intact Yeast Cells.

(Top) 2-(6-[7-nitrobenz-2-oxa-1,3-diazol-4-yl]-amino)hexanoyl (NBD)-phosphatidylserine (PS).
(Bottom) NBD-PC.
Internalization of NBD phospholipids by wild-type, *drs2*, and *drs2*-pYES2-*ALA1* yeast cells was assayed. The outer leaflet of the yeast cell plasma membrane was labeled with fluorescent phospholipid analogs, and internalization was measured as a function of time. Fluorescence intensities are indicated in arbitrary units (AU). Values are means ±SD for three independent experiments, each with duplicate samples. Transport of NBD-PS (top) and NBD-PC (bottom) is shown for wild type (filled squares), *drs2* (filled circles), and *drs2*-pYES2-*ALA1* (filled triangles). Background (fatty acid–free BSA was omitted from the assay) supernatant values are as follows: wild-type (open squares), *drs2* (open circles), and *drs2*-pYES-*ALA1* (open triangles) strains.

rapidly transported from the yeast surface in wild-type cells, although this transport was abolished in *drs2* cells (Tang et al., 1996) (Figure 3). However, as shown in Figure 3, the expression of *ALA1* in *drs2* cells restored internalization of NBD-PS from the outer leaflet of the yeast plasma membrane, a result indicating functional homology between the DRS2 and ALA1 proteins.

To assay the flippase activity more directly, we initially measured the transport of NBD phospholipids in reconstituted microsomes prepared from yeast cells expressing *DRS2*. The membrane-impermeable reagent sodium dithionite, which reduces the fluorescent NBD group of the NBD phospholipids into its nonfluorescent ABD form (McIntyre and Sleight, 1991), has been used to monitor transbilayer distribution of fluorescent lipids in vesicles (Suzuki et al., 1997). Using a similar approach, we measured the ATP-dependent changes in fluorescence of both the inner and outer leaflet of recon-

stituted NBD phospholipid–labeled yeast microsomes (Figure 4). Wild-type reconstituted yeast microsomal vesicles exhibited an ATP-dependent decrease in the amount of NBD-PS present in the inner leaflet, reflecting an outward-directed translocation of this phospholipid (Figure 4).

Several controls were performed to ensure that the lipid flipping in reconstituted yeast microsomes was dependent on the presence of the DRS2 protein (Table 2). NBD-PS transport was indeed markedly reduced in microsomal vesicles prepared from *drs2* yeast cells. As expected, reconstitution in the absence of protein led to symmetrical labeling of the two leaflets of the membrane vesicles. No substantial lipid flipping was observed during the assay in the protein-free reconstituted system, indicating that lipid transport is protein mediated. Aminophospholipid flipping was inhibited by orthovanadate, an inhibitor of P-type ATPases. Furthermore, no lipid flipping was observed in the reconstituted system in the presence of affinity-purified histidine-tagged plasma membrane H^{+}-ATPase (Lanfermeijer et al., 1998), indicating
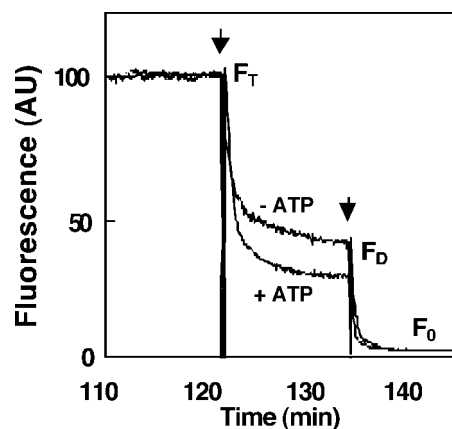

Figure 4. Measurement of NBD-PS Translocase Activity in Reconstituted Yeast Microsomal Vesicles.

The assay was based on the reduction of the fluorescent aryl-nitro group of NBD phospholipids into a nonfluorescent aryl-amino group. Fluorescence intensity was recorded for 10 min (F_T). The first arrow indicates addition of 30 μL of 1 M sodium dithionite in 1 M Tris-HCl, pH 10.0, to the reconstituted microsomes; the fluorescence was recorded after 10 min (F_D). Membrane-impermeable sodium dithionite reduces the fluorescent aryl-nitro group of NBD phospholipids present in the outer leaflet of the vesicle membrane (Suzuki et al., 1997). The second arrow indicates addition of 100 μL of 30% Triton X-100 to disrupt the microsomal vesicles and allow sodium dithionite to quench fluorescence from the inner leaflet–resident NDB phospholipids; the fluorescence was recorded (F_0). The fractions of NBD phospholipids present in the inner and outer leaflets were calculated from the following equations (Suzuki et al., 1997): percentage in the outer leaflet = $100([F_T - F_D]/[F_T - F_0])$; percentage in the inner leaflet = $100([F_D - F_0]/[F_T - F_0])$. −ATP, ATP was omitted from the assay; +ATP, ATP (3 mM) was included in the reaction mixture; AU, arbitrary units.

Table 2. NBD-PS Transport in Microsomal Vesicles Reconstituted in the Presence of Solubilized Membrane Proteins as Indicated

Controls	−ATP[a,b] (%)	+ATP[a,c] (%)
Yeast *DRS2* microsomes	48 ± 5	29 ± 4
Yeast *drs2* microsomes	51 ± 3	42 ± 6
Yeast *DRS2* microsomes + vanadate (200 μM)	46 ± 4	45 ± 3
No added protein	49 ± 3	51 ± 3
Plasma membrane H$^+$-ATPase	47 ± 3	46 ± 2

[a] Values are given as inner leaflet NBD-PS (%) and are the means ±SD of three measurements from three independent reconstitution experiments. Protein-to-lipid ratios were 1:15.
[b] ATP was omitted from the assay.
[c] ATP (3 mM) was added to start the reaction.

that lipid flipping is not an unspecific side activity of P-type ATPases in general.

After establishing the lipid transport assay, we attempted to measure aminophospholipid translocation in vesicles containing the ALA1 protein. In microsomal vesicles from *drs2* cells transformed with *ALA1*, the outward-directed translocation of NBD-PS was restored, indicating that PS translocation depends on ALA1 (Figure 5, top). No substantial ATP-dependent translocation of NBD-PC was observed with microsomes prepared from any of the three yeast strains used (Figure 5, middle), suggesting that ALA1 expression does not affect PC translocation.

Very small but significant (Student's *t* test, P < 0.05) ATP-dependent outward-directed transport of NBD-phosphatidylethanolamine (NBD-PE) was detected in microsomal vesicles from wild-type yeast cells but not from microsomes prepared from *drs2* cells (Figure 5, bottom). Moreover, expression of ALA1 in this strain restored ATP-dependent translocation of NBD-PE (Student's *t* test, P < 0.05), indicating that PE transport might also be affected by the ALA1 protein, albeit with a much lower efficiency than was PS.

ALA1 and Cold Tolerance of a Yeast *drs2* Mutant

To learn more about the physiological function of ALA1, we tested the effect of expressing *ALA1* in a *drs2* strain grown at 15°C. The yeast *drs2* mutant cannot grow below 23°C (Ripmaster et al., 1993). Figure 6 shows that *ALA1* complements the cold-sensitive phenotype of the mutant, supporting the notion that *ALA1* encodes a functional homolog of DRS2.

Cold Sensitivity in Transgenic Arabidopsis with Decreased ALA1

Because cold tolerance of yeast is influenced by the ALA1 protein, we investigated whether a similar function might exist for this polypeptide in Arabidopsis. Several homozygous

lines of *ALA1* antisense plants were generated. Among the 47 independent F$_4$ lines produced, four (lines 2, 7, 12, and 24) were selected for further analysis. All showed that the steady state amount of ALA1 mRNA was decreased by 60 to 80% as judged by semiquantitative reverse transcription–polymerase chain reaction (RT-PCR) (Figure 7).

When grown at normal temperatures (20 and 25°C for night and day periods, respectively), antisense plants retained wild-type rosettes, stem size, and number of siliques (Figure 8A and Table 3). However, when grown at chilling temperatures (8 and 12°C for night and day, respectively), all
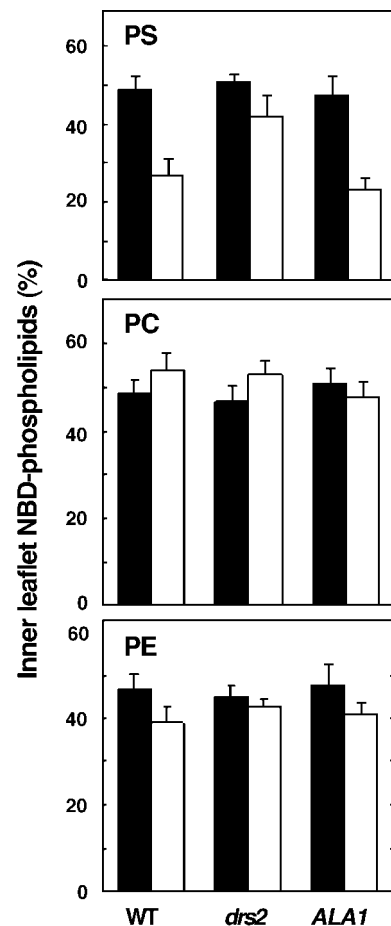
Figure 5. ATP-Dependent Translocation of NBD-Labeled Phospholipids by Reconstituted Microsomes Derived from Wild-Type, *drs2*, or *drs2*-pYES2-*ALA1* Yeast Strains.

(Top) NBC-PS.
(Center) NBD-PC.
(Bottom) NBD-PE.
Paired bars at left show results in wild-type yeast (WT); at center, *drs2* yeast; at right, *drs2* yeast expressing ALA1. Solid black bars show results without ATP; open white bars, results with ATP. Error bars indicate standard errors of the mean (*n* = 3).

four *ALA1* antisense lines had smaller rosettes, smaller stems, and fewer siliques than did the wild-type plants (line 7 is shown in Figures 8B and 8C and Table 3). This phenotype was still true when the plants reached the end of their life cycle, demonstrating that the growth of antisense plants was impaired rather than delayed. The phenotypes of the *ALA1* antisense plants strongly suggest that expression of this P_4 ATPase is important for cold tolerance of Arabidopsis.

ALA1 Expression in Arabidopsis Tissues

To examine the expression of the *ALA1* gene in Arabidopsis, we fused the *ALA1* promoter to the β-glucuronidase (*GUS*) reporter gene. Analysis of the GUS activity in transgenic plants transformed with this construct suggested that *ALA1* is expressed in a large number of tissues and cell types (Figure 9). In seedlings, the vascular tissue of the cotyledons, the root, and the cotyledonary node displayed high GUS activity (Figure 9A). Activity was low or absent in the emerging lateral roots, root hairs, and the hypocotyl. In the flower, GUS staining was apparent in stamens, particularly in the anthers (Figure 9B). In sepals, the reporter gene was expressed in vascular tissues, whereas expression was absent from petals.

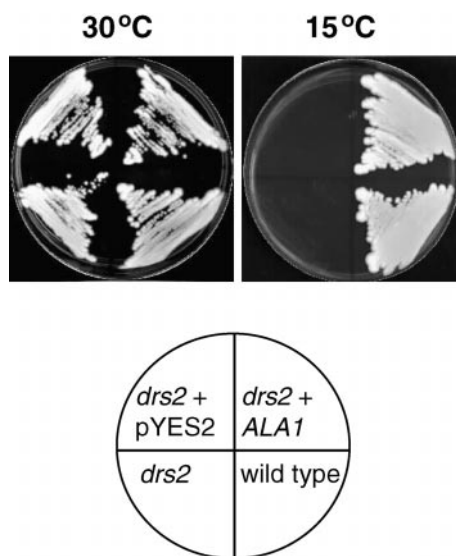To confirm these data, we isolated total RNA from roots,

Figure 6. Cold-Tolerant Phenotype of a *drs2* Yeast Mutant Complemented by *ALA1*.

The *drs2* null mutant (JWY2197; MATα *ura3-52 drs2::TRP1 leu2-1 his3-11 lys2-801*) was transformed with plasmid pYES2-*ALA1* (*ALA1*) or with the empty pYES2 vector. The wild-type yeast strain (DS94; MATα *ura3-52 trp1-1 leu2-3 his3-11 lys2-801*) served as a control. Yeast was plated onto amino acid–supplemented synthetic minimal medium containing 2% galactose. Cells were grown for 3 days at 30°C or for 14 days at 15°C.
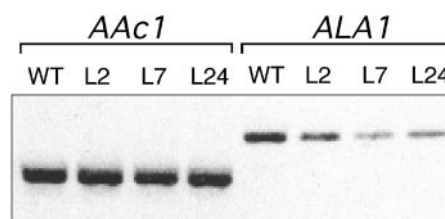

Figure 7. Decrease in Expression of *ALA1* in *ALA1* Antisense Arabidopsis.

ALA1-specific transcripts (*ALA1*) were amplified from total RNA derived from wild-type (WT) and different antisense plants (lines 2, 7, and 24 [L2, L7, and L24]) by RT-PCR. As an internal control, the actin *AAc1* transcript was amplified by RT-PCR.

stems, flowers, and rosette leaves. RT-PCR was used to generate cDNA and amplification products by using primers specific for *ALA1* and, as a control, primers corresponding to the actin AAc1 cDNA. Using this approach, we found that *ALA1* was expressed in all organs examined (Figure 10). Control experiments demonstrated that both the number of PCR cycles and the amount of RNA template used limited the amount of product obtained in a linear manner (data not shown). If reverse transcriptase was omitted during the RT step, no product was formed (data not shown), ruling out the possibility that a potential genomic DNA contamination in the RNA preparation influenced the results.

DISCUSSION

Identification of a Family of P_4 ATPases in Arabidopsis

By searching for *DRS2* homologs in the Arabidopsis genome, Axelsen and Palmgren (1998) identified a family (ALA) of relatively large membrane proteins with homology to P_4 ATPases. This family consists of at least 11 members in Arabidopsis, with two related genes also present in rice (data not shown). The exon structures of the various Arabidopsis P_4 ATPases suggest that both ancient gene duplications and more-recent duplication events have given rise to this novel family of putative transporters. Genome sequencing projects have also led to the identification of families of P_4 ATPases in eukaryotes such as yeast, nematodes, mice, and humans, whereas no bacterial homologs have been identified (Axelsen and Palmgren, 1998).

ALA1 Protein: A Putative Aminophospholipid Translocase

Two lines of evidence suggest that ALA1 functions as an aminophospholipid translocase. First, *ALA1* complements

the deficiency in lipid internalization by intact cells exhibited by the *drs2* yeast mutant. The complementation was assayed under conditions that limit endocytosis, suggesting that uptake is the result of transmembrane flipping. Second, in a reconstituted vesicle system, expression of *ALA1* increases the capacity for aminophospholipid translocation across the membrane.

Increasing evidence suggests that several other P$_4$ ATPases from animal and fungal systems are directly involved in aminophospholipid translocation. In animal cells, aminophospholipid translocases catalyze ATP-dependent aminophospholipid translocation and are sensitive to vanadate, an inhibitor of P-type ATPases (Seigneuret and Devaux, 1984; Daleke and Huestis, 1985; Zachowski et al., 1986). A candidate protein for this activity, the so-called ATPase II, has been purified from several sources, including chromaffin granules (Moriyama and Nelson, 1988), clathrin-coated vesicles (Xie et al., 1989), and the plasma membrane of erythrocytes (Morrot et al., 1990). The purified ATPase II is a PS-dependent and vanadate-sensitive ATPase, and membrane vesicles reconstituted with ATPase II purified from erythrocyte plasma membranes transport fluorescently labeled PS (Auland et al., 1994). Genes have been cloned encoding ATPase II of bovine chromaffin granules (Tang et al., 1996), bovine brain (Ding et al., 2000), and human tissues (Mouro et al., 1999). Several P-type ATPase consensus motifs have been identified in the sequences, hence identifying them as P$_4$ ATPases (Axelsen and Palmgren, 1998). Four isoforms of bovine brain ATPase II have been produced in a baculovirus expression system, and dephosphorylation of the phosphorylated enzyme intermediate has been demonstrated to depend strictly on the presence of PS (Ding et al., 2000), as expected for a P-type ATPase transporting this substrate.

The sequences of ATPase II are homologous to DRS2, a yeast P-type ATPase. The *drs2* mutant exhibits a decrease in the internalization of fluorescent-labeled PS supplied to the outer leaflet of the plasma membrane of intact cells (Figure 3) (Tang et al., 1996). Under certain conditions, *drs2* mutants exhibit no measurable deficiencies in lipid translocation (Siegmund et al., 1998; Marx et al., 1999). These negative results may indicate that other aminophospholipid translocases are present in yeast and can functionally replace DRS2 (Siegmund et al., 1998; Marx et al., 1999). This notion is supported by the presence of four P$_4$ ATPase genes (*YER166w*, *NEO1*, *YMR16cv*, and *YDR093w*; Swiss-Prot accession numbers P32660, P40527, Q12674, Q12675, respectively) in addition to *DRS2* (Swiss-Prot accession number P39524) in the yeast genome (Figure 2). Also, in some cases, the activity of the DRS2 protein may have escaped detection, depending on the experimental conditions. In our hands, the following criteria were critical for the lipid internalization assay: (1) cells used for experimentation had to be harvested in early mid-log-phase (OD$_{600}$ = 0.5). (2) The labeling of cells was optimal after 15 min; thus, the 1-min labeling time recommended by Tang et al. (1996) was insufficient.

Figure 8. Effect of Downregulation of *ALA1* in Arabidopsis.

Growth of wild-type (left) and *ALA1* antisense (line 7; right) plants at different temperatures.
(A) Normal temperature (20 to 25°C).
(B) Low temperature (8 to 12°C), lateral view.
(C) Low temperature (8 to 12°C), top view.
Bars = 5 cm.

Table 3. Growth Phenotypes of Wild-Type and *ALA1* Antisense Plants

Plants[a]	Growth Temperature °C	Stem Length (cm)	Rosette Diameter (cm)	No. of Siliques
Wild type	20–25	23.4 ± 3.9	7.2 ± 1.8	ND[b]
	8–12	22.1 ± 3.2	7.4 ± 2.1	348 ± 53[c]
Antisense line 7	20–25	21.8 ± 3.6	7.0 ± 1.6	ND
	8–12	8.1 ± 2.7[d]	2.8 ± 0.6[d]	77 ± 26[c]

[a] Measurements are the mean ±SD (*n* = 10).
[b] ND, not determined.
[c] *n* = 5.
[d] *n* = 12.

An asymmetric distribution of PS may affect the fluidity of plant membranes directly. However, PS accounts for <2% of total phospholipid extracted from leaves of numerous plant species (Delhaize et al., 1999). Because PS is a minor phospholipid in plant membranes, we cannot rule out the possibility that PS may influence cold sensitivity indirectly, possibly by interfering with elements of signal transduction pathways involved in cold adaptation. In animals, PS activates the signal transducer protein kinase C (Nishizuka, 1992), whereas in plants, this lipid activates a calcium-depen-
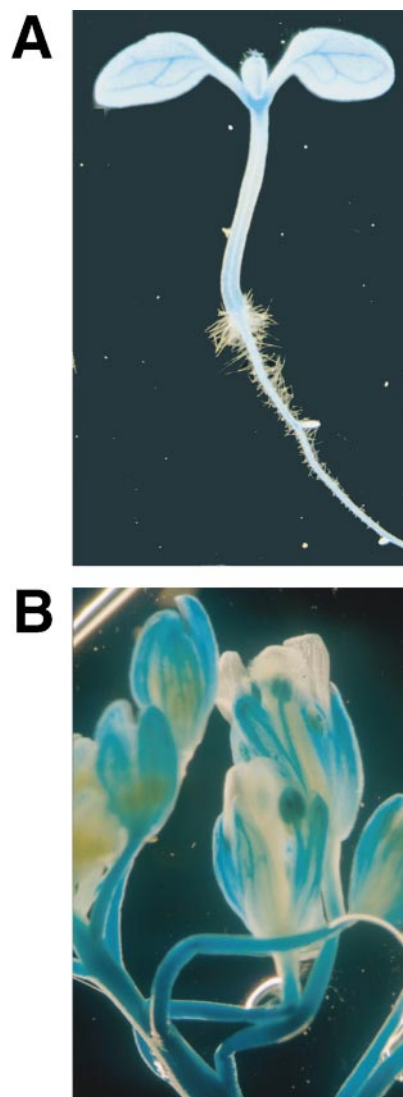
(3) The labeling had to be performed on ice; if labeling was performed at 30 or 23°C, temperatures that were used by others (Siegmund et al., 1998), it resulted in accumulation of the NBD lipid in *drs2* cells, probably as a result of endocytosis. (4) Measurements had to be restricted to a 12- to 15-min period; longer exposure times, such as the 30 to 35 min used by Siegmund et al. (1998) and Marx et al. (1999), led to an internalization of NBD-PS also in the *drs2* cells, albeit it occurred more slowly than it did in the wild type and without an immediate saturating uptake phase.

Because we have not characterized the activity of purified, reconstituted ALA1 protein, this polypeptide could in principle be involved indirectly in lipid flipping by influencing the amount or activity of an unidentified aminophospholipid transport system. However, the close homology between the sequences of the ALA proteins and those of ATPase II and DRS2 suggests a direct role in aminophospholipid translocation. Given the ability of ALA1 to complement lipid internalization in the yeast *drs2* mutant, we speculate that this protein is located in the plasma membrane. However, DRS2 is primarily localized to late Golgi vesicles in yeast (Chen et al., 1999), so an endomembrane localization of ALA1 cannot be ruled out. In this work, we have studied the function of ALA1 in more detail but have no evidence for the functions of ALA2 through ALA11. Perhaps specific expression of Arabidopsis P_4 ATPase isoforms is a means of ensuring optimal expressions of various specificities at specific subcellular locations and at different times of development.

Involvement of ALA1 in Cold Tolerance of Arabidopsis

For investigating the physiological role of ALA1 in the plant, ALA1 antisense Arabidopsis plants were generated. The smaller size of the antisense plants in comparison with wild-type plants grown at 8 to 12°C indicates that ALA1 plays a role in tolerance to chilling temperatures. The fact that these plants do not exhibit such a phenotype when cultivated at normal, nonchilling temperatures supports this hypothesis.

Figure 9. Activity of the *GUS* Reporter Gene in Transgenic Arabidopsis Containing the *pALA1–GUS* Fusion.

(A) GUS activity in a 6-day-old seedling.
(B) GUS expression pattern in flowers.

dent protein kinase (Szczegielniak et al., 2000). In animal and fungal plasma membranes, PS is normally distributed asymmetrically and is in general associated with the cytosolic face of the plasma membrane (Devaux, 1991; Schroit and Zwaal, 1991). The loss of this asymmetric distribution and the resulting appearance of PS at the cell surface allow phagocytes to identify cells that have undergone apoptosis (Fadok et al., 1992). Exposure of PS on the external face of the plasma membrane has also been associated with the apoptotic pathway in tobacco (O'Brien et al., 1998).

Whether the increased cold sensitivity of the ALA1 antisense plants is associated with alterations in transmembrane distribution of PE remains to be shown. Transbilayer redistribution of the aminophospholipid PE in response to cold has been reported before in poikilothermic animals (Miranda and Hazel, 1996). Several integral membrane enzyme activities, such as those of sarcoplasmic Ca^{2+}-ATPase (Navarro et al., 1984) and the transduction enzyme protein kinase C (Stubb and Slater, 1996), are reported to be highly sensitive to the amount of bilayer-destabilizing lipid, such as PE, present in the membrane. Perturbation of membrane-associated enzymes in cold stress, due to failure to achieve a transbilayer redistribution of PE, could impair the growth of *ALA1* antisense plants.

In conclusion, we have shown that ALA1, a putative aminophospholipid translocase, may play a role in cold tolerance in Arabidopsis. The actual mechanism by which ALA1 could participate in cold acclimation is not known. Differences in fatty acid compositions of particular membrane phospholipids, combined with transverse asymmetry, may result in differences in lateral diffusion and the local microviscosity of each hemileaflet of the membrane. Less-pronounced asymmetry would diminish this difference in fluidity of each leaflet. Local fluidity changes might affect the activity of important membrane proteins, and this in turn could influence the ability of the plant to adapt to cold. P$_4$ ATPases in Arabidopsis may play an important role in this process.

METHODS

Cloning ALA1

The 5' missing end of an *Arabidopsis thaliana* expressed sequence tag (N96084) with identity to the genomic clone AB005245 (GenBank accession number AB005245) was obtained by nested polymerase chain reaction (PCR) (Mundy et al., 1995), using the size-fractionated (3 to 6 kb) cDNA library CD4-16 as template (Kieber et al., 1993). The nucleotide sequence of the *ALA1* cDNA has been deposited in GenBank (accession number AF175769). The PCR-generated cDNA was directionally cloned into the yeast/*Escherichia coli* shuttle vector pYES2 (Invitrogen) for expression of *ALA1* under the control of the *GAL1* promoter, generating plasmid pYES2-*ALA1*. The *ALA1* cDNA from two independent PCR reactions was sequenced on both strands to ensure that no nucleotide substitutions had occurred during the PCR reaction.
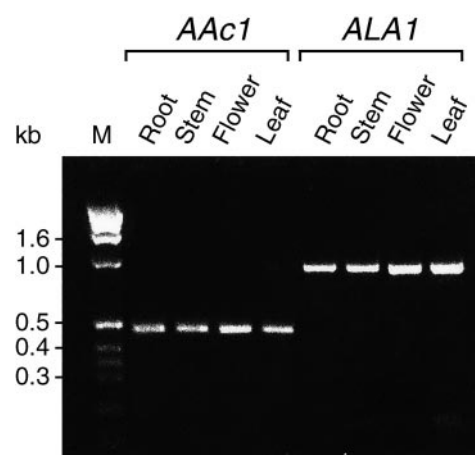

Figure 10. *ALA1* Expression in Various Organs of Arabidopsis.

ALA1-specific transcripts (*ALA1*) were amplified by RT-PCR from total RNA derived from different organs. The expected size of the resulting cDNA fragment was 839 bp. As an internal control, the actin *Aac1* transcript was also amplified by RT-PCR. Sizes of the molecular length standards (M) run in parallel are indicated in kilobases (kb) at the left.

Lipid Internalization by Intact Yeast Cells

Yeast (*Saccharomyces cerevisiae*) strains DS94 (wild type: MATα, *ura3-52*, *trp1-1*, *leu2-3*, *his3-111*, and *lys2-801*) (Tang et al., 1996) and JWY2197 (*drs2* null mutant: MATα, *ura3-52*, *drs2::TRP1*, *leu2-1*, *his3-11*, and *lys2-801*) (Tang et al., 1996) were transformed with the empty pYES2 vector or *pYES2-ALA1*. The lipid internalization assay was performed as described by Tang et al. (1996) but with some modifications. Thus, cells used for experimentation were harvested in early mid-log-phase (OD$_{600}$ = 0.5; Shimadzu UV-160 spectrophotometer, Kyoto, Japan), cells were labeled for 15 min and on ice, and measurements were restricted to a 12- to 15-min period. 2-(6-[7-Nitrobenz-2-oxa-1,3-diazol-4-yl]-amino)hexanoyl (NBD)-labeled phospholipids were purchased from Avanti Polar lipids (Lipids, Inc., Alabaster, AL).

Reconstitution of Yeast Microsomal Vesicles

Yeast cells were grown to an OD$_{600}$ of 0.5 in minimal medium containing 2% galactose and supplemented with amino acids. Membranes obtained from homogenized cells were subjected to differential and sucrose step-gradient centrifugation as described previously (Regenberg et al., 1995). Microsomes enriched in plasma membrane vesicles were collected from the 43 to 53% (w/w) sucrose interface. Mixed phospholipids (soybean asolectin type II-S; Sigma) were dispersed by vortex-mixing under argon for 10 min in the reconstitution buffer (10 mM Mes-KOH, pH 6.5, 50 mM K$_2$SO$_4$, and 20% [v/v] glycerol) at a final concentration of 30 mg/mL. Yeast microsomes (155 μg) were mixed with 2216 μg of asolectin and 116 μg of NBD-phosphatidylcholine (PC), NBD-phosphatidylserine (PS), or NBD-phosphatidylethanolamine (PE) (dried overnight under vacuum) in a final volume of 208 μL (lipid/protein mass ratio of 15:1). The protein/lipid mixture was solubilized by adding 12 μL of 1 M octylglucoside

(Boehringer Mannheim). Disposable 2-mL syringes fitted with glass wool at the bottom were filled with Sephadex G-50 gel (Pharmacia Biotechnology), equilibrated with reconstitution buffer, and centrifuged at 180g for 5 min at 4°C. The solubilized lipid/protein/detergent mixture (220 μL) was applied at the top of the column, and the column was centrifuged again for 7.5 min. This step removed the unincorporated NBD lipids and most of the detergent. Reconstituted membrane vesicles were then incubated for 30 min with 100 mg of Bio-beads (20 to 50 mesh; Bio-Rad) at room temperature in the presence of 1 mM phenylmethylsulfonyl fluoride and 10 μg/mL chymostatin to remove the remaining detergent.

Measurement of Phospholipid Flipping in Reconstituted Microsomal Vesicles

Reconstituted microsomes (30 μg of membrane protein) were incubated for 120 min at 25°C in a buffer containing 40 mM Tris-HCl, pH 7.4, 130 mM KCl, 4 mM MgCl$_2$, and 15 μM verapamil (to inhibit ATP-binding cassette transporters). ATP (3 mM) was added to start the reaction. Fluorescence emission of NBD phospholipids in both outer and inner leaflets of the microsomal vesicles was monitored essentially as described by Suzuki et al. (1997). The spectrofluorometer (Cary-17; Varian, Inc., Palo Alto, CA) was equipped with a fluorescence accessory to redirect light from the monochromator to the phototube. The monochromator was set at 470 nm, and a 495-nm sharp-cutoff glass filter (model GG945; Schott Glass Technologies, Duryea, PA) was placed in front of the phototube. When indicated, 10 mM sodium dithionite was added to reduce NBD phospholipids present in the outer leaflet of the microsomes. After the reaction, the microsomes were solubilized by adding 1% (w/v) Triton X-100 to allow dithionite to reduce the inner leaflet-resident NBD phospholipids.

Construction of a *pALA1–GUS* Fusion and an Antisense *ALA1* Construct

A promoter fragment of 2138 bp from the *ALA1* genomic sequence (*pALA1*, positions −2135 to +3) was amplified by nested PCR from a genomic library of Arabidopsis ecotype Columbia. In the first PCR reaction, the forward primer used was the synthetic oligonucleotide 5′-AAGTGAAGACAGAGTATTTAGCAG-3′; the reverse primer was 5′-CAATGGTTGATTAAAGCGTTGGAC-3′. In the second PCR reaction, the forward primer was 5′-GCAACTGCAGGTTAAACAAACTTGTAC-GAAAGCG-3′ (an introduced PstI site is underlined); the reverse primer was 5′-GAACCATGACGGATACGCTTAGAT-3′. The resulting fragment was digested with PstI and BamHI and subcloned into the PstI-BglII sites of the binary vector p3301 in front of the *E. coli GUS* gene terminated by the *nos* polyadenylation signal.

For antisense expression of *ALA1*, a 1235-bp BamHI (made blunt-ended)-SacI cDNA fragment (bp 3 to 1238) was cloned into the binary vector pCROX18 (a kind gift of J. Mundy) in the antisense orientation, leaving expression of the antisense fragment under the control of the strong 35S promoter.

Plant Transformation

Constructs for expression of the pALA1–GUS fusion and antisense ALA1 were introduced into *Agrobacterium tumefaciens* strain pGV3101 by electroporation and selected for resistance to kanamycin (50 μg/mL). Arabidopsis ecotype Columbia was transformed by vacuum infiltration, and seeds were selected by spraying with glufosinate ammonium (BASTA, Frankfurt, Germany) (*pALA1–GUS* fusion vector) or by selection on plates of MS medium (Murashige and Skoog, 1962) with kanamycin (50 μg/mL) (*ALA1* antisense vector). Individual transformed plants were selfed to select for homozygous plants.

Histochemical Localization of GUS Activity

Six-day-old seedlings grown under sterile conditions on an MS agar plate containing 1% sucrose (grown in 16 hr of light, 75% humidity, 100 μmol m^{-2} sec^{-1}) or flowers of 5-week-old soil-grown plants (also grown in 16 hr of light, 75% humidity, 100 μmol m^{-2} sec^{-1}) were rinsed with GUS buffer (100 mM sodium phosphate, pH 7.0, 1 mM EDTA, 1% Triton X-100, 3.5 mM K$_3$Fe[CN]$_6$, 3.5 mM K$_4$Fe[CN]$_6$, and 1 mg/mL 5-bromo-4-chloro-3-indolyl-[D-glucuronide]) before vacuum infiltration in the GUS buffer for 5 min. Plants were incubated at 37°C for 16 hr. Chlorophylls were removed with 70% ethanol, and the plants were photographed with an Olympus SZH binocular microscope (Olympus Optical Co., Hamburg, Germany).

RNA Analysis

With the RNeasy Plant Kit (Qiagen), Arabidopsis total RNA was extracted from soil-grown plants or from seedlings grown for 8 days in liquid medium (MS basic salt mixture supplemented with 1% [w/v] sucrose, with constant light). The RNA preparations were treated with RNase-free DNase (Promega) after isolation. Actin (*AAc1*)- and *ALA1*-specific transcripts were detected with reverse transcription (RT)–PCR by using the *rTth* RNA-PCR kit (Perkin-Elmer) according to the manufacturer's instructions. The primer pairs 5′-GTGCTC-GACTCTGGAGATGGTGTG-3′ and 5′-CGGCGATTCCAGGGAACA-TTGTGG-3′, and 5′-ACACTGCTTGGGCTTTAGG-3′ and 5′-TGC-TTGTTCACTAGGGGACTC-3′ were used to detect *AAc1* and *ALA1* transcripts, respectively, leading to amplification of 467-bp (*AAc1*) and 839-bp (*ALA1*) products from cDNA templates.

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