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RESEARCH ARTICLE

Expression and Regulation of *aERD2*, a Gene Encoding the KDEL Receptor Homolog in Plants, and Other Genes Encoding Proteins Involved in ER–Golgi Vesicular Trafficking

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***aERD2* and *aSAR1* of Arabidopsis are functional homologs of yeast genes encoding proteins essential for endoplasmic reticulum (ER)-to-Golgi transport. The regulation of these secretory pathway genes in yeast, mammals, and plants is not known. High levels of expression of *aERD2* and *aSAR1* were observed in roots, flowers, and inflorescence stems, with the highest levels being detected in roots. The *aSAR1* transcript levels were highest in young leaves and declined during leaf maturation. Low levels of *aERD2* were detected in both young and fully mature leaves when compared with roots. In situ hybridization showed that trichomes accumulate more *aERD2* transcript as the leaf expands, whereas *aSAR1* is expressed equally in all leaf cell types. Treating plants with tunicamycin, a drug that blocks N-glycosylation in the ER, or with cold shock, known to block secretory protein transport, led to a marked accumulation of *aERD2* and *aSAR1* transcripts. The Arabidopsis *ARF* gene, which encodes a GTPase probably involved in Golgi vesicle traffic, was not affected by these treatments. This study is an essential first step toward understanding the regulation of genes that encode proteins involved in vesicular trafficking.**

INTRODUCTION

The secretory pathway provides a common route for both proteins destined to be secreted and proteins that reside in the various organelles of the pathway itself. Membrane and lumenal proteins are transported between the various compartments of the secretory pathway by small carrier vesicles that bud from one compartment and fuse with another (Palade, 1975). Formation, budding, targeting, and fusion of these vesicles are mediated with the aid of cytosolic and membrane protein complexes (Rothman, 1994). The ability to discriminate between different protein populations requires specific recognition and/or sorting mechanisms to ensure correct compartmental localization along this pathway.

Specific C-terminal tetrapeptide motifs are found in many proteins that reside in the endoplasmic reticulum (ER) lumen: KDEL in mammalian cells (Munro and Pelham, 1987), HDEL in the yeast *Saccharomyces cerevisiae* (Pelham, 1988), and H/K/RDEL in plants (reviewed by Vitale et al., 1993). When the tetrapeptide is fused onto reporter proteins, it localizes them to the ER, showing that it is both necessary and sufficient for ER localization in mammals (Munro and Pelham, 1987), yeast (Dean and Pelham, 1990), and plants (Denecke et al., 1992). However, some of these K/HDEL proteins are post-translationally modified by enzymes in the Golgi apparatus (Dean and Pelham, 1990). This suggests that these proteins leave the ER and enter the Golgi apparatus, where they are sorted and returned. Genetic studies with yeast have identified the *ERD2* gene product (Erd2p) as the receptor for the retention signals of ER lumen proteins (Semenza et al., 1990). In yeast, the *ERD2* gene is essential for growth. At steady state, the receptor (Erd2p) localizes to the *cis* side of the Golgi apparatus and, upon ligand binding, redistributes to the ER. KDEL peptides bind to the receptor at slightly acidic pH (Wilson et al., 1993), suggesting that the pH gradient along the secretory pathway results in binding of the KDEL proteins in the Golgi apparatus and their release in the ER. The receptor is thought to contain seven membrane-spanning domains. Mutational analysis revealed that the seventh transmembrane domain of Erd2p is important for its targeting/recycling function (Townsley et al., 1993).

In contrast to our extensive knowledge of the secretory protein machinery in mammals and yeast, little is known about this system in plants. In addition, little is known about the regulation of these secretory pathway genes in yeast, mammals, and plants. Recently, several plant homologs of genes encoding proteins involved in ER-to-Golgi traffic in yeast have been isolated from plants (d'Enfert et al., 1992; Cheon et al., 1993) and found to complement the corresponding yeast mutants. We have recently isolated a cDNA clone from Arabidopsis (*aERD2*) that encodes a protein homologous to its yeast and

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mammalian counterparts (Lee et al., 1993). The *aERD2* gene functionally complements the yeast mutant lacking *ERD2*, whereas the human homolog does not (Lewis and Pelham, 1990). Nothing is known regarding the function of aErd2p in plants. As an initial step toward understanding the regulation of the secretory pathway in plants, we studied the expression of *aERD2* in relation to Arabidopsis genes *aPDI* (for protein disulfide isomerase), which encodes a protein residing in the ER, and *aSAR1* (for secretion-associated and Ras superfamily-related gene) and *aSEC12*, which encode homologs of proteins involved in transport between the ER and Golgi apparatus in yeast (Kaiser and Schekman, 1990; d'Enfert et al., 1992). These genes are compared with *aARF* (for ADP-ribosylation factor), which encodes a protein that is presumably involved in mediating traffic through the Golgi apparatus (Serafini et al., 1991).

RESULTS

Development, Expression, and Distribution of *aERD2*

We examined steady state levels of *aERD2* transcript in Arabidopsis leaves and roots at various stages of development, and in inflorescence stems and flowers. Total RNA samples were prepared from these tissues, separated by electrophoresis, and hybridized with various DNA probes. The expression of the Arabidopsis *aERD2* gene in roots was approximately twice that in flowers or in the inflorescence stem (Figure 1A). Very low levels of *aERD2* transcript (approximately sixfold less than in roots) were detected in both young and fully mature leaves (Figure 1A). The levels of *aERD2* transcripts in the roots were similar at different stages of development (2- and 12-week-old plants). Data in Figure 1 are from tissues of plants grown in agar cubes. Similar results were obtained using tissues of plants grown in soil or liquid media (in liquid-grown plants, only roots and leaves were analyzed), indicating that growth conditions did not affect *aERD2* transcript levels.

We wanted to determine whether the expression pattern of other genes that encode proteins involved in either ER-to-Golgi transport or between-Golgi cisterna is similar to that found with *aERD2*. The *SAR1* gene product in yeast encodes a small GTPase involved in the budding of transport vesicles from ER to Golgi (Nakano and Muramatsu, 1989). A functional homologous *aSAR1* cDNA isolated from Arabidopsis (d'Enfert et al., 1992) was used. Like *aERD2*, *aSAR1* was highly expressed in roots (Figure 1B), but in leaves the level of *aSAR1*, unlike *aERD2*, decreased when the leaf matured (Figure 1B). The amount of *aSAR1* mRNA in young leaves was similar to that found in the flowers and stems but three times less than that found in the roots. The relatively low transcript levels of genes encoding proteins that mediate trafficking between the ER and Golgi apparatus in the leaves are not unique to *aERD2* and *aSAR1*. In fact, similar results were also observed with *aSEC12* (i.e., low transcript levels in leaves and higher levels in roots; data not shown), which encodes a membrane protein that mediates vesicle budding from the ER (d'Enfert et al., 1992).

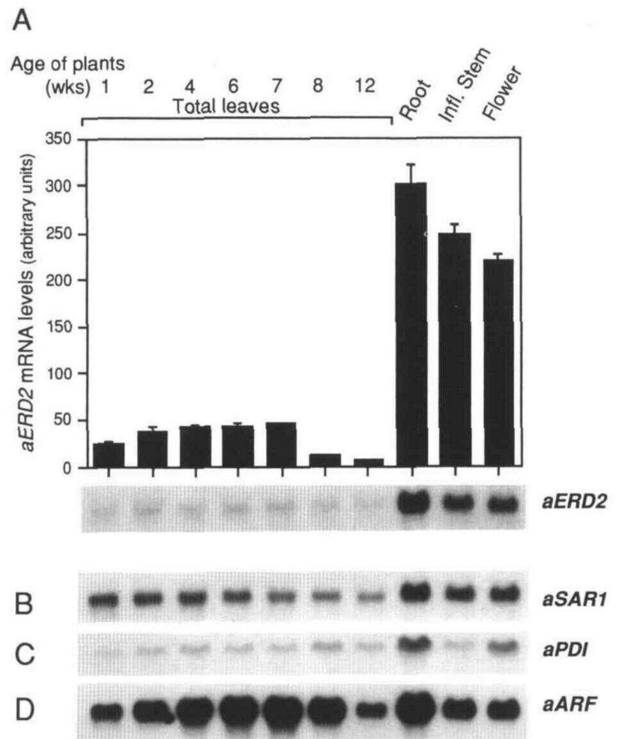

Figure 1. Tissue Expression of Genes Encoding Proteins That Mediate Vesicle Transport between the ER and Golgi Apparatus throughout Arabidopsis Development.

Whole leaves from Arabidopsis (ecotype RLD) plants grown on agar were collected at different times. Root, flower, and inflorescence (Infl.) stem tissues were collected from 12-week-old plants. RNA was isolated, and equal amounts were separated by electrophoresis on a formaldehyde-agarose gel and blotted onto a nylon membrane. The membrane was hybridized with *aERD2*, *aSAR1*, *aARF*, or *aPDI* ^{32}P-labeled probes. RNA gel blots were scanned with a PhosphorImager. The intensity of the signals was quantified by scanning densitometry. The data were derived from at least four independent experiments. Standard error values are indicated as bars.

(A) Histogram summarizing the relative amounts of *aERD2* transcripts. A representative autoradiogram of an RNA gel blot probed with *aERD2* is shown below the histogram.

(B) RNA gel blot probed with *aSAR1*.

(C) RNA gel blot probed with *aPDI*.

(D) RNA gel blot probed with *aARF*.

Two additional secretory pathway genes were chosen for this study. The first was a gene from Arabidopsis that encodes the homolog for ARF1, a small GTP binding protein involved in mediating traffic presumably through the Golgi apparatus (Serafini et al., 1991). The second control was the Arabidopsis gene *aPDI*, a resident KDEL-containing protein of the ER lumen that functions to form correctly disulfide bridges in membrane and soluble proteins (Noiva and Lennarz, 1992).

Unlike *aERD2*, *aSAR1*, and *aSEC12* transcripts, the mRNA of *aARF* is abundant and seems to be highly expressed in all of the tissues examined (Figure 1D; leaves, roots, stems, and flowers). The transcript level of *aPDI* was found to be four times lower in leaves throughout development when compared with roots (see Figure 1C).

These results indicate that root cells may have a higher secretory activity than leaves. However, the question remains whether the low level of the transcripts is found in all leaf cell types.

Distribution of *aERD2* mRNA in Plant Tissues

The spatial distribution of *aERD2* transcripts was examined in leaf tissues by in situ hybridization using a full-length *aERD2* antisense RNA probe. In 2-mm-wide leaves, *aERD2* mRNA transcript was found in all cell types at equivalent levels (Figure 2A). As the leaf matured (4 to 6 mm; ~3-week-old plants), *aERD2* distribution changed and trichomes had higher levels of this transcript relative to other cell types (Figure 2B). The negative control, the section probed with sense RNA, had no significant or unique labeling (Figure 2C).

In root tissues, a strong *aERD2* transcript signal was localized to the root tip and lateral root sections (Figure 2D), and mainly to the cortex and endodermis layers (Figure 2E). In the inflorescence stem, an *aERD2* signal was observed in the vascular bundles, epidermis, and adjacent cell layer (Figure 2G, negative control Figure 2H). Except for the anther, all flower parts (sepal, petal, carpel, and filaments) expressed the *aERD2* transcript (Figures 2I and 2J). As controls, *aPDI* and *aSAR1* antisense RNA probes were used and were equally expressed in all leaf cell types (Figures 3A and 3B). Unlike *aERD2*, the distribution of *aPDI* and *aSAR1* transcripts was not altered upon leaf maturation. Thus, the in situ studies indicated that certain cell types (root tips and trichomes of leaves) have higher secretory activity than other cell types have.

Induction of *aERD2* Transcript under Stress Conditions

Although *aERD2* can function as a yeast-sorting receptor (Lee et al., 1993), this alone does not prove that it performs an analogous role in plant cells. To verify its involvement in the secretory pathway, we treated plants under conditions that should have affected events along the secretory pathway. Tunicamycin, an inhibitor of core glycosylation in the ER, has been found in many organisms, including plants, to increase the levels of PDI and BiP, which are KDEL-containing proteins involved in the correct folding of proteins in the ER (Fontes et al., 1991; Shorrosh and Dixon, 1991; D'Amico et al., 1992). We speculated that treatments causing an increase in ER-soluble K/HDEL protein levels could affect the expression of the *aERD2* transcript as well. Therefore, Arabidopsis plants were treated with tunicamycin, and then roots and leaves were separated prior to RNA extraction. A more than twofold increase of *aERD2* mRNA levels was detected in leaves of plants treated with tunicamycin when compared with control plants (Figure 4A). The effect of tunicamycin treatment on the expression of *aERD2* in roots was not as pronounced, with an increase in transcript level of only one-half compared with that of the control (Figure 4A). The same blots were reprobed with an Arabidopsis *aPDI* clone to verify that the transcript level of this ER-resident protein also increased following treatment. Indeed, an increase of ~14-fold in levels of *aPDI* transcript was observed, as compared with levels in the control for both root and leaf tissues (Figure 4C). In tunicamycin-treated plants, the amount of *aSAR1* transcript also increased. A more than twofold increase in the level of *aSAR1* transcript was observed in roots and an over sixfold increase in the level in leaves when compared with those in control plants (Figure 4B). However, *aARF* transcript levels in roots and leaves were not affected by tunicamycin treatment (Figure 4D).

At a low temperature, proteins en route from the ER to the Golgi apparatus accumulate in an intermediate compartment in yeast and mammalian tissues (Saraste and Kuismanen, 1984). Arabidopsis plants placed for 12 hr at 8°C increased up to three times the level of *aERD2* mRNA in leaves and up to two times the level of transcript in roots when compared with levels in untreated plants (Figure 4A). Cold treatment led to a twofold increase of *aSAR1* transcripts in the leaves compared with those in the control but did not seem to affect *aSAR1* expression in the roots (Figure 4B). Low-temperature treatment did not significantly alter the transcript levels of *aARF* either in roots or leaves (Figure 4D).

Heat shock treatment was used to verify that the induction of *aSAR1* and *aERD2* was not the result of a general stress but rather of a specific one. Heat shock treatment (Figures 4A to 4D) had little effect on *aERD2*, *aARF*, and *aPDI* transcript levels in leaves (Figure 4) of Arabidopsis plants. In roots, heat shock treatment slightly altered *aERD2* and *aARF* transcript levels, whereas the transcript levels of *aSAR1* and *aPDI* were reduced (0.1- to 0.2-fold) compared with controls.

The specific induction of *aERD2*, *aSAR1*, and *aPDI* upon tunicamycin and cold treatments strongly suggests that these genes do indeed encode proteins that function between the ER and Golgi.

Expression and Distribution of *aERD2* in Trichome Mutants of Arabidopsis

The increased accumulation of *aERD2* transcripts in the trichomes upon leaf maturation indicates that some factors in trichome cells may regulate the expression of *aERD2* and possibly other genes encoding secretory proteins. To address this question, we first monitored *aERD2* levels in mutant Arabidopsis plants, *glabrous-1* (*gl1*), which lacked trichomes (Koornneef and Hanhart, 1983) or in transgenic plants, 35S-*R*, which produced extra trichomes compared with the wild type (Lloyd et al., 1992). The *R* gene from maize encodes a helix-loop-helix protein required to activate genes involved in anthocyanin

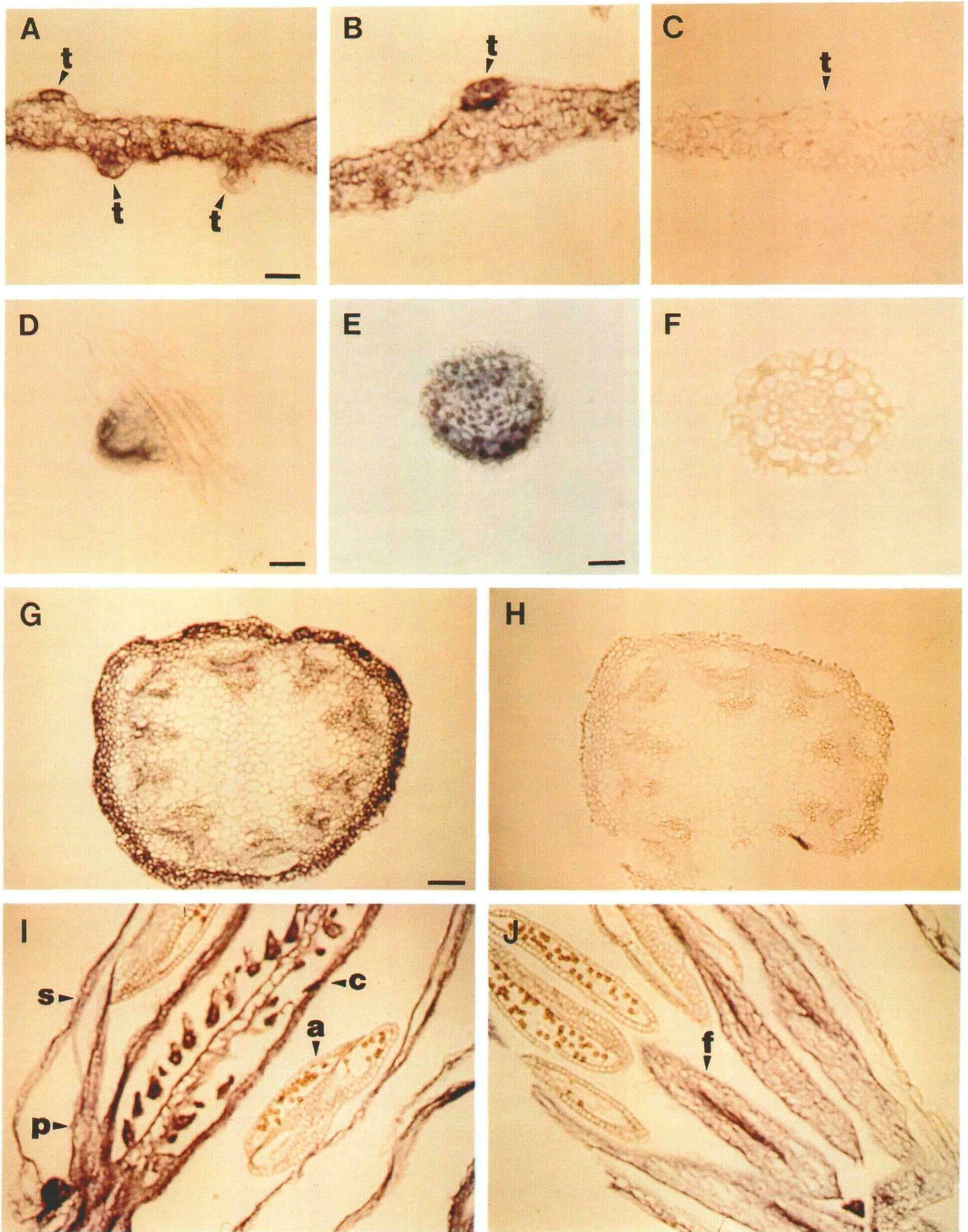

Figure 2. In Situ Localization of *aERD2* Transcripts.

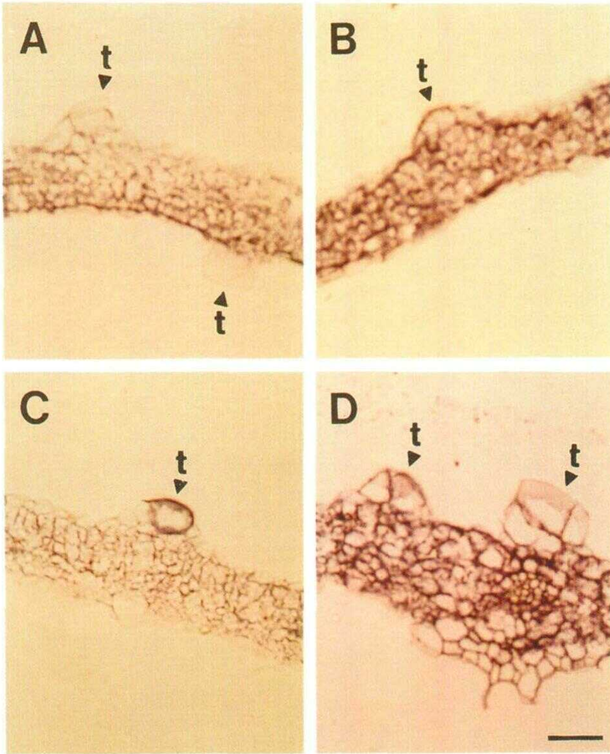

Figure 3. In Situ Localization of *aSAR1* and *aPDI* Transcripts in Wild-Type Leaves and *aERD2* mRNA Localization in Transgenic 35S-*R* Leaves.

(A) to **(C)** Transverse sections from Arabidopsis (ecotype RLD) leaves (6 mm long) were hybridized with dioxigenin-labeled antisense RNA of *aSAR1*, *aPDI*, and *aERD2*, respectively.

(D) Transgenic RLD transformed with 35S-*R* (a phenotype resulting in more trichomes) hybridized with *aERD2* antisense RNA. Bar = 32 μm. t, trichome.

biosynthesis. Based on RNA gel blot analysis, it was clear that leaves lacking trichomes (*gl1*) contain *aERD2* transcript but at slightly lower levels (~10% less) when compared with those of wild-type plants (Figure 5A). The steady state level of *aERD2* mRNA was strongly affected in transgenic plants expressing the maize *R* gene. The level of *aERD2* in the leaves of these transgenic plants (35S-*R*) was five times higher than that in leaves in wild-type plants and almost as high as levels in roots of wild-type plants (Figure 5A). The expression of the 35S-*R* gene in Arabidopsis also led to a 60 to 80% increase in the level of *aERD2* transcript in roots, as compared with that in roots of wild-type plants (Figure 5A). This result was surprising because roots have no trichomes. Interestingly, expression of the *GL1* gene (encoding a Myb-like transcription factor essential for trichome development; Larkin et al., 1994) under the control of the cauliflower mosaic virus 35S promoter in Arabidopsis plants led to only a slight increase in the level of *aERD2* transcript in leaves (~10%) when compared with that in the wild type. As expected, the level of *aERD2* transcript in roots of transgenic plants expressing 35S-*GL1* was unchanged (Figure 5A).

Our observation of high expression levels of *aERD2* transcript in leaves of the transgenic 35S-*R* plants could have resulted from more trichomes or from a more general process that led to activation of several genes, including *aERD2*. Thus, the blot shown in Figure 5 was reprobed with *aPDI* and *aSAR1*. The steady state levels of *aPDI* and *aSAR1* (Figures 5B and 5C, respectively) were not altered by introducing the 35S-*R* gene into Arabidopsis plants. The levels of their transcripts remained the same in wild-type plants, mutants lacking trichomes (*gl1*), 35S-*R* transgenic plants, and 35S-*GL1* transgenic plants. The in situ hybridization study of transgenic plants expressing the 35S-*R* gene revealed a strong *aERD2* signal in every cell type of the leaf when compared with the wild type (compare Figures 3C and 3D). The same intensity of hybridization was observed in trichome cells and all other leaf cell types (Figure 3D).

Arabidopsis Ecotypes Have a Single Gene for *aERD2*

The observation that *aERD2* gene expression can be induced (see Figure 4) and the existence of two closely related *ERD2*-like genes in mammalian cells (Lewis and Pelham, 1990, 1992) prompted us to question whether additional *ERD*-like genes exist in Arabidopsis. Only one band was observed on RNA gel blots when hybridized under low-stringency conditions with *aERD2* probe (data not shown). Extensive DNA gel blot analysis of Arabidopsis ecotypes RLD and Columbia (from which the cDNA was cloned) was performed. A single band (>2 kb)

Figure 2. (continued).

Sections from Arabidopsis (ecotype RLD) tissues were hybridized with dioxigenin-labeled antisense *aERD2* RNA.

(A) and **(B)** Transverse sections of leaves 2 and 6 mm long, respectively.

(C) Control section from a leaf hybridized with a sense *aERD2* probe.

(D) and **(E)** Longitudinal and transverse sections, respectively, of a root.

(F) Control section from a root hybridized with a sense *aERD2* probe.

(G) Transverse section of an inflorescence stem.

(H) Control section from an inflorescence stem hybridized with a sense *aERD2* probe.

(I) and **(J)** Longitudinal sections of a flower and stamen, respectively. Although inflorescence stems have trichomes, we were unable to detect them in the sections. a, anther; c, carpel; f, stamen filament; p, petal; s, sepal; t, trichome.

Bar in **(A)** = 32 μm for **(A)** to **(C)**; bar in **(D)** = 64 μm; bar in **(E)** = 128 μm for **(E)** and **(F)**; bar in **(G)** = 20 μm for **(G)** to **(J)**.

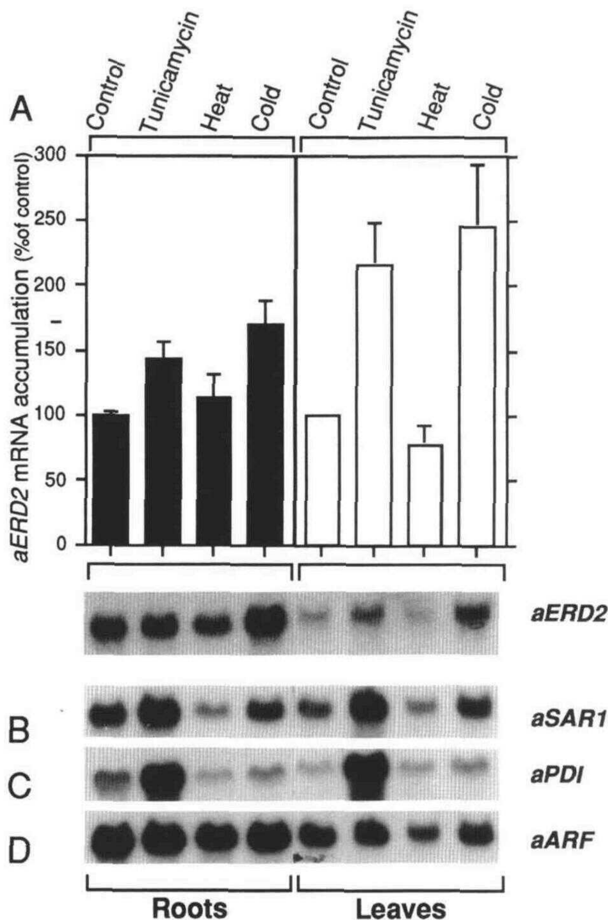

Figure 4. Induction of *aERD2* mRNA Levels under Various Stress Conditions.

Arabidopsis (ecotype RLD) seedlings (16 days) grown in liquid medium were treated with tunicamycin (20 µg/mL) for 12 hr, or were treated at 40°C (heat) for 2 hr or at 8°C (cold) for 12 hr. RNA was isolated from roots or leaves, and equal amounts were separated on a formaldehyde-agarose gel and blotted onto nylon membrane. The same membrane was hybridized either with Arabidopsis *aERD2*, *aPDI*, *aSAR1*, or *aARF* cDNA ^{32}P-labeled probes.

(A) Relative amounts of *aERD2* transcripts. RNA gel blots were scanned with a PhosphorImager, and the intensity of the signals was quantified using scanning densitometry. Control values from roots and leaves were defined as 100%. The data shown are the averages of five independent experiments. Standard error values are indicated as bars. A representative autoradiogram of the RNA gel blot probed with *aERD2* is shown below the histogram.

(B) RNA gel blot probed with *aSAR1*.

(C) RNA gel blot probed with *aPDI*.

(D) RNA gel blot probed with *aARF1*.

was detected in gel blots of Arabidopsis DNA digested with ClaI and HindIII (Figure 6, lane 8), representing the full open reading frame (ORF) with 35 nucleotides at the 5′ untranslated region. The size of this band suggested the presence of an intron. Two bands were detected when RLD and Columbia DNA were digested with HincII and HincII and SacI (Figure 6, lanes 2 and 3), or AccI and ClaI (data not shown), all of which recognized one site. DNA digested with endonucleases that recognize two sites in the cDNA resulted in similar banding patterns between RLD and Columbia; the endonuclease DraII yielded one major band (~1.8 kb) and two higher molecular weight minor bands (Figure 6, lane 1); XhoII resulted in one lower molecular band (~1.3 kb) and at least eight larger DNA fragments (Figure 6, lane 7), due to incomplete digestion of the genome DNA. As expected, two bands were observed upon digestion with either SacI and HindIII (Figure 6, lane 4) or SacI (data not shown), because SacI cuts once in the ORF of the cDNA. Digestion of the Arabidopsis DNA with endonucleases that do not cut the *aERD2* cDNA (i.e., XbaI or XhoI) resulted in one high molecular weight band hybridization signal (Figure 6, lanes 5 and 6 for RLD, and lanes 5 and 6 for Columbia).

DNA gel blot analysis strongly suggests that there is no polymorphism between the two Arabidopsis ecotypes and that *aERD2* is probably a single gene in both RLD and Columbia.

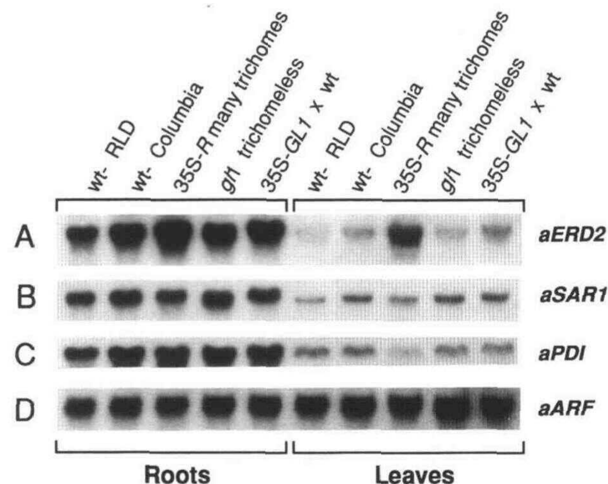

Figure 5. Effect of Mutants and Transgenic Plants with Varying Amounts of Trichomes on the Transcription Levels of *aERD2*, *aSAR1*, *aPDI*, and *aARF*.

Plants were grown on agar media. Roots and leaves were collected from 3-week-old Arabidopsis plants, wild-type (wt) ecotype RLD; wild-type ecotype Columbia; transgenic RLD expressing the anthocyanin *R* gene (35S-*R*) giving rise to many trichomes (Lloyd et al., 1992); Columbia mutant (*gl1*) lacking trichomes (Koornneef and Hanhart, 1983); transgenic Columbia expressing the *GL1* (35S-*GL1* × wild-type; Larkin et al., 1994). RNA was isolated, and equal amounts were separated by electrophoresis on a formaldehyde-agarose gel and blotted onto nylon membrane.

(A) Membrane hybridized with an *aERD2* cDNA ^{32}P-labeled probe.

(B) Membrane hybridized with an *aSAR1* cDNA ^{32}P-labeled probe.

(C) Membrane hybridized with an *aPDI* cDNA ^{32}P-labeled probe.

(D) Membrane hybridized with an *aARF* cDNA ^{32}P-labeled probe.

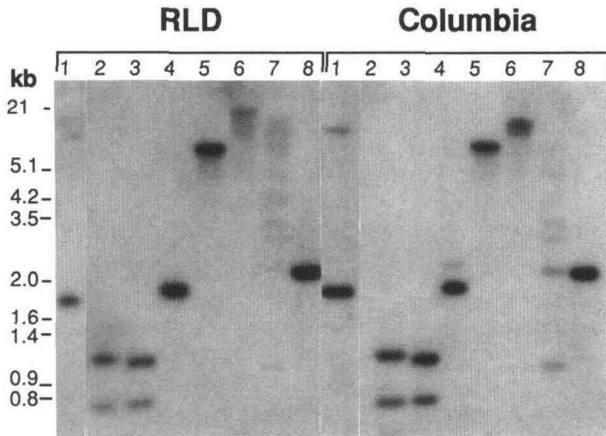

Figure 6. Arabidopsis Plants Have a Single Gene for *aERD2*.

Genomic DNA was isolated from the leaves of Arabidopsis ecotype RLD or Columbia. The DNA (5 μg) was digested with different endonucleases, separated by electrophoresis on an agarose gel, and blotted onto membrane. The endonucleases used are as follows: lanes 1, DraII; lanes 2, HincII; lanes 3, HincII and SacI; lanes 4, HindIII and SacI; lanes 5, Xba1; lanes 6, XhoI; lanes 7, XhoII; and lanes 8, HindIII and ClaI. The membrane was hybridized with a ^{32}P-labeled DNA probe generated from a 655-bp ORF of the Arabidopsis *aERD2* cDNA. Molecular length markers are given at left in kilobases.

DISCUSSION

We have studied the regulation of *aERD2* transcript during development and under various stress conditions. Very little is known in yeast and plant systems about the regulation and expression of secretory pathway genes. This is a first step in understanding the factors involved in regulating genes that encode proteins involved in vesicle traffic through the secretory pathway. This study also raised an old question regarding the differences in the secretory pathway and secretory activity between roots and leaves.

Differential Expression of Secretory Pathway Genes

In this study, we found that expression of the *aERD2* gene was highest in root tissues and lowest in leaves at various stages of growth. We found that other plant homologs of genes encoding proteins involved in ER-to-Golgi traffic were regulated in a manner similar to *aERD2* during development. With the exception of *aARF*, all genes tested (*aERD2* and *aSAR1*, see Figures 1A and 1B; *aSEC12*, data not shown) had the highest expression in root tissues and the lowest in expanding leaves. A similar observation is also reported for other plant species, in which a higher transcript level of an *aSAR1* homolog from tomato was detected in roots than in leaves (Davies, 1994).

What are the differences between roots and leaves that account for the variations in the transcript levels of *aERD2* (particularly high in root tips; see Figure 2D), *aSAR1*, and other genes we monitored? Both leaves and roots produce glycoproteins and cell wall matrix polysaccharides (synthesized in the Golgi apparatus), but unlike leaves, the root cap produces mucilage, a lubricant that helps to push the tip through the soil. The mucilage produced in the root Golgi apparatus is transported across the plasma membrane by the fusion of vesicles (Campbell and Greaves, 1990). In some plant species, mucilage secretion can reach up to 50 mg/g dry root weight, suggesting high secretory pathway activity. A further indication of high secretory activity comes from the architecture of the plant Golgi apparatus. Some root cells contain up to several hundred individual Golgi stacks dispersed throughout the cytoplasm (for a review, see Harris, 1986). It has been implied that an increase in Golgi stack number is associated with an increase in mucilage production in maize root caps (Iijima and Kono, 1992). A study by Staehelin et al. (1990) in Arabidopsis plants showed distinct morphological changes in the secretory system occurring during root cap cell differentiation. The authors attributed these tissue-specific variations in the Golgi stack architecture to the different functional activities of the secretory systems in different cell types. If there are differences between roots and leaves in the amount of proteins passing through the secretory pathway, it is reasonable to expect variations in the amounts of BiP and PDI that function in the correct assembly of proteins. Indeed, a higher level of PDI was observed in roots compared with leaves on the basis of total proteins (Shorrosh et al., 1993; Shimoni et al., 1995). Hence, is there higher secretory activity in the root than in leaf tissues? The strong signal of *aERD2* transcript in the root tips observed by in situ (Figure 2D), in combination with RNA gel blot analyses (Figure 1), may reflect a system higher in secretory activity in roots than in leaves.

We have demonstrated that stress conditions, especially cold, can markedly increase the levels of *aSAR1* and *aERD2* in leaves (Figure 4). These increments were always equal to or lower than the amounts of transcript in the roots of untreated plants. Furthermore, tunicamycin treatments that elevated *aERD2* levels in leaves did not significantly alter the already high levels in roots (Figure 4A). These observations suggest again that roots may already have a high level of secretory activity, so additional transcripts and presumably proteins are not required in response to stress. It should be noted that although mRNA fluctuations do not always reflect protein activity or amounts, the alterations in gene expression may reflect changes in the endogenous levels of aErd2p. A more direct protein analysis must be performed to substantiate such a protein/mRNA correlation.

The differences in *aERD2* transcript levels between roots and leaves could be due to tissue-specific regulation of the gene or to a second *ERD2*-like gene. Furthermore, the observation that *aERD2* gene expression can be induced and the existence of two *ERD2*-like genes in mammalian cells (Lewis and Pelham, 1990, 1992) led us to question whether additional

ERD2-like genes exist in plant cells. We could not detect more than one gene of *aERD2* in either Arabidopsis ecotype RLD or Columbia (Figure 6). Therefore, the low levels of *aERD2* transcripts in leaves compared with those in other tissues could be controlled by tissue-specific factors or stress regulatory elements that probably exist in the untranslated regions of the *aERD2* gene.

Increased Levels of *aERD2* in Trichome Cells

One of the unexpected observations of this study is the stronger hybridization signal of *aERD2* transcript in the trichome upon leaf maturation when compared with that of other leaf cell types (Figure 2B). Trichome cells had no such spatial regulation on two other secretory pathway genes examined (*aSAR1* and *aPDI*; Figures 3A and 3B). The significance of the relatively higher levels of *aERD2* transcript in trichomes is not known, but it may be that aErd2p is specialized for distinct secretory events in the trichomes. It is not known whether the trichome cells of Arabidopsis (which are not a glandular type) possess a higher secretory activity than do other leaf cells, and it is possible that Erd2p has a different role in these cells. Trichomes are specialized epidermal cells that are regularly distributed on the leaves of Arabidopsis plants. Trichomes are found on various parts of the plant, including the rosette and cauline (stem) leaves, the inflorescence stem, and the sepals of the flower; they originate from the developing leaf primordium. We verified by RNA gel blot and in situ hybridization analyses that *aERD2* is not exclusively expressed in trichome cells. In addition, the leaves of Arabidopsis mutants lacking trichomes (*gl1*) had levels of *aERD2* transcript similar to that in the wild-type plant (Figure 5A). Furthermore, overexpressing the *R* transcriptional activator led to an increase in the number of trichomes and an increase in *aERD2* transcript in both leaves and roots (Figure 5). Because roots lack trichomes, the increased steady state level of *aERD2* transcript is apparently due to other factors.

Conclusion

This study leads us to suggest that the secretory pathway is more active in roots than in leaves and can be induced under stress that affects vesicular traffic. Because the Arabidopsis genes *aERD2* and *aSAR1* can complement the corresponding yeast mutants (d'Enfert et al., 1992; Lee et al., 1993), these genes probably have a similar function in the secretory pathway in plants. The fact that tunicamycin and cold affect expression of specific secretory pathway genes also suggests the existence of a signal transduction pathway that mediates communication between the ER, the ER to Golgi, and the nucleus.

METHODS

Plant Growth and Treatment Conditions

Seed of *Arabidopsis thaliana* (~100 seeds) were sterilized and placed in a 125-mL flask containing 50 mL GM liquid medium (4.3 g/L Murashige and Skoog salts [Gibco BRL], 0.5 g/L 2-(*N*-morpholino) ethane-sulfonic acid, 10 g sucrose, 0.1 g/L myoinositol, 1 mg/L thiamine-HCl, 0.5 mg/L pyridoxine, and 0.5 mg/L nicotinic acid, adjusted to pH 5.7 with KOH). The sterile seeds were germinated and grown in flasks placed on a rotary shaker (50 rpm) in a 22°C incubator under a 12-hr fluorescent light/dark cycle for 16 days before stress treatments. For analysis of stress conditions, plants were placed either for 12 hr at 8°C (cold treatment) or for 5 min at 28°C, followed by 10 min at 37°C, followed by 105 min at 40°C (heat treatments), and then 10 hr at 22°C. Tunicamycin (20 μg/mL) dissolved in DMSO was added to each flask for 12 hr. Control plants were treated with 10 μL of DMSO. (An earlier study confirmed that DMSO alone has no effect on *aERD2* mRNA levels in roots or leaves.) At the end of the treatments, roots were separated from leaves and rinsed twice with sterile water; the plant tissues were frozen (−80°C) until extraction. To study transcript levels and distribution at various stages of plant development, seed of *A. thaliana* ecotype RLD or Columbia either were germinated in GM-agar cubes and grown as described previously or were germinated in soil and grown in a controlled environment chamber at 22°C under a 12-hr day/night cycle: 120 $\mu mol/m^{-2}/sec^{-1}$ light intensity, 70% relative humidity. Plant tissues (from roots, leaves, flowers, and inflorescence stems) were collected at various time points as indicated and stored frozen (−80°C) until extraction.

RNA Isolation and Gel Blot Analysis

Frozen plant tissues (up to 1 g) were ground under liquid nitrogen to a fine powder in a mortar with a pestle. Plant materials were then extracted by further grinding with 4 mL of extraction buffer (0.1 M Tris-HCl, pH 8; 1% SDS, 1% deoxycholate-sodium, 20 mM EDTA) and 10 μL of β-mercaptoethanol. Equal volumes of phenol-chloroform (50/50 [v/v], pH 8) were added to the homogenates (Sambrook et al., 1989). Samples were vortexed (5 min) and placed at 4°C on a shaker for up to 90 min, while other samples were extracted. Samples were centrifuged (12,000*g* for 20 min at 4°C), and the RNA-containing aqueous phase (4 mL) was mixed gently with 0.35 mL of 2 M KCl (to a final concentration of 0.16 M KCl). After 1 hr at 4°C, samples were centrifuged (12,000*g* for 20 min at 4°C), and the supernatants were collected. RNA was precipitated by the addition of one-quarter volume of 10 M LiCl (to a final concentration of 2 M, according to Hall et al., 1978) during the 30-min incubation at −80°C. After centrifugation (12,000*g* for 20 min at 4°C), the RNA pellet was washed twice with 5 mL of 70% EtOH, air dried, and then resuspended in 1 mL of sterile water before NaOAc/EtOH precipitation. The pellet was washed as described previously and allowed to air dry before it was dissolved in 0.2 mL of sterile water. The amount of RNA was determined using a spectrophotometer. RNA samples (30 μg RNA per lane) were denatured with formamide-formaldehyde, separated on formaldehyde–1.5% agarose gel (Sambrook et al., 1989), and transferred by capillary blotting action with 10 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate) for 16 to 18 hr at room temperature onto Hybond-N nylon membrane (Amersham). RNA was fixed to the membrane by 5-min UV light

irradiation (210 nm) followed by 2 hr at 80°C. The ^{32}P probes (1 × 10^6 cpm/mL) were made from various cDNAs (see following discussion) using random primers (Boehringer Mannheim), [α-^{32}P]-dATP, nucleotides, and the Klenow fragment of DNA polymerase I, according to Sambrook et al. (1989). The membranes were prehybridized and hybridized according to Taylor and Green (1991). After hybridization, membranes were washed twice at room temperature with 2 × SSC, 0.5% SDS followed by two to three washes with 0.2 × SSC, 0.5% SDS at 65°C. All RNA analyses were the results of at least four independent experiments. Every blot was scanned by PhosphorImager, and the intensity of the signals was quantified using a scanning densitometer of a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). The same blot was used with other probes after stripping the DNA probe and confirming stripping by autoradiogram. Stripping was performed by pouring 100 mL of boiling solution (0.1 × SSC, 0.1% SDS) on the membrane. After 15 min at room temperature, the membrane was stripped again.

cDNA Probes

The cDNAs used in this study were as follows: an HindIII-ClaI 655-bp fragment of Arabidopsis *aERD2* cDNA (Lee et al., 1993); a NotI 950-bp fragment of Arabidopsis *aSAR1* (for secretion-associated and Ras superfamily) cDNA (also called *artSAR1*; d'Enfert et al., 1992); and a NotI ~1450-bp fragment of Arabidopsis *aSEC12* (also called *Stl12*; d'Enfert et al., 1992). Both cDNAs were kindly provided by C. d'Enfert. The SalI 700-bp fragment of Arabidopsis *aPDI* (for protein disulfide isomerase) cDNA or Arabidopsis *aARF* (for ADP-ribosylation factor) cDNA was provided by T. Newman of the Arabidopsis genome project (MSU-DOE Plant Research Laboratory, Michigan State University, East Lansing). The *aPDI* cDNA clone used is similar to alfalfa *PDI* (Shorrosh and Dixon, 1991) with 386 score (according to the Blast program) and shares 68% amino acid identity with the alfalfa functional protein, having the exact amino acids comprising the two regions of the PDI active sites. The *aARF* cDNA clone is similar (90% amino acid identity) to another Arabidopsis *ARF* (Regad et al., 1993).

In Situ Hybridization

Arabidopsis leaves no larger than 6 mm were fixed for 2 hr in 50% EtOH, 3.7% formaldehyde, 5% glacial acetic acid. The leaves were washed three times in 50% EtOH for 30 min to remove the fixative. Dehydration, clearing, and paraffin embedding were performed as described by De Block and Debrouwer (1993).

Sense and antisense digoxigenin-labeled RNA were transcribed in vitro from linearized pBS-*aERD2*, pSP-*aPDI*, and pBS-*aSAR1* using either T7, T3, or SP6 RNA polymerase and a mix of nucleotides and digoxigenin-UTP (Boehringer). Plant sections (10 μm) were cut, hybridized, washed, and RNase treated. The sections were treated for 2 hr with blocking solution (TBS, 0.1% Tween 20, 3% BSA). Signal was detected using the chromogenic reagents nitro-blue tetrazolium chloride and 5-bromo-chloro-3-indolyl phosphate after 1 hr incubation with anti-digoxigenin antisera (De Block and Debrouwer, 1993). The reaction was stopped after 16 hr.

DNA Gel Blot Analysis

Genomic DNA was extracted (according to Dellaporta et al., 1983) from Arabidopsis leaves of 2-week-old plants growing in GM-agar cubes. Genomic DNA (5 μg) was digested overnight in the presence of 1 mM spermidine with various restriction enzymes in 20 μL reaction volume. DNA samples (5 μg/lane) were separated on a TBE-0.8% agarose gel. The gel was irradiated with UV light for 3 min before being washed with 0.25 M HCl followed by alkaline denaturation. After neutralization, the DNA was transferred by capillary blotting action with 10 × SSC for 20 hr at room temperature onto Hybond N nylon membrane (Amersham). DNA was fixed to the membrane, hybridized, and probed as described previously.

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