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Molecular Analysis of an Auxin Binding Protein Gene Located on Chromosome 4 of Arabidopsis

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We have isolated a cDNA clone from Arabidopsis, At-ERabp1, for the Arabidopsis auxin binding protein located in the lumen of the endoplasmic reticulum (ER). This cDNA clone codes for a protein related to the major auxin binding protein from maize, Zm-ERabp1. A single open reading frame, 594 bases in length, predicts a protein of 198 amino acid residues and a molecular mass of 22,044 D. The primary amino acid sequence contains an N-terminal hydrophobic signal sequence of 33 amino acids. We demonstrated by in vitro studies that the At-ERabp1 protein is translocated into ER-derived microsomes. The protein was processed, and the cleavage site for the N-terminal signal peptide was determined by radiosequencing. The mature protein is composed of 165 amino acid residues, with a molecular mass of 18,641 D. The At-ERabp1 protein contains potential *N*-glycosylation sites (Asn^{46}-Ile-Ser and Asn^{130}-Ser-Thr). In vitro transport studies demonstrated cotranslational glycosylation. Retention within the lumen of the ER correlates with an additional signal located at the C terminus and represented by the amino acids Lys^{196}-Asp-Glu-Leu, well known to be essential for active retrieval of proteins into the lumen of the ER. DNA gel blot analysis of genomic DNA revealed single hybridizing bands, suggesting that only a single At-ERabp1 gene is present in the Arabidopsis genome. Restriction fragment length polymorphism mapping indeed revealed a single locus mapping to chromosome 4.

INTRODUCTION

The small crucifer Arabidopsis is widely used as a model to study plant gene expression and function. Particularly useful for both classical and molecular genetic studies are its small genome size, the relative lack of repeated DNA within its genome, the short generation time, and the small plant size (Redei, 1975; Meyerowitz, 1987). In addition, the presence of an extensive genetic map, including a restriction fragment length polymorphism (RFLP) linkage map, and the availability of developmental mutants make this plant very useful for the molecular and functional analysis of plant genes (Koornneef et al., 1983; Chang et al., 1988; Nam et al., 1989). In particular, the steadily increasing collection of Arabidopsis mutants impaired in hormone synthesis or response will contribute greatly to the analysis of the molecular mechanisms of phytohormone action (for review, see Scott, 1990).

The phytohormone auxin has been implicated in the control of various aspects of growth and development in higher plants, including cell division, stem elongation, xylem differentiation, gravitropism, and senescence (for reviews, see Davies, 1987; Palme et al., 1991). It has been demonstrated that plant cells respond rapidly to the action of auxins by alteration of the abundance of specific mRNAs (Hagen, 1989) or by changes in the electric gradients across plasma membranes (Barbier-Brygoo et al., 1989, 1991). Receptor-like proteins have been assumed to play an important role in recognition and transmission of the auxin signal. Auxin binding proteins, thought to represent potential auxin receptors, have been identified in several monocotyledonous and dicotyledonous plants and were detected in cellular fractions corresponding to the endoplasmic reticulum (ER), the vacuole, and the plasma membrane (for reviews, see Cross, 1985; Venis, 1985; Jones, 1990; Napier and Venis, 1991). Recently, several auxin binding proteins were purified to homogeneity (Shimomura et al., 1986; Palme et al., 1990; Feldwisch et al., 1991). The primary structure of one of these auxin binding proteins, the "major" auxin binding protein from maize coleoptiles, was deduced from the nucleotide sequences of several cDNAs (Hesse et al., 1989; Inohara et al., 1989; Tillmann et al., 1989). The primary amino acid sequence of this maize ER-located auxin binding protein, Zm-ERabp1, includes a hydrophobic N-terminal signal sequence. An additional motif, well recognized as a signal for retrieval of proteins into the lumen of the ER and consisting of the amino acids Lys-Asp-Glu-Leu (KDEL), was found at the C terminus of this protein (Hesse et al., 1989; Pelham, 1990). The identification of this motif was consistent with the microsomal location of the Zm-ERabp1 protein and with its release from the microsomal fraction as a soluble protein.

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Except for the fact that expression of the Zm-ERabp1 gene was shown to be developmentally regulated, there is no evidence pointing to a receptor function of this gene (Hesse et al., 1989). This is mainly because maize cannot readily be transformed. Thus, approaches commonly used to identify functions of genes, e.g., overproduction of the encoded product or inactivation at any of several levels, cannot yet be addressed routinely in maize. Although a large array of interesting developmental and hormonal mutants has been identified in maize, the lack of efficient regeneration procedures has further prohibited complementation analysis of relevant mutants with the genes of interest (Sheridan, 1982). Efficient gene transfer and regeneration methods have been established for Arabidopsis. Hence, this organism provides unique properties to enable the study of putative phytohormone receptor genes using the repertoire of reverse genetic analysis. Our goal was to isolate a gene from Arabidopsis related to the Zm-ERabp1 gene from maize. We report here the nucleotide sequence, RFLP mapping, and molecular analysis of a cDNA clone from Arabidopsis encoding an auxin binding protein that is related to an auxin binding protein from maize.

RESULTS

Isolation of cDNA Clones Encoding an Auxin Binding Protein from Arabidopsis

Previously, we have isolated cDNA clones encoding auxin binding proteins from maize (Hesse et al., 1989). One of these clones was used as a heterologous probe in an attempt to isolate a related clone from Arabidopsis. This strategy stood a reasonable chance of success because auxin binding proteins from plants as distantly related as maize, cockspur weed, tobacco, or mung beans share common epitopes that can be detected by immunoblotting using antisera raised against maize Zm-ERabp1 (Napier and Venis, 1990; K. Palme and T. Hesse, unpublished results). However, using Zm-ERabp–specific cDNAs as probes, we were not able to detect any hybridizing bands in RNA gel blots containing mRNA isolated from Arabidopsis plants. This indicated either strong sequence divergence between the maize and Arabidopsis genes or low abundance of the corresponding Arabidopsis-specific transcript.

A cDNA library using λGEM2 as a vector was prepared from poly(A)$^+$ mRNA isolated from aerial parts of Arabidopsis plants. Replica filters were screened at low stringency using Zm-ERabp–specific cDNAs as probes. Ten positive phages were obtained from screening 100,000 recombinant phages. Two positive phage isolates, λAt-ERabp4 and λAt-ERabp9, with ~750- and ~850-bp inserts, respectively, were selected for further analysis. The inserts were subcloned, and the plasmids containing these inserts were designated pAt-ERabp4 and pAt-ERabp9.

DNA Sequence Analysis of At-ERabp1 cDNA

DNA sequences were determined in each case for both strands after generating subclones with exonuclease III. Both clones contained an open reading frame with similarities to the Zm-ERabp1 coding sequence. As both Arabidopsis clones ended in the presumed N-terminal leader region, we used a polymerase chain reaction to analyze whether our Arabidopsis cDNA library contained a full-length At-ERabp1 cDNA clone. Using a primer corresponding to nucleotides 103 to 125 of pAt-ERabp9 and to a genomic At-ERabp1 clone, we were able to isolate a 154-bp DNA fragment from the cDNA library. DNA sequence analysis revealed sequence identity of this fragment to the 5′ upstream region of the genomic At-ERabp1 clone isolated from a genomic Arabidopsis library (S. Schwonke and K. Palme, unpublished data). The nucleotide sequence of the clone obtained, pAt-ERabp1, is shown in Figure 1. The 5′ noncoding region is 9 bp long. A large open reading frame initiates

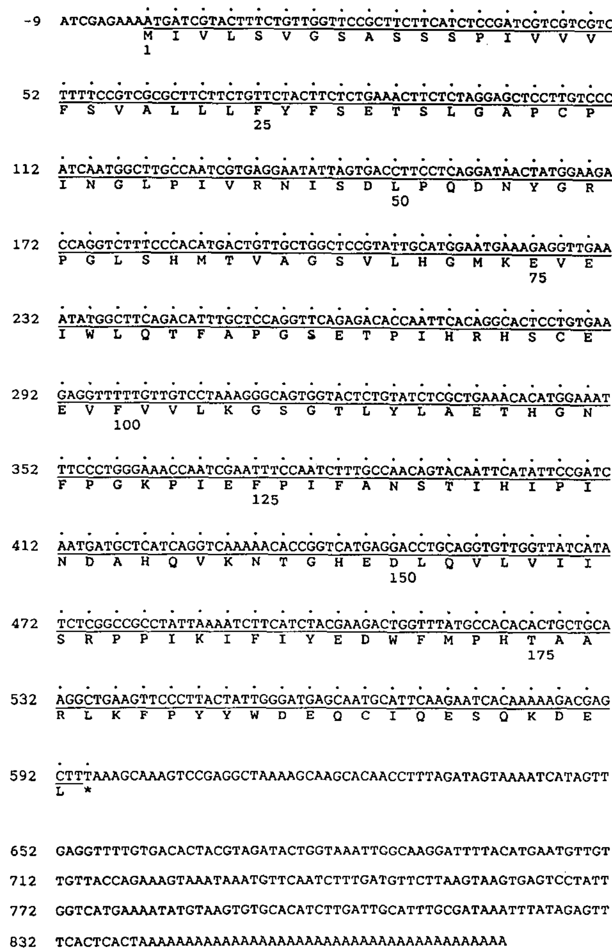

Figure 1. Nucleotide Sequence and Deduced Amino Acid Sequence for Arabidopsis At-ERabp1 Protein 1.

Asterisk denotes stop codon.

with the AUG triplet of nucleotide residues +1 to +3. The sequence immediately surrounding the translation start site AGAAAAUGA matches neither the Kozak consensus sequence CC(A/G)CCAUGA nor the plant gene initiation consensus sequence AACAAUGGC (Lütcke et al., 1987; Kozak, 1989). A translational termination codon (TAA) occurs in frame after nucleotide 594. The 3′ noncoding region is 246 bp long, excluding the poly(A) tail, and contains a putative polyadenylation signal 25 bp upstream from the poly(A) tail.

Amino Acid Sequence of the Arabidopsis At-ERabp1 Protein

DNA sequence analysis of pAt-ERabp1 revealed the presence of an open reading frame encoding a mature protein of 198 amino acids with a calculated molecular mass of 22,044 D (Figure 1). Alignments between the deduced Arabidopsis and maize amino acid sequences demonstrated a high degree of similarity (73.5%), as shown in Figure 2. The N-terminal signal sequences of both proteins showed only very weak homology. Similarity comparisons, however, exluding the signal sequences, demonstrated >80% similarity for the mature proteins. The amino acid sequence comparison indicated several blocks of identical amino acids (marked in black in Figure 2). The most variable regions are at the N terminus of the mature proteins around amino acid residues 39 to 46, and 114 to 151 for the maize proteins, and 34 to 43 and 111 to 147 for the Arabidopsis proteins. Potential glycosylation sites are located at asparagine residues in position 46 and 130, respectively, following the general rule of Asn-X-Ser/Thr. A tetrapeptide sequence (Lys[196]-Asp-Glu-Leu, KDEL) is found at the C terminus, a domain known to bind to the KDEL receptor responsible for the retrieval of these proteins to the lumen of the ER (Pelham, 1990). A search of various data bases did not reveal significant homology to any other known protein.

Analysis of in Vitro Processing of the At-ERabp1 Protein

When At-ERabp1 was transcribed by *Escherichia coli* RNA polymerase and the resulting mRNA translated in a wheat germ cell-free system, a major polypeptide with a molecular mass of ~22 kD was obtained, as shown in Figure 3 (lane 1, unprocessed At-ERabp1). This protein is sensitive to digestion with proteinase K (Figure 3, lane 2). In the presence of dog pancreas microsomes, processing of the At-ERabp1 is observed, resulting in a band with a molecular mass of 18 kD (Figure 3, lane 3, processed At-ERabp1). In addition, a 20-kD form of the At-ERabp1 protein appears [processed and glycosylated At-ERabp1 (pG), in Figure 3]. This protein corresponds to the processed and glycosylated form of At-ERabp1 as shown by glycopeptidase F digestion (compare lanes 3 and 4 and lanes 5 and 6 in Figure 3). After removal of the glycan residue, the protein (pG) has a higher electrophoretic mobility

Figure 2. Amino Acid Sequence Alignment of ERabp1 Proteins from Maize and Arabidopsis.

Black areas identify identical amino acid residues, and dashes represent missing amino acid residues at a particular position.

and is converted to the processed form. Surprisingly, only the processed and glycosylated form of At-ERabp1, pG, was protected against proteinase K digestion (Figure 3, lanes 3 and 5). However, after digestion with proteinase K in the presence of sodium deoxycholate, all proteins including pG were degraded, indicating that protection against protease digestion (Figure 3, lanes 2, 5, and 6) was due to successful translocation inside of microsomes.

These results indicate that the translocation of At-ERabp1 was aborted after processing, resulting in release to the cytoplasmic side of microsomal membranes. A small portion of pG, however, was successfully translocated across the membrane, indicating that processing and uptake correlate with *N*-glycosylation.

Two possible cleavage sites for a signal peptidase were predicted at amino acid residue 33 and 40 (von Heijne, 1983). We determined the actual cleavage sites for a signal peptidase by N-terminal amino acid sequence analysis of the processed At-ERabp1 protein. At-ERabp1–specific RNA was synthesized in vitro and translated in a wheat germ cell-free system in the presence of ^{3}H-leucine and ^{35}S-methionine and in the presence of maize endosperm microsomes. After translation, radiolabeled proteins were separated by SDS-PAGE and blotted onto polyvinylidene difluoride membrane. Radiolabeled bands were excised from the blot and subjected to automated Edman degradation. The result, shown in Figure 4, demonstrates that radioactivity was released, corresponding to the leucine residues at positions 41, 50, and 60, and methionine at position 63. This suggests that cleavage of the signal peptidase occurs between Gly[33] and Ala[34].

At-ERabp1 Protein Encoded by a Single Nuclear Gene

DNA gel blot analysis (Southern, 1975) of Arabidopsis genomic DNA cleaved with various restriction enzymes revealed several bands with lengths between 3.3 and 7 kb hybridizing with the At-ERabp1 cDNA probe, as shown in Figure 5. The complete At-ERabp1 gene is most likely located within a 7-kb

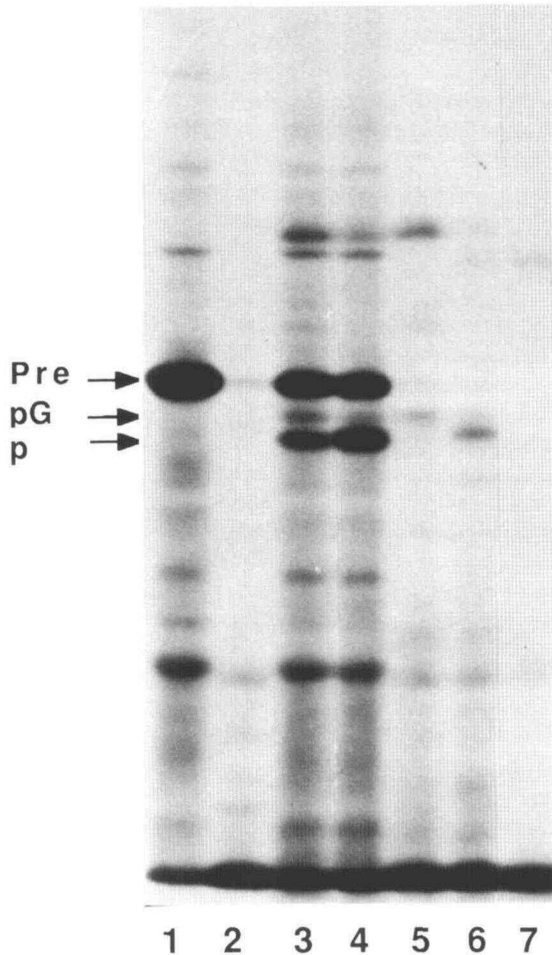

Figure 3. *In Vitro Translocation of the At-ERabp1 Protein.*

The At-ERabp1 cDNA cloned in the pDS6 vector was transcribed using RNA polymerase from *E. coli*, and the RNA was translated in a wheat germ extract in the absence (lanes 1 and 2) or presence (lanes 3 to 7) of microsomes (MIC). Aliquots of the translation mixtures were digested post-translationally with either proteinase K in the absence (lanes 2 and 5) or presence (lane 7) of sodium deoxycholate (PK) or with endoglycopeptidase F (PNGase F; lanes 4 and 6). In the sample for lane 6, digestion with glycopeptidase F was performed after digestion with proteinase K. Enzyme digestions were performed as detailed in Methods. Proteins were separated by SDS-PAGE (14% polyacrylamide gel). Fluorography was for 4 hr. Pre, unprocessed At-ERabp1; pG, processed and glycosylated At-ERabp1; p, processed At-ERabp1.

genomic fragment, which is consistent with the isolation of a genomic clone displaying similar size characteristics. The bands shown in Figure 5 indicate that probably only a single gene encoding the At-ERabp1 protein is present in the Arabidopsis genome. Several mutations are known in Arabidopsis that affect hormonal responses and have been mapped on Arabidopsis chromosomes. As a first step to determine whether At-ERabp1 is closely linked to one of the known mutant alleles, we determined the genetic map position of the At-ERabp1 gene using the RFLP mapping method (Chang et al., 1988). The At-ERabp1 gene revealed a BglII polymorphism, and 103 individuals, representing 206 chromatids, were scored and analyzed by the mapmaker program. The final localization of the At-ERabp1 gene in relation to the rest of the RFLP markers on chromosome 4 are shown in Figure 6. The mapping data suggest that At-ERabp1 is represented by a unique gene.

DISCUSSION

Using low-stringency hybridization, we have isolated an Arabidopsis cDNA clone encoding an auxin binding protein

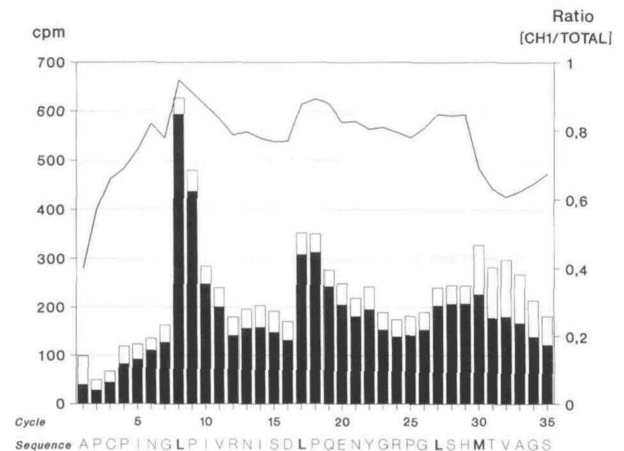

Figure 4. Partial Amino Terminal Sequence Analysis of in Vitro Translated and Processed At-ERabp1.

At-ERabp1 mRNA was translated in a wheat germ lysate in the presence of L-4,5-^{3}H-leucine and L-^{35}S-methionine and maize endosperm microsomes. Separation was by SDS-PAGE. Proteins were blotted to polyvinylidene difluoride membrane and located by autoradiography. Radiolabeled and processed At-ERabp1 protein bands were excised and subjected to Edman degradation. Radioactivity released at each cycle was monitored by liquid scintillation counting. To distinguish radioactivity from the two isotopes, windows were defined for counting (channel 1, 0 to 397 nm; channel 2, 397 to 670 nm). Luminescence from ^{3}H is found exclusively in channel 1 (black bars), whereas those from ^{35}S are found in both channels (white bars). The line above the bars shows the ratio of channel 1, to channel 1 + channel 2. Release of L-4,5-^{3}H-leucine in a given cycle correlates with an increase in the ratio; release of L-^{35}S-methionine correlates with a decrease in the ratio.

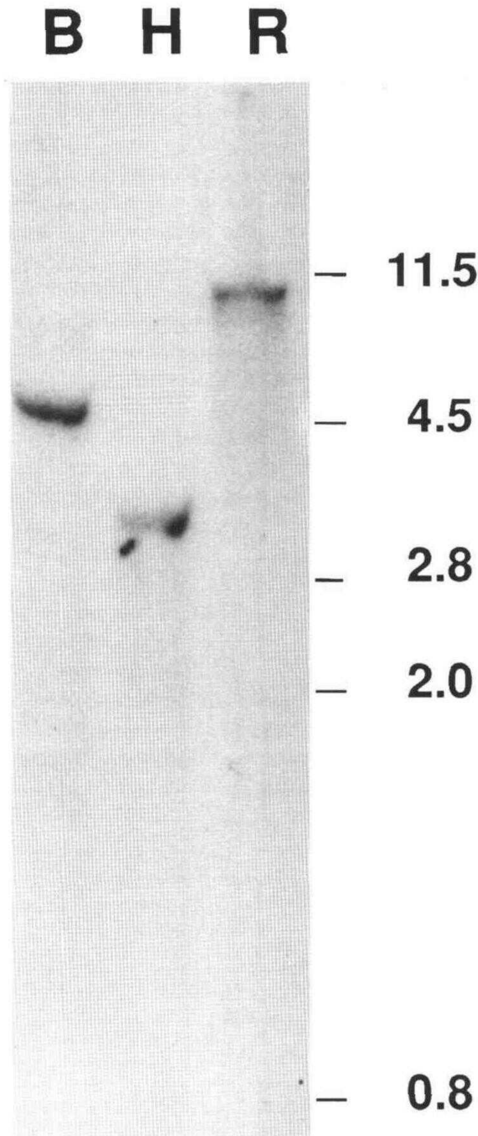

Figure 5. DNA Gel Blot Analysis of the At-ERabp1 Gene.

Twenty micrograms of genomic DNA from Arabidopsis was digested with the restriction enzymes BamHI (B), HindIII (H), and EcoRI (R). Genomic DNA was electrophoresed in a 1% agarose gel and transferred to a Hybond membrane. The blot was probed with pAt-ERabp1, which is described in detail in Methods. DNA length markers used (λPst digest) are shown on the right.

related to the maize auxin binding protein Zm-ERabp1. Diverse terminology has been used previously to describe this protein and the gene encoding this protein, e.g., the site I protein, the axr[1] gene, or the ABP1 gene (Hesse et al., 1989; Inohara et al., 1989; Tillmann et al., 1989; Napier and Venis, 1990). For consistent nomenclature and to avoid confusion with genes encoding novel auxin binding proteins that are presently characterized in several laboratories, we have renamed members of this gene family according to their cellular localization and the plant species from which they were isolated. Therefore, members of the gene family encoding maize auxin binding proteins that are located in the lumen of the ER have been termed Zm-ERabp. Similarly, related Arabidopsis proteins will be termed At-ERabp.

The Arabidopsis cDNA described here encodes a protein with an N-terminal signal sequence. Like the maize protein, the primary sequence of the Arabidopsis protein At-ERabp1 contains no classical membrane-spanning segment, suggesting that the protein is not inserted in any cellular membranes. This finding together with the identification of the C-terminal ER retrieval sequence KDEL indicated that the Arabidopsis protein is probably imported into the ER. Furthermore, analogous to other eukaryotic systems, the KDEL motif found at the C terminus of At-ERabp1 is likely to be responsible for continuous retrieval of this protein from salvage compartments by KDEL receptors (Pelham, 1990). Translocation of secretory proteins or ER resident proteins is typically triggered by signal sequences 15 to 40 amino acids long. Such signal sequences mediate the interaction between the ribosome and the signal recognition particle, a cytosolic ribonucleoprotein complex (Rapoport, 1990). Interaction with the signal recognition particle receptor results in targeting of the ribosome to the rough ER and initiation of import to the ER. It was found

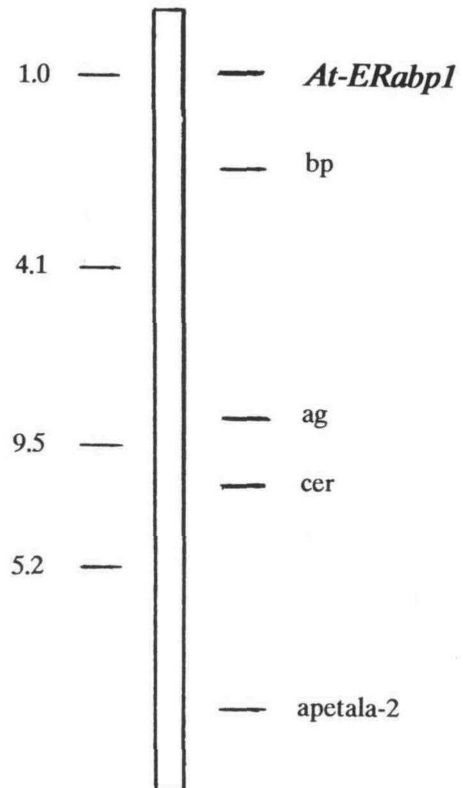

Figure 6. RFLP Mapping of the At-ERabp1 Gene.

Chromosomal localization of RFLP marker genes and the At-ERabp1 gene are shown.

that plant and mammalian translocation systems share common features and in many cases can substitute for each other (Prehn et al., 1987; Campos et al., 1988). Because signal sequences can vary significantly in their amino acid composition and in the position of the hydrophobic core sequence, it was not surprising to observe that the signal peptide sequences shown in Figure 2 differ in both amino acid composition and position of the hydrophobic core sequences. According to statistical rules established for prediction of signal peptidase cleavage sites (von Heijne, 1983), the At-ERabp1 amino acid sequence should contain two possible sites for processing. We, therefore, analyzed translocation and processing in vitro.

When the At-ERabp1 cDNA was introduced into a coupled in vitro transcription-translation system, the At-ERabp1 protein was processed in the presence of either maize endosperm microsomes or dog pancreas microsomes. The translocation process was dependent on signal recognition particles because dog pancreas signal recognition particle preparations inhibited the cell-free synthesis of At-ERabp1 in the absence of microsomes and stimulated processing of the protein when translation was done in the presence of microsomes (data not shown). Our results further indicated that only a small portion of the At-ERabp1 protein is translocated and glycosylated. This was a surprising result because >80% of the At-ERabp1 protein was found to be localized on the cytoplasmic face of the microsomal vesicles after cleavage by the signal peptidase. This result was confirmed by post-translational centrifugation of the microsomes both in the absence and presence of denaturing agents such as Na_2CO_3 or urea (data not shown). Our results suggest that translocation of the At-ERabp1 preprotein was aborted by an unknown mechanism, resulting in dissociation from the translocation machinery. Similar observations have been made for the targeting of the hepatitis B virus precore protein to the ER, where 70 to 80% of the processed protein was not associated with membranes but was localized in the cytoplasm. The remaining 20 to 30% appeared, however, to be correctly translocated to the lumen of the ER (Bruss and Gerlich, 1988; Garcia et al., 1988). Because the catalytic site of the signal peptidase is thought to reside on the luminal side of the ER, a significant part of the At-ERabp1 protein must have been translocated across the ER membrane to allow processing. Similar to the hepatitis precore protein, we must assume that the abortion of translocation must be determined by some unusual feature of the signal peptide or of the At-ERabp1 protein because all other substrates tested, including the Zm-ERabp1 protein, were efficiently translocated (results not shown). We therefore think that the At-ERabp1 signal sequence or other parts of the protein may have some not yet understood functions in the translocation process. This finding could be relevant for the in vivo function of this protein; however, further speculations on the functional role have to await determination of the precise function for this protein.

Our data further show that the At-ERabp1 protein was glycosylated. Although it cannot be decided which of the canonical glycosylation sites identified in the primary sequence is used, it is interesting to note that only one of the sites (e.g., Asn^{130}-Ser-Thr), flanked symmetrically by proline residues at a distance of four amino acids, has a high statistical probability to favor the formation of a β-turn. The importance of the structure-forming potential of the proline residues has been noted earlier in studies analyzing the glycosylation of synthetic glycosyl acceptor peptides (Bause, 1983). It was found that the presence of two prolines at sufficient distance from the glycosylation motif could promote the formation of a loop structure for the catalytically essential orientation of the Asn-Ser-Thr motif. We found that the position of both proline residues is exactly conserved in the At-ERabp1 and Zm-ERabp1 proteins. Furthermore, the maize protein that contains a unique glycosylation site only at Asn^{133} was found earlier to be efficiently glycosylated by the addition of a high mannose-type glycan (Hesse et al., 1989).

The ER is the site at which newly synthesized proteins enter the vacuolar system; this system includes the ER itself, the Golgi system, intracellular organelles, and a variety of intermediate transport compartments. Analysis of the luminal ER content, the reticuloplasm, has demonstrated that this organelle contains a high concentration of proteins likely to be used for calcium storage. Several ER resident proteins with a C-terminal KDEL motif such as GRP94 or calreticulin have polyacidic clusters of amino acids within their C-terminal amino acid sequence that are probably sites of low-affinity calcium binding (Fliegel et al., 1989; Smith and Koch, 1989). The preferential distribution of these sites toward the C terminus suggests that this zone might perform a specialized function for calcium binding. It was proposed that these acidic regions might be anchors for calcium bridges between ER proteins, or ER proteins and phospholipids, and form a loose matrix in the region of the transverse tubules (Meissner, 1975; Smith and Koch, 1989; Koch, 1990). Such a matrix could be important for the calcium storage capability of the ER with reversible sol-gel transformations and thus provide an explanation for oscillations of cytosolic calcium concentrations upon stimulation (Koch, 1990). Structural comparisons between the maize and Arabidopsis auxin binding protein sequences revealed a high content of proline residues in the primary sequence as well as clusters of acidic amino acids enriched in the C-terminal region; these features are related to those observed in the reticulin sequences (Fliegel et al., 1989; Smith and Koch, 1989). It is worth noting here that auxin-induced conformational changes have been observed in Zm-ERabp1, resulting in an inhibition by auxin of binding of a monoclonal antibody directed against the C terminus of this protein (Napier and Venis, 1990). It was found that the ability of auxins and other related synthetic compounds to induce a conformational change also fits the relative binding characteristics of Zm-ERabp1 for these compounds. The question therefore arises whether changes in the calcium concentration could result in binding of calcium to acidic amino acids along the C-terminal area and induce oligomerization of this protein and probably changes in the auxin binding characteristics.

Although no biological function has yet been found for the At-ERabp1 gene, it was interesting to find out that the

At-ERabp1 gene is located on an RFLP (1.2 cM) together with the *hy4* locus on chromosome 4 using RFLP mapping. The *hy4* allele belongs to one of several genes, *hy1* to *hy6* (Koornneef et al., 1980; Chory et al., 1989), that are defined by mutations that cause the failure of homozygous mutant seedlings to respond properly to light. Of the six complementation groups isolated, several mutants were found to display pleiotropic effects that were caused by changes in structure or concentration of photoreversible phytochrome in adult plants (Parks et al., 1989). However, mutants corresponding to the *hy4* locus were found to express increased hypocotyl elongation growth under strong illumination; this transient phenotype was confined to a limited period during development. Because light-dependent changes in the auxin binding activity have been found (Walton and Ray, 1981), it will now be possible to determine whether any relation between this gene and the *hy4* locus exists in transgenic Arabidopsis plants overexpressing the At-ERabp1 gene.

METHODS

Enzymes and Chemicals

Restriction enzymes, T4 DNA ligase, and reagents for cDNA synthesis were obtained from either Bethesda Research Laboratories or Boehringer Mannheim (Germany). The sequenase enzyme was purchased from United States Biochemical Corp. T7 DNA polymerase was purchased from Pharmacia, Sweden. All enzymes were used as indicated by the manufacturer. If not stated otherwise, the cloning methods that were used are described in Maniatis et al. (1989) or Ausubel et al. (1989). Deoxynucleotides, dideoxynucleotides, and sequence primers were obtained from Boehringer Mannheim; $\gamma^{32}P$-labeled nucleoside triphosphates, ^{35}S-dATP, L-^{35}S-methionine, and L-4,5-^{3}H-leucine, and other radiochemicals were purchased from Amersham International.

Screening of cDNA Libraries

An *Arabidopsis thaliana*–specific cDNA library (Promega) was analyzed. Recombinant phages (100,000 of them) were screened with 20% formamide at 42°C using a digoxigenin-labeled Zm-ERabp4 gene-specific probe (K. Palme, T. Hesse, and C. Garbers, unpublished results) from *Zea mays*. Each nylon filter was washed once for 5 min at room temperature in 5 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate) and 0.1% SDS, twice for 15 min at 42°C in 5 × SSC and 0.1% SDS, and once for 15 min at 42°C in 0.1 × SSC and 0.1% SDS. Phages from plaques yielding positive signals were detected by digoxigenin staining. Digoxigenin labeling was performed using a kit from Boehringer Mannheim.

DNA Sequence Analysis

DNA from recombinant clones was isolated according to standard procedures and characterized by restriction and hybridization analysis. DNA sequence analysis was carried out by the dideoxy chain termination method following subcloning in pUC118 and pUC119. Nested deletions were created using exonuclease III. In all cases, DNA sequences were determined on both strands. For data handling, a VAX computer was used with the University of Wisconsin (Madison) Genetics Computer Group program.

DNA Blot Hybridization

Genomic DNA was isolated according to Bedbrook (1981) and separated on 1% agarose gels. After transfer to a nylon membrane (Hybond N, Amersham International), the blot was hybridized with a nick-translated probe, washed, and exposed to Kodak XAR-5 film using intensifying screens.

RFLP Mapping

RFLP mapping was done as described by Chang et al. (1988) using DNAs isolated from a cross between Landsberg *erecta* and Niederzenz ecotypes. A genomic clone encoding the complete At-ERabp1 gene (S. Schwonke and K. Palme, unpublished results) from Arabidopsis was used to probe genomic DNA blots to reveal DNA polymorphisms between appropriate ecotypes of Arabidopsis. RFLP mapping data was analyzed using the MAPMAKER computer program developed by Lander et al. (1987) and modified for the Macintosh by Les Proctor (Du Pont).

In Vitro Transcription-Translation System

The At-ERabp1 cDNA was cloned into pDS6 (Stüber et al., 1984), and the resulting plasmid was transcribed in vitro with *Escherichia coli* RNA polymerase. mRNA was translated in a wheat germ cell-free system (Roberts and Patterson, 1973; Stüber et al., 1984). To analyze translocation and processing, dog pancreas or maize endosperm microsomes were added to the translation reaction. Dog pancreas microsomes were prepared and treated with staphylococcal nuclease essentially as described by Walter and Blobel (1983). Maize endosperm microsomes were isolated and used as described by Campos et al. (1988).

Protease Digestion

Aliquots of the translation reaction were incubated for 30 min at 4°C with either proteinase K (0.09 mg/mL) or proteinase K and sodium deoxycholate (1%). Proteolysis was inhibited by the addition of phenylmethylsulfonyl fluoride (2 mg/mL).

Glycopeptidase Digestion

Aliquots of the translation reaction were incubated in 150 mM $NaPO_4$, pH 7.5, 10 mM EDTA, 1% Triton X-100, 0.2% 2-mercaptoethanol, and 0.8 units glycopeptidase F from *Flavobacterium meningosepticum* (PNGase F; Boehringer Mannheim) for 5 hr at 37°C. After precipitation with trichloroacetic acid, the pellets were washed with acetone, dissolved in SDS sample buffer, and analyzed by SDS-PAGE.

Radiolabeling of At-ERabp1 Protein and Amino Acid Sequence Analysis

mRNA was translated in a wheat germ cell-free system containing microsomes treated with maize endosperm nuclease and both L-^{35}S-methionine (1300 Ci/mmol) and L-4,5-^{3}H-leucine (163 Ci/mmol). After translation, the samples were separated by SDS-PAGE (14% polyacrylamide gel) and blotted onto a polyvinylidene difluoride membrane; the membrane was exposed for autoradiography. Radioactive bands were excised and stored at −20°C until radiosequencing was performed. Radiosequence analysis was performed by automated serial Edman degradation using an Applied Biosystems Inc. (Foster City, CA) 477 gas phase sequenator. The sequencer was operated using polybrene coated GF/C discs and the AT-sequencer program omitting the conversion cycle. Radioactive samples released during each degradation cycle were collected. Radioactivity was determined in 10 mL of Rotizint (Roth, Karlsruhe, Germany) using a Beckman Instruments LS 7500 liquid scintillation counter.

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