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RESEARCH ARTICLE

The Tobacco Homolog of Mammalian Calreticulin Is Present in Protein Complexes in Vivo

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The analysis of protein sorting signals responsible for the retention of reticuloplasmins (RPLs), a group of soluble proteins that reside in the lumen of the endoplasmic reticulum (ER), has revealed a structural similarity between mammalian and plant ER retention signals. We present evidence that the corresponding epitope is conserved in a vast family of soluble ER resident proteins. Microsequences of RPL60 and RPL90, two abundant members of this family, show high sequence similarity with mammalian calreticulin and endoplasmin. RPL60/calreticulin cofractionates and costains with the lumenal binding protein (BiP). Both proteins were detected in the nuclear envelope and the ER, and in mitotic cells in association with the spindle apparatus and the phragmoplast. Immunoprecipitation of proteins from in vivo–labeled cells demonstrated that RPL60/calreticulin is associated with other polypeptides in a stress- and ATP-dependent fashion. RPL60/calreticulin transcript levels increased rapidly in abundance during the proliferation of the secretory apparatus and the onset of hydrolase secretion in gibberellic acid–treated barley aleurone cells. This induction profile is identical to that of the well-characterized ER chaperones BiP and endoplasmin. However, expression patterns in response to different stress conditions as well as tissue-specific expression patterns indicate that these genes are differentially regulated and may not act in concert.

INTRODUCTION

The endoplasmic reticulum (ER) is one of the largest organelles of the eukaryotic cell. It is built up by a dynamic network of cisternae and tubules and is believed to contain a single internal space, the ER lumen. The ER is a unique protein folding environment that allows proteins to become glycosylated, to acquire disulfide bridges, and to be modified in other ways, such as proteolytic processing, oligomerization, and hydroxylation. Normal function of the ER in protein synthesis, maturation, and transport requires the coordinate action of a large family of intrinsic components. These include proteins located in the ER membranes and the ER lumen (reviewed in Vitale et al., 1993). Soluble ER resident proteins are collectively referred to as reticuloplasmins (Koch, 1987; Macer and Koch, 1988) because of their high abundance in the ER lumen. Several genes coding for ER resident proteins in plants have been cloned and sequenced (Vitale et al., 1993). The plant lumenal binding protein (BiP) has been shown to carry out the same function as in yeast or mammalian cells. This was shown either by genetic complementation experiments (Denecke et al., 1991) or by direct biochemical analysis (Pedrazzini et al., 1994). Protein disulfide isomerase (PDI), a protein involved in the formation of disulfide bridges, has also been shown to contain a functional homolog in plants (Shorrosh and Dixon, 1991). This process is restricted to the lumen of the ER in eukaryotic cells and is an essential step in the folding or assembly of a large population of proteins. However,

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it is clear that the family of proteins involved in protein synthesis, maturation, and transport consists of many other members (Vitale et al., 1993). We are interested in a further characterization of plant ER proteins to study mechanisms related to the maintenance of ER function in protein biosynthesis.

The constant transport of membranes and proteins from the ER to the Golgi, needed to maintain anterograde transport of products to their final locations, causes a constant leakage of ER resident proteins to more distal compartments of the endomembrane system. The eukaryotic cell has developed efficient means to limit this leakage to a minimum to maintain adequate levels of intrinsic ER components at the site where they perform their function. ER localization signals prevent soluble ER proteins from being transported to the cell surface or to the tonoplast membrane by directing them to the retrograde transport route in an early Golgi compartment (Vitale et al., 1993). It has become evident that a variety of C-terminal tetrapeptide sequences are able to cause the accumulation of recombinant proteins in the ER lumen of mammalian cells (Andres et al., 1990, 1991; Mazzarella et al., 1990; Haugejorden et al., 1991) or plant cells (Denecke et al., 1992, 1993).

Several lines of evidence support the view that upstream sequences beyond the C-terminal tetrapeptide can influence or may be part of the structure of reticuloplasmin retention signals (Haugejorden et al., 1991; Denecke et al., 1992) and that retention cannot be predicted based on the primary sequence. This notion is illustrated by the fact that the auxin binding protein ABP1 contains the KDEL motif but does not colocalize with abundant ER proteins (Napier et al., 1992) and is secreted via the Golgi apparatus (Jones and Herman, 1993). With the help of monoclonal antibodies, a conserved epitope was identified at the C terminus of plant and mammalian reticuloplasmins as well as ER-retained chimeric phosphinothricin acetyltransferase (PAT) fusion proteins containing a variety of C-terminal tetrapeptides (Denecke et al., 1992, 1993). Tetrapeptides as different as KDEL, HDEL, and RDEL can thus result in the formation of an identical structure, which is recognized by the sorting machinery and deviates PAT from the secretory default pathway (Denecke et al., 1990). Most likely, this structure contains (or depends on) other amino acids that precede the tetrapeptide. Moreover, the higher order structure of the protein has to support exposure of the C terminus on the surface of the protein. The exact sequence requirements for the formation of a functional ER localization signal of the tetrapeptide and the context of the C terminus cannot be deduced from the primary sequence of the known ER proteins, but the monoclonal antibody 1D3 (Vaux et al., 1990) can be used to determine the presence or absence of such a signal (Denecke et al., 1992, 1993).

This article describes the detection of a large family of polypeptides, identified by direct immunologic recognition of the ER localization signal for soluble proteins. One of the identified proteins, a potential homolog of mammalian calreticulin, has been analyzed in detail with respect to its possible function in the plant ER.

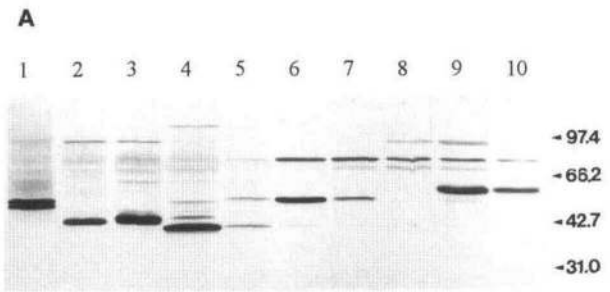
RESULTS

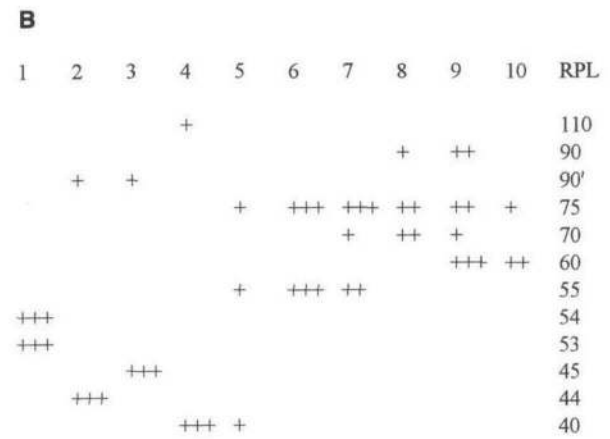
Direct Immunologic Recognition of ER Resident Proteins

We wanted to use the specific recognition of ER retention signals by monoclonal antibody 1D3 as a means to identify new soluble ER resident proteins. For that purpose, microsomes were isolated from germinating tobacco seed 4 days after the start of imbibition because this tissue was previously shown to contain the highest BiP mRNA levels of all tobacco tissues analyzed (Denecke et al., 1991). Preliminary microsequence data of abundant microsomal proteins suggested that homologs of mammalian ER proteins had been identified (Denecke et al., 1993). We have now refined the extraction procedure to obtain a higher enrichment for soluble proteins. Soluble proteins were extracted by an osmotic shock treatment followed by a gentle mechanical disruption by pipetting the resuspended pellet through a Pasteur pipette. This allowed us to increase the sensitivity of the detection method.

Figure 1A shows the immunoblot analysis of fractions obtained from ion exchange fractionation of acidic microsomal proteins. ER localization signals were detected with the help of the 1D3 monoclonal antibody. Figure 1B summarizes the data and describes the proteins for future analysis. Proteins in Figure 1A, lane 9, were further purified by a single gel filtration step, which provided essentially pure fractions of reticuloplasmins RPL60 and RPL90, as estimated from Coomassie blue–stained gels and subsequent reverse phase chromatography. Interestingly, the mobility of RPL60 on gel filtration columns was higher than that of RPL90, which indicates that RPL60 is a homopolymer in its native form. RPL75 cross-reacts with BiP antisera, and its general properties when subjected to ion exchange chromatography correspond well with the model that BiP binds to a variety of nascent polypeptides under unstressed growth conditions (Denecke et al., 1993). Figure 1C shows a set of microsequence data corresponding to RPL60 and RPL90 that were obtained by microsequencing peptides from the purified proteins. RPL90 appears to be highly similar to mammalian endoplasmin and almost identical to the proposed barley homolog of endoplasmin (Walther-Larsen et al., 1993). Due to this overall homology, we propose that RPL90 is the tobacco homolog of endoplasmin. RPL60 is very similar to mammalian calreticulin, as shown from the sequence alignments. These sequences were used to clone the cDNA fragment constituting the mature part of this protein from tobacco (see the following discussion).

RPL40 designates a protein that cross-reacts only with 1D3 ascites antibodies and not with cell culture supernatants of 1D3. This protein is not recognized by a polyclonal anti-KDEL antiserum (KX5-KDEL; Vaux et al., 1990), which appears to be specific for the retention signal and recognizes both KDEL- and HDEL-containing proteins in plants, in a very similar way to 1D3 (J. Denecke, unpublished observations). RPL40 was





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          ----->                                        ------->
RPL60:     EVPFEESFNDG  KGIQTSEDYRFFAISAEIPEFSNKT  KKPEDWDDQEDGEW
MOCRP:  DPAIY*K*Q*L**  **L***Q*A**Y*L**KFEP****G  *******EEM****

RPL90:  KFEFQAEVSRLMDIIINSLYS  KADGAFAISEDVGNEPLK  KLYVRRVFISDEF
BAENP:  **********************  ****S******TW****G  ************
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Figure 1. Identification of a Family of Soluble ER Resident Proteins.

(A) Protein gel blot analysis of acidic protein fractions from ion exchange chromatography. Numbers above the lanes refer to different fractions. The size markers are given at right in kilodaltons.

(B) Classification of several identified putative plant reticuloplasmins (RPLs). Numbers above the lanes refer to the same fractions as shown in **(A)**. The numbers at right beneath RPL refer to the apparent molecular weights as estimated from SDS-PAGE and the included size markers. The relative abundance for each lane separately is indicated by (+).

(C) Alignments of microsequences from RPL60 and RPL90 with the corresponding regions in mouse calreticulin (MOCRP; Smith and Koch, 1989) and barley endoplasmin (BAENP; Walther-Larsen et al., 1993) arranged from the N terminus toward the C terminus. Identical residues are indicated by an asterisk. The arrows above the sequences EVFFEE and KKPEDWD indicate regions that were used for the design of degenerated primers for PCR amplifications. The first microsequence of RPL60 is the N-terminal sequence. The N terminus of RPL90 was blocked and could not be determined. The second microsequence of RPL60 and the first microsequence of RPL90 have been reported by Denecke et al. (1993).

the only protein detected with monoclonal antibody 5D3, an anti-idiotypic antibody thought to mimic the structure of the retention signal and could thus exhibit binding affinity to the sorting receptor (Vaux et al., 1990). The binding activity of 1D3 produced in ascites could thus be due to the presence of anti-idiotypic antibodies. RPL40 is present in the ER fraction and is not membrane associated. Its function remains unknown. The remaining polypeptides that were identified based on affinity to 1D3 (Figures 1A and 1B) are currently being purified to obtain microsequence information.

RPL60 Has High Sequence Similarity with Calreticulin

Microsequence data combined with sequence information from mammalian reticuloplasmins were used to design the appropriate degenerated oligonucleotides for polymerase chain reaction (PCR). In addition, the two C-terminal tetrapeptide sequences, encoding KDEL and HDEL, known to occur in proteins retained in the plant ER (Vitale et al., 1993) were used to design the 3′ primers. By using DNA fragments encoding the chimeric proteins PAT-KDEL, PAT-HDEL, PAT-RDEL, and PAT-SDEL (Denecke et al., 1992), we optimized reaction conditions that would allow us to amplify genes encoding a specific tetrapeptide preceding the stop codon (see Methods).

To amplify specifically RPL60 and related genes, we used the microsequence information to design the upstream primers. One of the microsequences obtained from purified RPL60 contained the sequence KKPEDWD, which is 100% conserved between the tobacco protein and mammalian calreticulin. Because this sequence motif is repeated two to three times in mammalian calreticulin and calnexin, we have chosen this sequence to design the 5′ primer for PCR, designated CRP1. The DNA template population was derived from RNA prepared from tunicamycin-treated tobacco seedlings. First-strand cDNA was synthesized from poly(A)$^+$ RNA, and aliquots of this reaction mixture were directly used for PCR amplifications. Two amplification products were obtained of the expected lengths (490 and 600 bp) based on the mammalian sequence, but only in combination with the HDEL-specific 3′ primer and not with the KDEL-specific primer. This result strongly suggested that the calreticulin homolog in tobacco contains the HDEL sequence motif, as has been observed for tobacco BiP (Denecke et al., 1991).

In a similar way, we also cloned the cDNA that encodes the entire mature part of RPL60. The degenerated oligonucleotide CRP2 was designed using the microsequence data from the extreme N terminus (EVFFEE), and PCR amplification resulted in the specific amplification of a 1200-bp fragment in combination with the HDEL primer only. This DNA fragment was also of the expected length based on the assumption that the plant homolog of calreticulin has a similar primary structure. Amplification with CRP1/HDEL using the gel-purified CRP2/HDEL amplification product as template resulted in the amplification of the same products as with total first-strand

cDNA, confirming the presence of the repetitive KPEDWD motif in the large fragment.

All PCR fragments were sequenced, and the deduced amino acid sequences encoded by the open reading frames were compared with the available data bases using the IntelliGenetics program (Mountain View, CA). Internal microsequence data of RPL60 could be confirmed, even though small differences in the amino acid sequences suggest the existence of several isoforms of RPL60 similar to the case of BiP (Denecke et al., 1991). The presence of the HDEL- and signal peptide–encoding regions on the same transcript has been confirmed by sequencing PCR amplification products obtained with specific internal primers in combination with 3′ and 5′ cDNA anchors (J. Denecke, unpublished data). The complete nucleotide sequence of the tobacco RPL60 cDNA presented in Figure 2 has been submitted to GenBank and has been assigned accession number X85382.

The derived amino acid sequence of the full-length clone appeared to have high sequence similarity with mammalian calreticulin (Smith and Koch, 1989) and calnexin from mammals (Wada et al., 1991) and plants (Huang et al., 1993) (Figure 2A). The alignment shows the mature portions of the proteins, because the signal peptides do not contain any sequence similarity. The deduced molecular weight of RPL60 is more similar to that of calreticulin than that of calnexin. RPL60 and mouse calreticulin contain three repeats of the conserved motifs IxDxxxxKPEDWD and GxWxxPxIxNP, whereas calnexin has an additional repeat for each of the motifs. Unlike calnexin, RPL60 lacks a transmembrane domain in the C-terminal region, providing a second argument for a closer similarity with calreticulin. In the case of mouse calreticulin, 53% amino acid sequence identity with tobacco RPL60 was observed. A consensus site for N-linked glycosylation was found at the N terminus of RPL60. This site is not conserved in mammalian calreticulin.

To compare the degree of sequence conservation of RPL60 among different plant species with that of BiP, we cloned and sequenced RPL60 and BiP cDNA from maize, barley, and Arabidopsis, using the same strategy as that described previously based on specific 5′ primers and HDEL. Genomic DNA gel blot analysis provided evidence that both RPL60 and BiP are encoded by multigene families in Arabidopsis (L.E. Carlsson, unpublished results). These results are similar to those reported for the tobacco BiP gene family (Denecke et al., 1991). In this study (Figures 2B and 2C), we describe only one representative member of either RPL60 or BiP. RPL60 shows high sequence conservation with somewhat more divergence in the C-terminal region preceding the HDEL retention motif (Figure 2B). This pattern of conservation is also observed when comparing the BiP clones of tobacco (Denecke et al., 1991), maize (Fontes et al., 1991), barley, and Arabidopsis (this study, Figure 2C), but the hypervariable region at the C terminus is smaller. The C-terminal region of RPL60 contains a large proportion of negatively charged amino acids and may form an α-helix based on secondary structure predictions.

While this manuscript was being reviewed, a cDNA clone of barley was reported (Chen et al., 1994) that has high homology with mammalian calreticulin. The mature part of this protein has 79.3% sequence identity with RPL60. The C terminus is identical to the barley sequence shown in Figure 2B.

RPL60, a New Marker for the ER in Plant Cells

Antibodies against RPL60 were obtained from a recombinant PAT fusion containing the C-terminal 104 amino acids of RPL60, as has been described for tobacco BiP (Botterman et al., 1991; Denecke et al., 1991). In both cases, this strategy was used to avoid the generation of toxic antibodies that interact with the conserved parts of these proteins, which are present in the same endomembrane system by which immunoglobulin chains are synthesized, assembled, and secreted.

The antibodies we obtained recognize an abundant 60-kD protein with high specificity (Figure 3). Cell fractionation experiments using protoplasts from tobacco leaves demonstrated that the bulk of cellular RPL60 cofractionated with the ER marker BiP (Figure 4). We could also confirm that the recognized protein corresponds to the microsequenced protein RPL60 described in Figure 1 (data not shown). The results confirm a predominant ER localization of RPL60, as predicted from the recognition by 1D3 and the presence of the HDEL consensus site.

Since the anti-RPL60 antiserum does not cross-react with other proteins in germinating tobacco seed (Figure 3), we decided to use this serum for microscopic immunolocalization. For visualization of the primary antibody, we used an indirect method based on the peroxidase–antiperoxidase technique, which results in a red insoluble precipitate near the site of antibody binding. DNA is stained blue with this technique. Figure 5A shows cotyledon cells incubated with either preimmune serum (left panel) or anti-RPL60 antiserum (right panel). No precipitate could be detected in the preimmune-treated cells, whereas a clear red precipitate in the perinuclear cytoplasm was observed in the cells incubated with anti-RPL60 antiserum (right panel, lower cells). In cells in the mitotic stage of the cell cycle, the red precipitate appears closely associated with the spindle figure (right panel, upper cell). A similar mitotic cell in the left panel (upper cell to the right) shows no red precipitate. A faint, spotted, red precipitate distributed over the entire cells also suggests a cytoplasmic localization of RPL60 (right panel). Although no details can be revealed with this procedure, these results confirm the specificity of the antiserum (Figure 3) and establish a nonrandom cellular distribution of RPL60.

To obtain further insight into the subcellular localization of RPL60, we stained sections of germinating tobacco seed with anti-RPL60 or anti-BiP antisera in combination with fluorescent anti–rabbit IgG antibodies. This staining results in a green fluorescence at the site of antibody binding. In many interphase cells, this procedure resulted in a clear fluorescence of the

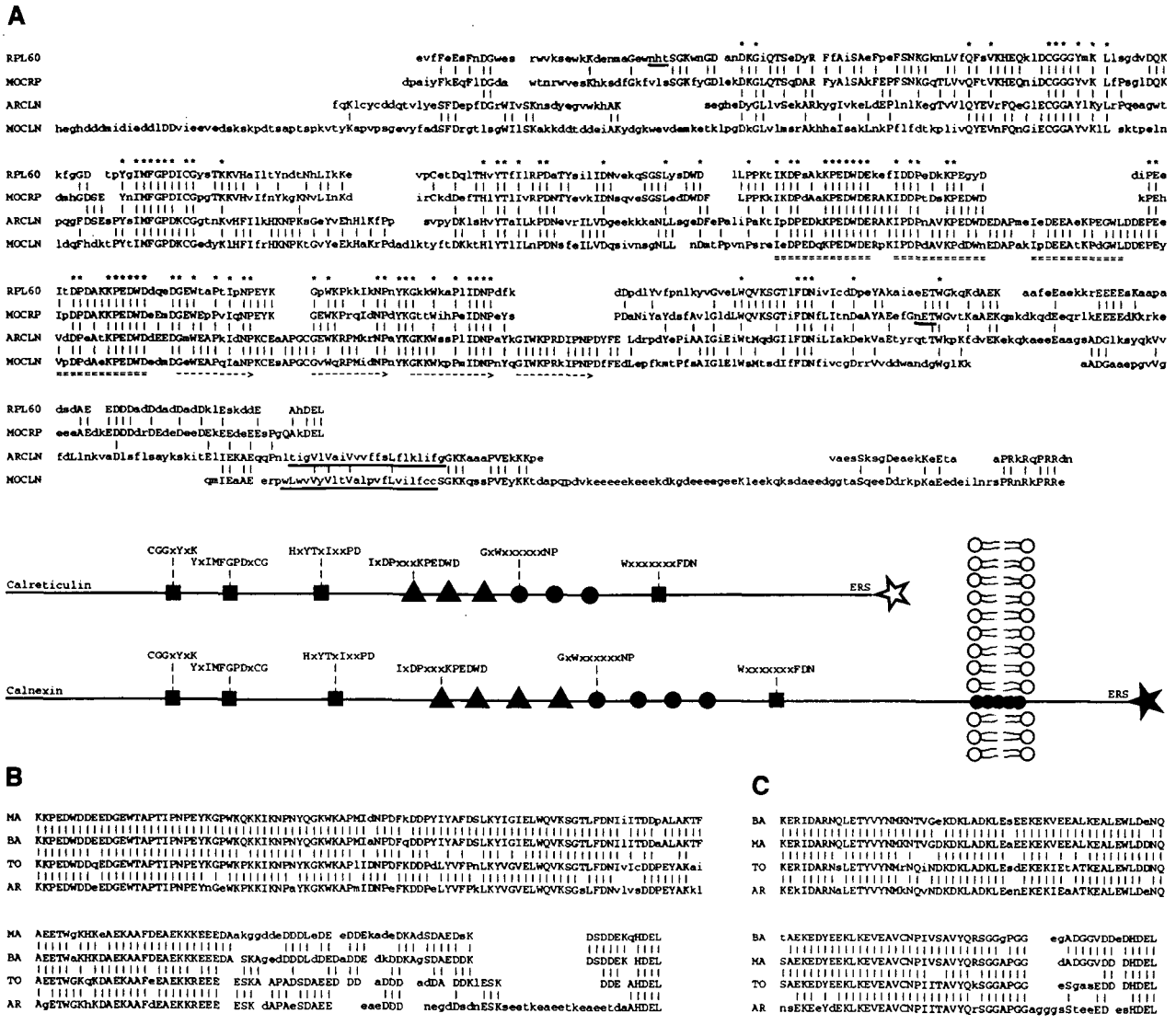

Figure 2. Sequence Alignments.

(A) Amino acid sequence alignment of RPL60, mouse calreticulin (MOCRP; Smith and Koch, 1989), Arabidopsis calnexin (ARCLN; Huang et al., 1993), and mouse calnexin (MOCLN; Wada et al., 1991). Lowercase letters are nonconserved residues. Vertical lines as well as uppercase letters highlight sequence conservations. Asterisks indicate residues conserved in all four proteins. The double-dashed-underlined regions indicate the first proline-rich repetitive motif, which is also shown as solid triangles in the schematic presentation below the sequence data. The dashed arrows indicate the second proline-rich repeat, which is shown as solid circles below. The boldface underlined regions indicate the consensus sites for N-linked glycosylation in the two calreticulin sequences and the transmembrane domains of the two calnexin sequences. Calreticulin and calnexin are schematically shown below. Boxes represent conserved domains that appear once in either protein. Triangles and circles show the repetitive sequence motifs that appear three times in calreticulin and four times in calnexin. The transmembrane domain of calnexin is shown as five overlapping circles, and the ER retention signals (ERS) are indicated by a star and located at the C terminus, as known for calreticulin (open star) and assumed for calnexin (solid star). The calnexin ERS has not yet been characterized, but it differs from the signals for ER localization used by soluble proteins such as calreticulin (Vitale et al., 1993).

(B) Sequence alignment of the C-terminal portion of RPL60 and homologs from maize (MA), barley (BA), tobacco (TO), and Arabidopsis (AR). Note the hypervariable region preceding the HDEL tetrapeptide at the C terminus.

(C) Sequence alignment of the C-terminal portion of BiP homologs of the same four plant species given in **(B)**. Note that the hypervariable region preceding the HDEL tetrapeptide is shorter than that of RPL60.

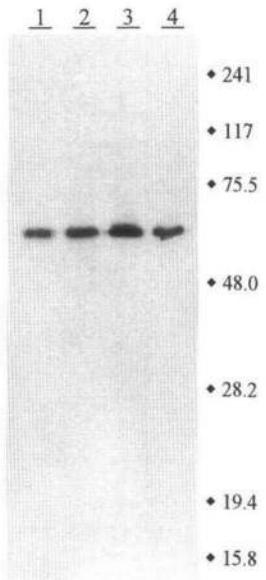

Figure 3. Specificity of the Anti-RPL60 Serum.

Protein gel blot of extracts from germinating tobacco seed harvested 1 day (lane 1), 2 days (lane 2), 3 days (lane 3), and 5 days (lane 4) after the start of imbibition. Numbers at right refer to the molecular mass of the protein standards given in kilodaltons. Proteins were detected using the anti-RPL60 antibody.

nuclear envelope. This was observed using anti-RPL60 antiserum (Figure 5B) as well as anti-BiP antiserum (Figure 5C), whereas in sections incubated with preimmune serum no perinuclear labeling was seen above the weak fluorescent background of the tissue (Figure 5D). At higher magnification, RPL60 was also detected in a polygonal cytoplasmic network apparently radiating from the nucleus (Figure 5F, left cell). In anaphase/metaphase cells, specific labeling of RPL60 was again obvious in the spindle figure (Figure 5F, right cell). Identical staining patterns were observed with the anti-BiP antiserum (Figure 5G), whereas the preimmune serum control at similar magnification showed no specific labeling (Figure 5E). At telophasic stages of the cell cycle (Figures 5H and 5I), RPL60 labeling was also observed in the phragmoplast, a microtubule array involved in cell plate formation (Goosen-de Roo et al., 1984).

RPL60 Is Present in Complexes with Other Polypeptides in Vivo

A clearly defined function of calreticulin has not yet been established. However, the closely related protein calnexin was recently shown to have a chaperone function (David et al., 1993; Margolese et al., 1993; Ou et al., 1993; Jackson et al., 1994; Rajagopalan et al., 1994). Because RPL60 and calreticulin exhibit a high degree of sequence similarity with calnexin, it is conceivable that RPL60/calreticulin may have a related function. To determine whether RPL60 exhibits polypeptide binding properties, tobacco leaf protoplasts were subjected to various forms of stress known to lead to the accumulation of malfolded proteins in the ER lumen. During these incubations, the cells were labeled with ^{35}S-methionine, followed by extraction and immunoprecipitation of RPL60 and associated proteins with antibodies to RPL60. Figure 6 (lanes 1 to 4) shows that several labeled polypeptides coprecipitated with RPL60 and that coprecipitation could be influenced by the type of stress applied to the cells. Treatment with tunicamycin led to a decrease in the apparent molecular mass of RPL60 from 60 to 54 kD. The consensus site for N-linked glycosylation shown in Figure 2A may be utilized in vivo, and lack of glycosylation may cause the observed reduction in molecular mass. This is supported by the fact that RPL60 binds to concanavalin A Sepharose (J. Denecke, unpublished observations).

A 75-kD protein was found to be associated with RPL60 under all conditions but more prominently in tunicamycin-treated and heat shock–treated cells. A 32-kD protein was found to be associated in tunicamycin-treated cells only. DTT-treated and heat shock–treated cells showed RPL60-dependent coprecipitation of a 35-kD protein, whereas a 26-kD protein coprecipitated only with RPL60 from heat shock–treated cells. In the experiments described previously, all cellular RPL60 was precipitated. The portion of RPL60 present in protein complexes is likely to be only part of this. Moreover, each coprecipitating protein should individually be even less abundant. It should also be noted that the apparent abundance is dependent on the number of methionine residues in a polypeptide, which is unknown for all protein bands except for RPL60.

We also demonstrated that coprecipitation of all polypeptides could be suppressed in the presence of ATP and Mg^{2+} (Figure 6, lanes 5 to 8). Binding to the 32-kD protein appeared to be more tight and difficult to release (Figure 6, lane 6). It

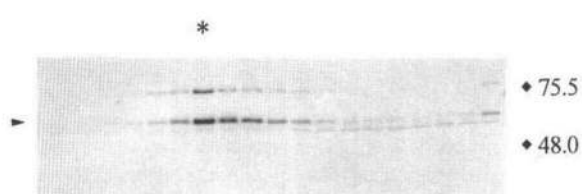

Figure 4. *Cofractionation of RPL60 and BiP.*

Protein gel blot of cell fractions obtained from sucrose density gradients (15 to 42.5%). Samples were taken from the bottom of the gradient and loaded from left to right. The sample at the right side of the panel is the top layer of the cleared microsomal extract (12% sucrose), which contains cytosolic proteins as well as lumenal proteins released from microsomal vesicles during cell disruption. BiP and RPL60 were detected with a mixture of anti-BiP and anti-RPL60 antisera. RPL60 is indicated with an arrowhead. BiP runs close to the 75.5-kD molecular mass marker. The peak fraction for both proteins (indicated with an asterisk) was measured and had a refractive index of 1.1175 to 1.1222.

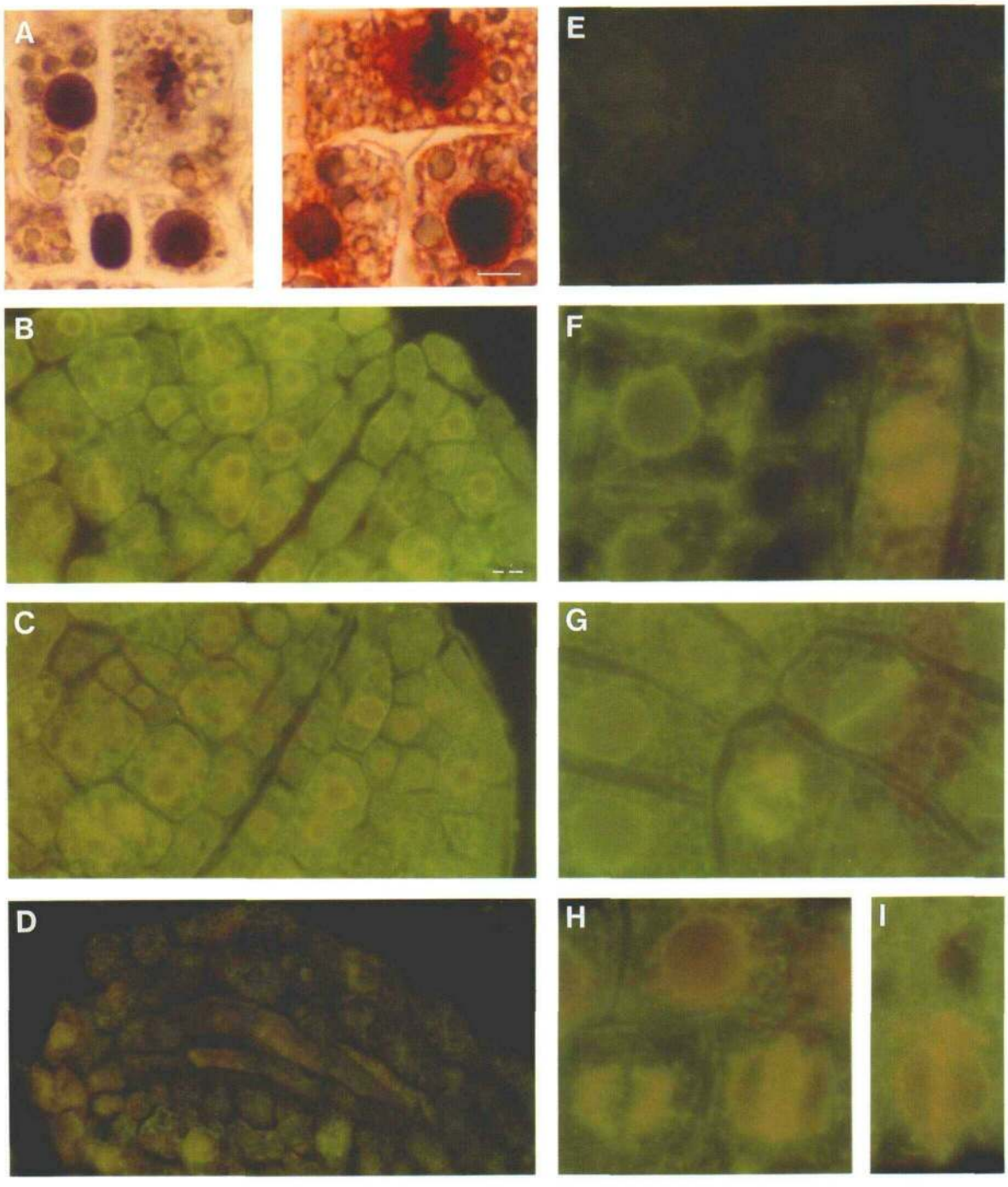

Figure 5. Visualization of the Plant ER by Immunofluorescence.

Sections of germinating tobacco seeds 3 days after imbibition in Murashige and Skoog medium are shown. Stainings were done with antisera against RPL60 and BiP and with preimmune serum. Identical plant material was analyzed by protein gel blots using RPL60 antiserum to demonstrate the specificity of the serum in this tissue (Figure 3, lane 3). Specificity of the anti-BiP antiserum in plant cells was shown previously by Denecke et al. (1991). The scale bar in **(A)** = 5 μm in **(A)** and **(E)** to **(I)**. The scale bar in **(B)** = 10 μm in **(B)** to **(D)**.

(A) Cotyledon cells shown at ×1000. The staining with preimmune serum (left panel) and anti-RPL60 antiserum (right panel) was visualized by the peroxidase–antiperoxidase technique. Both panels contain a mitotic cell in the upper right corner. Note the absence of red in the left panel and the presence of red staining of the perinuclear cytoplasm and the spindle figure in the right panel.

(B) to **(D)** Cotyledon cells stained with anti-RPL60 antiserum **(B)**, anti-BiP antiserum **(C)**, and preimmune serum **(D)** are shown at ×400. Note the staining of the nuclear envelopes in **(B)** and **(C)** and the absence of staining in **(D)**.

(E) to **(I)** Sections stained with preimmune sera **(E)**, anti-RPL60 antiserum **([F], [H], [I])**, and anti-BiP antiserum **(G)** shown at ×1000. Note the characteristic staining pattern of the dividing cell in **(F)** (stained with anti-RPL60 antiserum) and **(G)** (stained with anti-BiP antiserum). Dividing cells at different stages are shown in **(H)** and **(I)**.

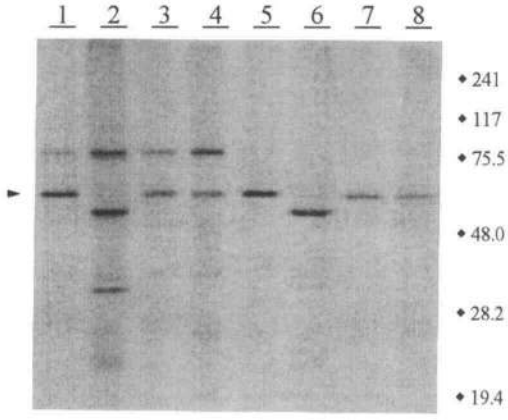

Figure 6. Detection of RPL60 Protein Complexes.

Protein gel analysis of in vivo–labeled proteins immunoprecipitated with anti-RPL60 antisera. Immunoprecipitations were done in the absence (lanes 1 to 4) or in the presence (lanes 5 to 8) of ATP and Mg^{2+}. Extracts were prepared from control cells (lanes 1 and 5), tunicamycin (20 μg/mL)-treated cells (lanes 2 and 6), DDT (5 mM)-treated cells (lanes 3 and 7), and heat shock–treated cells (lanes 4 and 8). The arrowhead indicates RPL60. Note the apparent molecular weight of RPL60 is reduced in lanes 2 and 6.

is also possible to elute the bound proteins from immunoprecipitated RPL60 complexes by several washes with ATP and Mg^{2+} (J. Denecke and K.M.C. Sinjorgo, unpublished results). This result demonstrates that coprecipitation is not due to cross-reactivity to other proteins in the extract and confirms that the antibody is specific (shown in Figure 3). Particularly, it confirms that the 54-kD protein in Figure 6, lanes 2 and 6, can only be RPL60.

Coordinate Induction of RPL60, BiP, and Endoplasmin in Barley Aleurone Cells

To obtain indirect information about the possible function of RPL60, we wanted to compare its expression with that of ER proteins whose function as chaperones is well established. We have initiated this analysis using the aleurone layer of barley grains as a model system because it is one of the few plant tissues with a primary function in protein secretion. When induced by gibberellins (GAs), hydrolases such as α-amylase are secreted by the aleurone. Furthermore, GA-induced hydrolase secretion by the aleurone involves a pronounced proliferation or reorganization of the ER (Jones, 1969a, 1969b, 1980; Evins and Varner, 1971; reviewed in Jones, 1985). These characteristics allowed us to analyze the regulation of RPL60 expression in relation to ER chaperones, such as BiP and endoplasmin. Because α-amylase can constitute >50% of the newly synthesized protein in this tissue (Higgins et al., 1982), we compared the expression profile of these reticuloplasmins with that of α-amylase.

We chose a batch of barley cultivar Triumph, in which hydrolase secretion by the aleurone layer is completely dependent on the presence of GA_3 (Wang et al., 1994). We isolated RNA from barley aleurone after incubation of the tissue for various periods of time in the presence and absence of GA_3. RNA gel blots were made and subsequently hybridized with the probes for barley RPL60, BiP (the sequences of the isolated barley clones correspond to Figures 2B and 2C), endoplasmin (Walther-Larsen et al., 1993), and high-pI α-amylase (Rogers, 1985). The latter appeared most suitable because the expression of the corresponding gene is strictly controlled by GA_3 (Huang et al., 1984; Rogers, 1985). Figure 7A shows the time course of GA_3-dependent accumulation of high-pI α-amylase mRNA. In the absence of GA_3, no transcript was present. At the time point at which α-amylase transcripts become detectable, a pronounced rise in the mRNA abundance was observed for RPL60, BiP, and endoplasmin. These transcripts showed a basal level in noninduced aleurone, consistent with a role for the encoded proteins in protein biosynthesis by the ER. Prolonged incubation with and without GA_3 did not result in a further rise of the observed mRNA levels (M.J. van Zeijl and K.M.C. Sinjorgo, unpublished results). Figure 7B shows that the GA_3 dose–response dependence of the accumulation of the reticuloplasmin mRNAs was very similar to that of the α-amylase. For all mRNAs, a pronounced increase in their abundance was observed at GA_3 concentrations of 10^{-8} M and above (Figure 7B). However, a weak increase of all reticuloplasmin mRNA levels was already visible at GA_3 concentrations of 10^{-9} M when no α-amylase transcripts could be detected even after longer exposures.

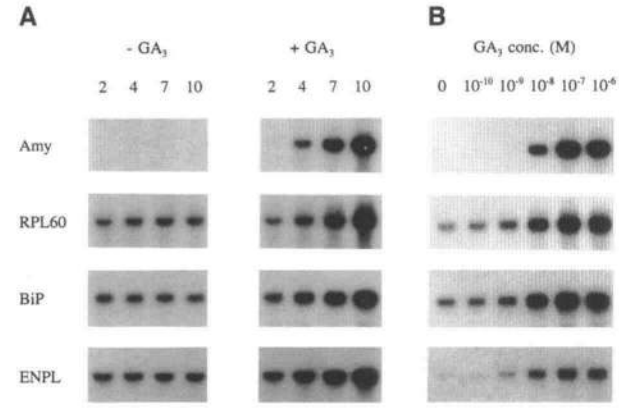

Figure 7. Correlation of Secretory Activity in Barley Aleurone Cells with RPL60, BiP, and Endoplasmin Transcript Abundance.

(A) Time-dependent accumulation of GA_3-stimulated and uninduced basal transcript levels corresponding to α-amylase (Amy), RPL60, BiP, or endoplasmin (ENPL). Numbers above the lanes refer to the time in hours after adding GA_3 (0.1 μM, +) or after mock treatment (−). **(B)** Dose–response relation for GA_3. Transcript levels were measured 8 hr after adding GA_3. The different probes are as indicated in **(A)**. Numbers above the lanes refer to the molarity of GA_3 in the incubation medium.

Tissue-Specific Regulation of RPL60, BiP, and PDI

To characterize RPL60 further, we compared its expression profile with that of BiP and PDI in different tissues of tobacco plants. Roots, leaves, stems, petals, anthers, stigmas, styles, and germinating seed (4 days after the start of imbibition) were analyzed. Tissues were chosen as in previous experiments on the expression of tobacco BiP (Denecke et al., 1991), except that young roots of axenically grown tobacco plants 10 days after transplantation were used. Figure 8A shows that the general pattern of expression was similar but not identical for the three genes. For all transcripts, the lowest abundance was observed in leaves. Higher levels were found in roots and stems and particularly in flower tissues and germinating seed. The higher BiP expression levels in roots compared with previous experiments (Denecke et al., 1991) are due to the fact that young roots were chosen. In young root tissues, the proportion of actively dividing cells is high. RPL60 expression was very similar to that of BiP but not of PDI. PDI mRNA levels were very low in stems, and the levels in petals, styles, and germinating seed were not as much increased compared to the corresponding levels in leaves as those of BiP and RPL60.

Figure 8B shows the expression profile of the three reticuloplasmins in stigmas and styles at different developmental stages (see Methods). In stigmas, transcript levels were higher in young (ST1) and old (ST4, pollinated) tissues, and in styles, transcript levels decreased rapidly after pollination (SY3). In all cases, expression profiles were similar except for a lower abundance of PDI transcripts in styles compared with RPL60 and BiP. This confirms the data shown in Figure 8A, in which pools of different developmental stages were analyzed. The pool of styles was strongly biased for SY3, which explains the low transcript levels compared with the stigma in Figure 8A. In summary, the data illustrate that tissue-specific expression patterns are similar but not identical for the three reticuloplasmins.

Treatment with Tunicamycin Does Not Lead to a Coordinate Induction of RPL60, BiP, and PDI

Tunicamycin inhibits N-linked glycosylation, which results in the malfolding of a subset of glycoproteins whose glycans are necessary for their correct folding. The presence of this set of malfolded proteins in the lumen was proposed as the trigger for enhanced transcription of the BiP gene (Kozutsumi et al., 1988). The increased binding of BiP to malfolded protein aggregates is thought to result in a reduction of the free BiP concentration in the lumen, which may be monitored by a transmembrane receptor kinase (Cox et al., 1993; Mori et al., 1993). Unlike the GA_3-mediated stimulation of total secretory protein synthesis by the ER in aleurone cells, tunicamycin treatment leads only to the induction of a subset of ER chaperones, which can dissolve protein aggregates in the ER lumen rather than induce the entire protein synthesis and transport machinery

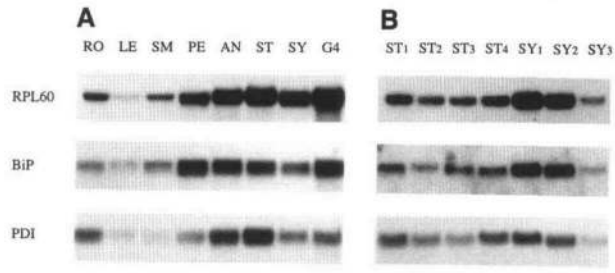

Figure 8. Expression Profile of RPL60, BiP, and PDI in Different Tobacco Tissues.

(A) Gel blot analysis of RNA extracted from different plant tissues including roots (RO), leaves (LE), stems (SM), and flower tissues. The latter were extracted as mixtures of developmental stages as described by Koltunow et al. (1990). Petals (PE) are mixtures of stages 3 to 12, anthers (AN) are mixtures of stages −3 to 8, stigmas (ST) are mixtures of stages 1 to 12, and styles (SY) are mixtures of stages 3 to 12. Germinating seeds were extracted 4 days after the start of imbibition (G4). The different probes are as indicated on the left.

(B) Gel blot analysis of RNA extracted from stigmas and styles at different developmental stages. ST1, stages 1 to 8; ST2, stage 9; ST3, stages 10 to 11; ST4, stage 12, pollinated; SY1, stages 3 to 8; SY2, stages 9 to 11; SY3, stage 12, pollinated.

of the endomembrane system. We used tobacco protoplasts as a model system.

Transcripts were detected using probes to RPL60, the tobacco BiP isoform BLP2 (Denecke et al., 1991), and tobacco PDI (J. Denecke and L.E. Carlsson, unpublished results). As shown in Figure 9A, RPL60 mRNA accumulated only modestly in response to treatment with tunicamycin compared with the strong induction seen for BiP and PDI. BiP transcripts accumulated very quickly and reached almost maximum levels after 2 hr. We also observed a decrease of induced BiP transcript levels during prolonged incubations with tunicamycin (Figure 9A, 9 hr). PDI transcripts accumulated more slowly than BiP transcripts, and no decrease was seen after prolonged incubations. The basic β-1,3-glucanase GN1 (Castresana et al., 1991), a pathogenesis-related protein synthesized and transported to the vacuole by the endomembrane system in protoplasts, was used as a negative control. Its mRNA levels were not significantly increased during this treatment. The data show that RPL60, BiP, and PDI transcript levels are not induced coordinately and that RPL60 in particular is much less induced by inhibition of N-linked glycosylation in the ER lumen.

Stress- and Hormone-Specific Regulation of RPL60, BiP, and PDI

To define further the similarities and differences in expression profiles, we wanted to investigate gene expression under a variety of conditions that might cause an increase in the secretory activity of plant cells. Similarly, we were interested in analyzing the effect of conditions that cause the accumulation of

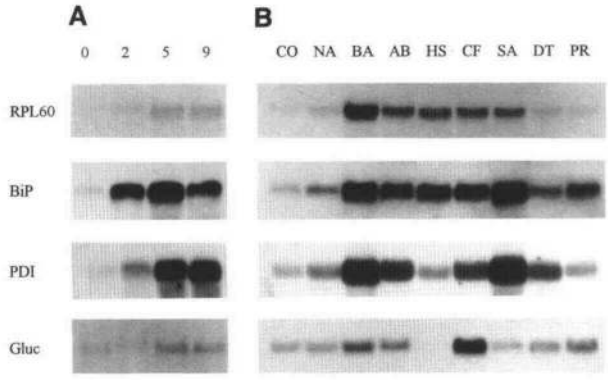

Figure 9. Stress Induction of RPL60, BiP, and PDI Transcripts in Tobacco Protoplasts and Leaves.

(A) Gel blot of RNA extracted from tobacco protoplasts at different times during tunicamycin treatment. Numbers above the lanes refer to the time in hours after adding tunicamycin (20 μg/mL) to the culture medium. Transcripts were detected of RPL60, BiP, PDI, and a basic β-1,3-glucanase (Gluc).

(B) Gel blot analysis of RNA extracted from tobacco leaves in control plants (CO), protoplasts (PR), and plants subjected to various forms of stress or treatments with hormones such as NAA (NA), BAP (BA), abscisic acid (AB), heat shock (HS), culture filtrate of *E. carotovora* (CF), salicylic acid (SA), or DDT (DT). See Methods for a detailed description of the treatments. The different probes are as indicated in **(A)**.

malfolded proteins other than those that accumulate due to underglycosylation. Because leaves show low basal transcript levels for these genes, we used this tissue as a model system and subjected leaves to various forms of stress or exposure to phytohormones.

Figure 9B illustrates that RPL60, BiP, and PDI differ in their overall response to the various stimuli. RPL60 transcript levels were increased during treatment with cytokinin, abscisic acid, heat shock, cell wall–degrading enzymes of plant pathogenic bacteria, and salicylic acid. Cytokinin treatment led to the highest transcript levels. BiP mRNA levels were induced by the same treatments, but salicylic acid treatment led to high mRNA levels similar to those observed for treatment with cytokinin. In contrast to RPL60, BiP levels were also induced in protoplasts prepared from leaves. An important result is that BiP appears to be heat shock induced, in contrast to previous experiments (Denecke et al., 1991). However, in those experiments, germinating seeds were used in which the basal level of BiP transcripts was already high compared with that in leaves (see Figure 8A). Most likely, an additional induction by heat shock was masked by these high basal levels. The pattern of PDI expression differs from both RPL60 and BiP in the absence of induction by heat shock. In contrast to BiP, no induction was seen in protoplasts, whereas treatment with DTT led to an increase of PDI mRNA levels.

No correlation of expression levels could be seen for any of the known tobacco pathogenesis-related proteins, which are synthesized by the endomembrane system under various stress conditions. This is shown with the basic β-1,3-glucanase that is specifically induced by treatments with cell wall–degrading enzymes of *Erwinia carotovora* but not by treatment with salicylic acid after similar short incubations (12 hr). A correlation with the enhanced synthesis of a specific abundant secreted protein such as α-amylase in the secretory aleurone tissue (Figure 7) therefore could not be performed. We concluded that genes encoding the different ER proteins are not regulated in a coordinated fashion.

DISCUSSION

Identification of a Sorting Signal: A Powerful Method To Identify New Proteins of a Specific Cell Compartment

The specific immunological recognition of the sorting signal responsible for the accumulation of soluble ER resident proteins in plants and mammalian cells (Vaux et al., 1990; Denecke et al., 1992) was used to develop a methodology to characterize the lumenal content of the plant ER (Denecke et al., 1993). Preliminary microsequence data for two abundant ER proteins suggested that homologs of mammalian BiP, calreticulin, and endoplasmin had been identified (Denecke et al., 1993). We have now optimized the extraction procedure for reticuloplasmins and classified a family of soluble acidic proteins, isolated from microsomal pellets by osmotic shock and concentrated by anion exchange fractionation. This method allowed us to identify soluble proteins containing ER localization signals that are less abundant than the well-characterized ER proteins BiP, calreticulin, and endoplasmin. We are currently studying other proteins identified by this method, and until now, no protein localized in any compartment other than the ER has shown itself recognizable by 1D3. This immunological recognition may lead to the identification of ER proteins whose retention signals are not recognized from the primary sequence alone (Vitale et al., 1993). Our data and the fact that the structural features of the ER localization motif are very conserved (Denecke et al., 1992) indicate that immunological identification of the retention motif is a powerful method to identify new ER proteins. A similar methodology may also be used for sorting signals other than ER localization motifs.

RPL60/Calreticulin, a Novel Marker for the ER in Plants

A cDNA covering the entire coding region of the mature part of RPL60 was obtained, and the deduced amino acid sequence clearly established that RPL60 is a potential homolog of calreticulin. The overall sequence identity between the plant and the mammalian proteins is as high as in the case of BiP (Denecke et al., 1991), endoplasmin (Walther-Larsen et al., 1993), and PDI (Shorrosh and Dixon, 1991). Due to the lack of yeast mutants for calreticulin and the fact that no clear role

has been established for this protein, it is not possible to perform functional complementation experiments. However, the high sequence similarity leads us to propose that RPL60 is the plant homolog of mammalian calreticulin and that it should therefore be called tobacco calreticulin.

Antibodies prepared against the C-terminal portion of the protein gave the same pattern of immunolabeling as that observed for BiP, whose localization in the ER is beyond doubt. Staining patterns that would be typical for the Golgi apparatus (Satiat-Jeunemaitre and Hawes, 1992; Henderson et al., 1994) were not observed. However, cell fractionation and immunofluorescence microscopy are unsuitable techniques for excluding the possibility that ER resident proteins such as calreticulin and BiP can leak out to the Golgi apparatus.

Interestingly, calreticulin and BiP are detected in microtubule arrays of dividing cells. It has been shown that the ER is arranged parallel to the microtubules during cell division in plant cells (Schmiedel et al., 1981), and our data support these observations. The concentration of RPL60 and BiP at the cell plate may reflect a high level of activity of the endomembrane system after cell division to synthesize cell wall polysaccharides and proteins at the newly formed cell wall.

Is Calreticulin a Calcium Storage Protein or a Chaperone?

Calreticulin is a highly abundant reticuloplasmin in both mammalian and plant cells. Due to its calcium binding capacity, this protein could be involved in calcium immobilization in the ER lumen and have a role in signal transduction mechanisms that are based on the release of calcium by the ER. In mammalian cells, the protein is known to bind calcium through a low-capacity/high-affinity region and a high-capacity/low-affinity region (Michalak et al., 1992). In the plant protein, the high-affinity region is extremely conserved. The low-affinity region is at the C terminus of the protein, and on the amino acid level there is less similarity except for the conserved predominance of acidic residues within this region. The barley homolog of calnexin is 79.3% identical to the tobacco homolog and was shown to possess calcium binding activity similar to that reported for mammalian calreticulin (Chen et al., 1994). It is unclear in any eukaryotic system whether the calcium binding activity is involved in calreticulin function. Our data indicate that RPL60/calreticulin is a homomultimeric glycoprotein in its native form, although the number of subunits is unknown.

It has been shown that radiolabeled calreticulin of mammalian cells binds to proteins blotted on nitrocellulose membranes and that the interaction occurs mainly through its C-terminal domain (Burns and Michalak, 1993). Here, we present evidence that plant calreticulin is present in protein complexes in vivo. Native complexes could be immunoprecipitated, complex formation was shown to be stress dependent, and complexes could be dissociated by addition of ATP and Mg^{2+}. These observations indicate that complex formation is of biological significance. The polypeptides that form complexes with calreticulin have not yet been identified. It is unclear how ATP is involved in the dissociation of calreticulin complexes, because the sequence of the cDNA clone does not reveal any obvious consensus sites for ATP binding. The 75-kD protein has the same molecular mass as BiP and is associated even when cells are grown in the absence of stress; this calreticulin protein complex is the most abundant one. This finding would explain the ATP-mediated release of the 75-kD protein from calreticulin. However, it cannot explain the ATP-dependent coprecipitation of the other polypeptides, because BiP has never been shown to bind to several polypeptides simultaneously. In particular, the 32-kD protein that interacts with calreticulin to some extent even in the presence of ATP indicates that calreticulin is forming complexes independent of the presence of the 75-kD protein. Clearly, further work is required to determine whether the presence of calreticulin in protein complexes reflects a chaperone activity, as suggested for calnexin.

Is Calreticulin a Soluble Counterpart of Calnexin?

The primary structure of calreticulin is very similar to that of calnexin, an ER resident type I transmembrane protein. Calnexin has a longer C terminus comprising the transmembrane domain and a charged cytoplasmic tail, and its lumenal portion could be regarded as a membrane-anchored form of calreticulin (see Figure 2A). Calnexin was shown to associate with secretory glycoproteins (Ou et al., 1993) but with a binding specificity different from that reported for the ER lumenal molecular chaperone BiP. Moreover, complexes were shown to be formed with membrane proteins through transmembrane interactions that are postulated to be unavailable to the major soluble ER chaperone BiP (Margolese et al., 1993). Whereas calnexin binds to a variety of newly synthesized proteins, prolonged association was observed for unassembled ER-retained T-cell receptor β chains (David et al., 1993). Also, the ability of calnexin to protect and retain major histocompatibility complex class I assembly intermediates was proposed to contribute to the efficient intracellular formation of class I peptide complexes (Jackson et al., 1994). Engineered calnexin that lacks the ER retention motif for type I membrane proteins and localizes to the Golgi apparatus was able to redirect normally ER-retained CD3 ε-chains to the Golgi in coexpression experiments in vivo (Rajagopalan et al., 1994). The latter report provided the most direct evidence for an in vivo chaperone function, and calnexin may thus be involved in protein assembly and perhaps in the retention within the ER of unassembled protein subunits in vivo. The high similarity between the lumenal portion of calnexin and calreticulin suggests that calreticulin may have a similar function in protein assembly.

Other Functions for Calreticulin?

Mammalian calreticulin was shown to contain a putative nuclear targeting signal (PPKKIKDPD), and the possibility of a

nuclear localization has been suggested by Michalak et al. (1992). However, the corresponding sequences in the tobacco protein (PPKTIKDPS) and the barley protein (PPKEIKDPS) do not have such a consensus site. Moreover, the probability that the signal peptide of calreticulin would escape retrieval by the signal recognition particle, which can occur either cotranslationally or post-translationally, is not very high, and to our knowledge, this situation has never been reported for any protein. This view is challenged by the recent observation that purified calreticulin is capable of inhibiting nuclear hormone receptor activity by binding to the KXFFKR DNA binding domain of steroid hormone receptors (Burns et al., 1994; Dedhar et al., 1994). These authors also report the detection of calreticulin in nuclear extracts and in the cytoplasm. Our studies do not exclude this possibility. Clearly, further research is required to define the function of calreticulin in vivo, but the subcellular localization and the primary sequence suggest a function in the lumen of the ER/nuclear envelope rather than in the nucleoplasm.

Regulation of Transcript Levels of Calreticulin and Other ER Proteins

We have studied the mRNA levels of calreticulin and other ER proteins in several conditions under which the activity of the ER changes. During seed germination, hydrolase secretion is needed for a relatively short time to mobilize starch reserves from the endosperm. The barley aleurone undergoes dramatic morphological changes at the onset of hydrolase secretion, one of which is the proliferation/reorganization of ER membranes (Jones, 1969a, 1969b, 1980; Evins and Varner, 1971; reviewed in Jones, 1985). Calreticulin, BiP, and endoplasmin represent a large proportion of the steady state content of the ER lumen and could thus be regarded as a measure for ER abundance. The GA_3-mediated induction of secretory enzyme synthesis in barley aleurone cells was accompanied by a coordinated increase in the mRNA concentrations for calreticulin, BiP, and endoplasmin. Both the timing and the GA_3 dose dependence of reticuloplasmin mRNA correlate with that of α-amylase, an abundant secretory protein we used as a representative marker to monitor secretory activity in these cells. These observations correspond well with ultrastructural studies and measurements of lipid metabolism which have indicated that ER proliferation occurs (Jones, 1969a, 1969b; Evins and Varner, 1971). However, this observation could also indicate merely a higher secretory activity of the ER. The latter possibility is supported by measurement of cytochrome *c* reductase levels, a classic marker of the ER in qualitative cell fractionation. In purified microsomes of GA_3-treated aleurone cells, a putative BiP cognate was found to enrich relative to cytochrome *c* reductase compared with microsomes prepared from ABA-treated cells (Jones and Bush, 1991). Hence, it remains to be determined whether the ER proliferates or merely reorganizes at the onset of hydrolase secretion in response to GA_3.

ER chaperones such as BiP are thought to be regulated via a feedback mechanism termed the malfolded protein response. The tunicamycin-mediated induction of BiP and PDI was proposed to be achieved by such a feedback regulation, and recently, a transmembrane receptor kinase located in the endomembrane system of yeast was implicated in this process (Cox et al., 1993; Mori et al., 1993). Results from our experiments revealed that calreticulin, BiP, and PDI are not always coordinately regulated. Treatment of tobacco cells with tunicamycin mainly induced BiP and PDI, whereas calreticulin expression was almost unaffected under these conditions. Other stress treatments were also shown to result in differential responses. To explain these results, we postulate that signal transduction pathways other than the malfolded protein response must exist to induce the enhanced synthesis of different ER proteins under specific stress conditions. The cloning of genes encoding other ER resident proteins involved in protein biosynthesis by the endomembrane system will provide the necessary target genes to study signal transduction starting from the lumen of the endomembrane system and to study regulatory mechanisms that ensure correct function of the ER under various conditions.

METHODS

Protein Purification and Microsequencing

Microsomal fractions were prepared from germinating tobacco seed 3 days after the start of imbibition, as described by Denecke et al. (1992). Soluble proteins were extracted by osmotic shock treatment in a buffer containing 50 mM Tris-HCl, pH 7.5, 2 mM EDTA, 10 mM β-mercaptoethanol, and 1 mM phenylmethylsulfonyl fluoride, during 5 min on ice. The pellet was resuspended by gently pipetting the solution through a Pasteur pipette. The suspension was centrifuged at 16,000 rpm during 10 min in an Eppendorf centrifuge (Eppendorf-Netheler-Hinz GmbH, Hamburg, Germany), and the membrane pellet was resuspended once more, extracted once more as described previously, and then discarded. The supernatants were pooled, frozen, thawed, and centrifuged as given previously, and the supernatant was dialyzed against 20 mM Tris-HCl, pH 7.5. The dialyzed solution was centrifuged, and the supernatant was loaded on a Mono-Q column using the Pharmacia FPLC system. Proteins were eluted with a continuous salt gradient (buffer B was 20 mM Tris-HCl, pH 7.5, 1 M NaCl), and fractions were analyzed by protein gel blotting using the monoclonal antibodies 1D3 and 5D3 and polyclonal antibody KX5-KDEL (Vaux et al., 1990). Fraction 9 in Figure 1 was concentrated by spin dialysis using centricon 10 microconcentrators (Amicon Inc., Beverly, MA), and gel filtration was performed on a Superose 6 column (Pharmacia) with 20 mM Tris-HCl, 100 mM NaCl as the mobile phase.

1D3-reactive gel filtration fractions containing either RPL60 or RPL90 were brought to pH 2 with trifluoroacetic acid (TFA) and concentrated by reverse phase chromatography on a C4 Brownlee column (2.1 × 30 mm) (Applied Biosystems Inc., Foster City, CA). Buffer A was 0.12% TFA in water. Buffer B was 90% acetonitrile, 10% water, 0.1% TFA. The column was developed with a gradient of 2 to 62% buffer B during 60 min. The flow rate was 0.1 mL/min. The chromatogram was monitored with a Waters Diod array detector (Millipore AB. Waters

Chromatography Division, Sundbyberg, Sweden) at 214 nm. The purified fraction was concentrated in the Savant spin (Savant Instruments Inc., Farmingdale, NY). The protein pellet was resuspended in 6 M guanidine hydrochloride, diluted threefold with 20 mM Tris-HCl, pH 8.0, and digested with *Achromobacter lyticus* Lys C protease according to Riviere et al. (1991). Peptides were purified by reverse phase chromatography as described previously. Purified peptides were applied to a 470A gas phase sequencer with online HPLC analysis essentially according to the manufacturer's instructions (Applied Biosystems Inc.).

First-Strand cDNA Synthesis for Amplification by the Polymerase Chain Reaction (PCR)

First-strand cDNA was synthesized starting from 1 μg of poly(A)$^+$ RNA using the Stratagene cDNA synthesis kit. The reaction mixture (50 μL) was diluted twice with distilled water, boiled for 5 min, and centrifuged for 5 min in an Eppendorf centrifuge at maximum speed. The supernatant was kept and is referred to as the cDNA mix. For maize coleoptiles, barley root tips, and germinating seed of *Arabidopsis thaliana*, the same procedure was followed except that 20 μg of total RNA was used for the first-strand cDNA synthesis.

Oligonucleotides and PCR Amplifications

The sequences of the oligonucleotides are given in the 5′ to 3′ direction: CRP1, AARAARCCNGARGAYTGGGA; CRP2, AGCTGCAGARGTNTTYTTYGARGA; KDEL, CGTTATCTTACAGYTCRTCYTT; HDEL, CGTTATCTTACAGYTCRTCRTG; PDI1, TGGTGYGGNCAYTGYAA; PDI2, CACCAAGGNGCRTARAAYTC; and BiP1, TTYGCAGARGARGAYAARAA. For all amplifications, 40 cycles of 30 sec at 95°C, 30 sec at 45°C, and 2 min at 71°C were done using Dynazyme *Taq* polymerase (Finnzmes OY, Espoo, Finland) and the buffer recommended by the manufacturer. A reaction mixture of 30 pmol/50 μL was used of each degenerated oligonucleotide, and a reaction mixture of 0.2 μL/50 μL was used of the aforementioned cDNA mix. Specificity for KDEL or HDEL amplification was tested with 10 ng of plasmids carrying the chimeric genes described by Denecke et al. (1992). These genes encode phosphinothricin acetyltransferase (PAT)-HDEL, PAT-KDEL, PAT-RDEL, and PAT-SDEL. Amplifications were done in the presence of HDEL or KDEL oligonucleotides in combination with a 5′ primer that covers the N terminus of the coding region. Under the aforementioned reaction conditions, HDEL amplification did not result in amplification products using PAT-KDEL, PAT-RDEL, and PAT-SDEL plasmids. Similar specificity was observed for KDEL. CRP1 and CRP2 were combined with HDEL for the cloning of RPL60 cDNA molecules. Protein disulfide isomerase 1 (PDI1) (WCGHCK) was used in combination with KDEL for the cloning of tobacco PDI. PDI1 and PDI2 (EFYAPWC) were used to amplify an internal fragment from the gel-purified fragment obtained with PDI1/KDEL. The PCR amplification products were found to be highly conserved with the recently cloned alfalfa PDI cDNA (Shorrosh and Dixon, 1991). The complete tobacco PDI sequence will be published elsewhere. Binding protein (BiP) homologs from barley and Arabidopsis were isolated by PCR using oligonucleotide BiP1 (FAEEDKK) in combination with the HDEL primer.

Cloning and Sequencing

DNA fragments of the appropriate size from the PCRs were ligated into an EcoRV site in the plasmid pGEM5z, yielding pRPL60/1 (490-bp fragment), pRPL60/2 (600-bp fragment), and pRPL60/3 (1200-bp fragment). Calreticulin- and BiP-encoding DNA fragments from different plant species as well as the tobacco PDI amplification products were cloned in the same vector. The plasmids obtained were prepared using the Qiagen midiprep kit (Diagen, Stockholm, Sweden) and sequenced with the dideoxy method using the 2.0 Sequenase kit (Amersham).

Plasmid Constructs

All DNA manipulations were performed according to established procedures (Sambrook et al., 1989). The *Escherichia coli* strain used for plasmid construction was MC1061 (λ) (Casadaban and Cohen, 1980). pRPL60/1, which contains DNA sequences encoding the last 104 amino acids of tobacco RPL60, and the expression vector pGSFRC1 (Botterman et al., 1991) were cut with BglII and filled in using the Klenow fragment of DNA polymerase I. Both plasmids were then cut with PstI and ligated. The plasmid then encoded a fusion protein between PAT and calreticulin.

Purification and Characterization of the PAT-RPL60 Fusion Protein

The PAT-RPL60 fusion protein was synthesized under control of the inducible λ P_R promoter using the *E. coli* strain NF1 carrying pLC101. The strain was grown at 28°C to OD 0.6. The promoter was then induced by switching the temperature to 42°C. This was done by adding an equal volume of fresh Luria-Bertani medium (Sambrook et al., 1989) at 56°C, followed by further incubation at 37°C for 4 hr. The cells were harvested, spun down, and frozen. The bacterial pellet was dissolved in 20 mL of extraction buffer (50 mM Tris, 2 mM EDTA, 10 mM β-mercaptoethanol), and the suspension was sonicated and centrifuged for 30 min at 18,000 rpm and then frozen. After thawing and centrifugation, the DNA in the supernatant was removed by streptomycin sulfate precipitation, and the supernatant was applied to an anion exchange column, Mono-Q 10/10, using the Pharmacia FPLC system. Proteins were eluted using a linear salt gradient of 0 to 1 M in 20 mM Tris-HCl, pH 7.5. Fractions were analyzed for PAT activity according to Denecke et al. (1992), and the fractions with the highest PAT activity were pooled and applied on an S300 gel filtration column (Pharmacia). The fractions containing PAT activity were concentrated on a Mono-Q 5/5 column and analyzed on SDS-PAGE. The purified protein was dialyzed against PBS and used as such for immunization.

Antibody Production

Two rabbits were immunized with the purified chimeric protein. Aliquots (1 mg/mL) of purified fusion protein were sent to Agrisera AB (Umea, Sweden), and two rabbits were injected during 3-week intervals. Serum was recovered by consecutive bleedings 6 days after the third, fourth, and fifth injections.

Cell Fractionation

Protoplasts (2.5×10^7) were washed in 250 mM NaCl, pelleted, and resuspended in 2 mL of fractionation buffer (12% sucrose, 100 mM Tris-HCl, pH 7.5, 1 mM EDTA). Resuspended cells were gently disrupted by passing through a Pasteur pipette, and cell debris was spun down

at 1800*g* for 5 min in a swing-out rotor. Supernatant (1.5 mL) was layered on a sucrose gradient (15 to 42.5%) and centrifuged at 150,000*g* for 2 hr. Twenty fractions were taken from the bottom of the gradient.

Plant Material and Culture

Imbibition of barley grains (cv Triumph, crop 1988), isolation of aleurone layers, and further incubations were done as described by Sinjorgo et al. (1993) using a medium of 20 mM succinic acid, 20 mM $CaCl_2$, pH 4.2. *Nicotiana tabacum* cv Petit Havana SR1 plants (Maliga et al., 1973) were grown axenically and in the greenhouse under conditions described by Denecke et al. (1991), and germinating seeds for RNA extraction were prepared as described by Denecke et al. (1992). Protoplasts of tobacco leaves from axenically grown plants were isolated and kept in culture as described by Denecke et al. (1989). Six-week-old axenically grown tobacco plants were sprayed with 10 mL of aqueous solutions containing 0.2% Tween 20 and either naphtalene–acetic acid (2 μg/mL), 6 benzyl-aminopurine (2 μg/mL), abscisic acid (50 μM), salicylic acid (5 mM), or DDT (5 mM). Every leaf of each plant was sprayed from both sides. *Erwinia carotovora* culture filtrate containing pectinolytic and cellulolytic enzymes was prepared by centrifuging an overnight culture (grown at 28°C for 15 hr after diluting a preculture at OD 0.4 1:100 in fresh Luria-Bertani medium) and filter sterilizing the supernatant. Ten milliliters of this culture filtrate, supplemented with 0.2% Tween 20, was sprayed as described for the other treatments. Heat shock treatment was done by incubating the glass jar with the axenically grown plant at 42°C for 4 hr. All other treatments were done for 12 hr.

Tobacco tissues extracted for RNA gel blot analysis (Figure 9) represent roots from 10-day-old axenically grown plants, leafs and stems from 6-week-old axenically grown plants, and flower tissues from greenhouse-grown plants.

Immunocytochemistry

Tobacco seeds were imbibed in Murashige and Skoog medium (Murashige and Skoog, 1962) for 3 days at 28°C. Germinating seeds were fixed by incubation in 3.7% paraformaldehyde in PBS at 8°C overnight. Dehydration was done using a series of watery solutions with increasing ethanol concentrations. The germinating seeds were then incubated in xylene and embedded in Histowax (tissue-embedding medium, pellet melting point 56 to 58°C; Histolab, Gothenburg, Sweden). Sections (5 to 10 μm) were placed on cromogelatin-coated microscope slides, deparaffinized in xylene, and rehydrated in a series of watery solutions with decreasing alcohol concentrations, distilled water, and finally PBS. For immunofluorescence staining, the sections were blocked for 10 min with normal goat serum diluted 1:10 in PBS containing 4% (w/v) BSA. Detection of calreticulin was done by a 1-hr incubation at room temperature with the rabbit antisera (diluted 1:200 in PBS containing 4% BSA), followed by a 30-min incubation with fluorescein isothiocyanate–labeled anti-rabbit IgG antiserum from swine (Dakopatts, Copenhagen, Denmark) diluted 1:200 in the same buffer. After several washes in PBS containing 4% BSA, the sections were mounted in Fluoromount (Southern Biotechnology Associates Inc., Birmingham, AL). For the indirect method (based on peroxidase activity), the sections were treated with 0.3% H_2O_2 in PBS for 20 min to prevent further endogenous peroxidase activity. Blocking of unspecific binding and primary antibody incubations was done as for the immunofluorescence staining. Following rinse in PBS, sections were incubated for 30 min with swine anti-rabbit antibodies (Dakopatts) diluted 1:40 in PBS with 4% BSA. Finally, the sections were incubated for 30 min with a complex of horseradish peroxidase and rabbit peroxidase antibodies diluted 1:200 in PBS with 4% BSA followed by development for 15 min in 0.8 mM 3-amino-9-ethyl carbazole, 11% DMSO, 0.02 M sodium acetate, pH 5.0, to which 30% H_2O_2 was added to a final concentration of 0.005%. For counter staining, Mayers hematoxylin (Histolabs Products AB, Frvlunda, Sweden) was used followed by a 10-min wash in water. Specimens were mounted in glycin–gelatin. Sections were studied in a Nikon optiphot microscope (Nikon Corp., Tokyo, Japan). Photographs were taken with Fuji (Tokyo, Japan) or Kodak 400 ASA film.

Detection of Protein Complexes

Tobacco leaf protoplasts were produced as described by Denecke and Vitale (1995) and incubated at a cell density of 10^6 protoplasts per mL in 15-mL Falcon tubes (Becton Dickinson and Co., Lincoln Park, NJ) (1 mL per tube). Protoplasts were incubated at room temperature in the presence of tunicamycin (20 μg/mL) or DDT (5 mM). Heat shock treatment was done at 42°C. All treatments were done for 3 hr and in the presence of ^{35}S-methionine (100 μCi/mL). All other manipulations were done essentially as described by Denecke and Vitale (1995).

RNA Isolation

Total RNA and poly(A)$^+$ RNA were prepared from different tobacco tissues or cells as described by Dean et al. (1985). Total RNA from maize coleoptiles were kindly provided by K. Palme (Max-Planck-Institut, Cologne, Germany). For the work with the barley aleurone cells, 20 aleurone layers were incubated in 4 mL of incubation buffer for 10 hr as described previously. The aleurone layers were ground in a mortar with a pestle in liquid N_2, and RNA was isolated as described by Hensgens and Van Os-Ruygrok (1989).

RNA and Protein Gel Blotting

Gel blots of glyoxylated RNA (10 μg per slot) were prepared according to the established procedures of Sambrook et al. (1989) on Hybond-N nylon membrane, as suggested by the manufacturer (Amersham). Radioactive-labeled DNA probes for DNA and RNA gel blot analyses were prepared from gel-purified DNA fragments using a multiprime labeling system (Amersham). A high-pI α-amylase cDNA probe was prepared by isolation of the 153-bp BamHI-HinfI fragment from plasmid pM/C (Rogers, 1985). The barley endoplasmin probe was prepared from the complete cDNA sequence as described by Walther-Larsen et al. (1993). Barley BiP and calreticulin and tobacco calreticulin and PDI were prepared from the complete inserts in pGEM5z as described previously. The tobacco BiP probe was identical to the one described by Denecke et al. (1991). The basic glucanase GN1 (Castresana et al., 1991) probe was prepared from pGN1 (kindly provided by Dr. D. Inzé, Laboratory of Genetics, University of Ghent Belgium). Hybridization was performed at 65°C in 50 mM Tris, pH 8.5, 10 mM EDTA, 0.5% SDS, 1 M NaCl, 0.1% sodium pyrophosphate, 0.2% Ficoll (Pharmacia), 0.2% polyvinylpyrrolidone, 0.2% BSA, and 0.5 mg/mL sonicated herring sperm DNA. After hybridization, the blots were washed at 65°C twice in 5 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate) plus 1% SDS, twice in 2 × SSC plus 1% SDS, and finally twice in 0.2 ×

SSC plus 1% SDS. All blots were rehybridized with a pea ribosomal RNA probe to check for differences in loading.

Protein gel blots were done as described by D'Halluin et al. (1992).

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