Transport of Proteins to the Plant Vacuole Is Not by Bulk Flow Through the Secretory System, and Requires Positive Sorting Information

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Abstract. Plant cells, like other eukaryotic cells, use the secretory pathway to target proteins to the vacuolar/lysosomal compartment and to the extracellular space. We wished to determine whether the presence of a hydrophobic signal peptide would result in the transport of a reporter protein to vacuoles by bulk flow; to investigate this question, we expressed a chimeric gene in transgenic tobacco. The chimeric gene, *Phalb*, used for this study consists of the 1,188-bp 5′ upstream sequence and the hydrophobic signal sequence of a vacuolar seed protein phytohemagglutinin, and the coding sequence of a cytosolic seed albumin (PA2). The chimeric protein PHALB cross-reacted with antibodies to PA2 and was found in the seeds of the transgenic plants (~0.7% of total protein), but not in the leaves, roots, or flowers. Immunoblot analyses of seed extracts revealed four glycosylated polypeptides ranging in molecular weight from 29,000 to 32,000. The four polypeptides are glycoforms of a single polypeptide of M_r 27,000, and the heterogeneity is due to the presence of high mannose and endoglycosidase H–resistant glycans. The PHALB products reacted with an antiserum specific for complex plant glycans indicating that the glycans had been modified in the Golgi apparatus. Subcellular fractionation of glycerol extracts of mature seeds showed that only small amounts of PHALB accumulated in the protein storage vacuoles of the tobacco seeds. In homogenates made in an isotonic medium, very little PHALB was associated with the organelle fraction containing the endoplasmic reticulum and Golgi apparatus; most of it was in the soluble fraction. We conclude that PHALB passed through the Golgi apparatus, but did not arrive in the vacuoles. Transport to vacuoles is not by a bulk-flow mechanism, once proteins have entered the secretory system, and requires information beyond that provided by a hydrophobic signal peptide.

THE maintenance of diverse functional compartments in eukaryotic cells depends on proper targeting of proteins to their correct cellular locations. Uptake of proteins into the nucleus, mitochondria, chloroplasts, or peroxisomes involves direct recognition by the organelle of proteins synthesized on free polysomes and released in the cytosol. Proteins which are synthesized on the rough ER may be either secreted from the cell or retained in distinct locations, such as the ER, Golgi complex, plasma membrane, or vacuole/lysosome (see Pfeffer and Rothman, 1987). Entry into this secretory pathway depends on the presence of a hydrophobic, amino-terminal, signal peptide that is usually cotranslationally cleaved from the polypeptide. In plant cells, proteins destined for export as well as vacuolar proteins have such signal peptides and traverse the secretory pathway together, at least as far as the Golgi complex. Both extracellular proteins (Gardiner and Chrispeels, 1975; Melroy and Jones, 1986; Mitsui et al., 1985) and vacuolar proteins (Chrispeels, 1983) of plant cells are known to pass through the Golgi complex, but it is not known what determines their final destination. Plant vacuoles resemble yeast vacuoles and mammalian lysosomes in their enzymic complement and function (Matile, 1975). Transport of acid hydrolases to the lysosomes of mammalian cells (von Figura and Hasilik, 1986; Kornfeld, 1987) and to the vacuoles of yeast (*Saccharomyces cerevisiae*) cells (Johnson et al., 1987; Valls et al., 1987) requires active sorting information in addition to a signal peptide. Absence of this sorting information or disruption of the sorting process results in the transport of these hydrolases by bulk flow (Kelly, 1985) along the default pathway (Rothman, 1987; Wieland et al., 1987). Mistargeting occurs when the pH of the transport pathway is increased by treatment with chloroquine (Gonzalez-Noriega et al., 1980). The same treatment disrupts transport along the regulated secretory pathway (Moore et al., 1983; Wagner et al., 1986). Treatment of cells with the sodium ionophore, monensin, which raises the pH of the *trans*-Golgi complex, also causes mistargeting (see Tartakoff, 1983). In addition,

mistargeting occurs when a vacuolar protein is over expressed, resulting in its secretion (Stevens et al., 1986).

Recent results from our laboratory show that expression of the gene for a plant vacuolar protein in yeast results in the transport of this protein to the yeast vacuole (Tague and Chrispeels, 1987). This result indicates a high degree of similarity between the targeting signals and transport machinery of yeast and plant cells. Vacuoles of plant cells have both a lytic and a storage function; they contain numerous acid hydrolases as well as storage proteins, lectins, and other plant defense proteins (Etzler, 1985; Higgins, 1984; Matile, 1978). The extracellular matrix of the plant cell (cell wall), and the intracellular spaces also contain numerous acid hydrolases as well as structural proteins (Lamport and Catt, 1981). It is not known whether transport to the vacuoles or secretion into the extracellular matrix is by a bulk-flow mechanism or requires sorting information. Many of the extracellular and vacuolar proteins are glycoproteins with Asn-linked glycans and targeting could be mediated by the protein and/or the carbohydrate domain of the protein. However, many vacuolar proteins have no Asn-linked glycans, indicating that such glycans have no essential targeting information (Chrispeels et al., 1987). Mistargeting and transport along the bulk-flow pathway occurs when animal cells are treated with the sodium ionophore monensin, and similar results have been obtained with plant cells. When cotyledons of both peas (Craig and Goodchild, 1984) and jackbeans (Bowles et al., 1986) were treated with monensin, storage proteins and lectins that normally accumulate in protein bodies were transported to the plasma membrane.

Developing embryos of dicotyledonous plants constitute an excellent system to examine protein transport, because large amounts of storage proteins accumulate in specialized protein storage vacuoles, also called protein bodies. We found that the expression of two bean (*Phaseolus vulgaris*) embryo storage protein genes in transgenic tobacco resulted in the correct targeting of their gene products, phaseolin and leucoagglutinating phytohemagglutinin (PHA-L),[1] to the protein bodies in embryos and endosperm of tobacco seeds (Greenwood and Chrispeels, 1985; Sturm et al., 1988). To find out if targeting to vacuoles requires positive sorting information, we made a chimera consisting of a cytosolic protein (presumed to be devoid of targeting information), the signal peptide of a vacuolar protein, and the regulatory sequences (5′ and 3′) of seed proteins. Such a protein should be expressed in the seeds of transgenic tobacco, enter the ER, and be transported along the default pathway. The chimeric gene (*phalb*) used for these experiments consists of the promoter and signal peptide-coding sequence of the bean vacuolar protein PHA-L and the coding and 3′ sequence of a pea (*Pisum sativum*) cotyledon cytosolic albumin (PA2) (Harris and Croy, 1985; Higgins et al., 1987). PHA-L is a major polypeptide of the seed lectin of the common bean (*P. vulgaris*) and accounts for ~3% of the total protein in mature seeds. Its gene, *dlec2* (Hoffman and Donaldson, 1985), has been sequenced and shown to be expressed in the developing seeds of transgenic tobacco (Voelker et al., 1987). In this heterologous system, the polypeptide is posttranslationally processed, assembled into oligomers, and correctly targeted to the protein storage vacuoles (Sturm et al., 1988). The PA2 protein is a relatively sulfur-rich protein present in the cytosol of pea (*P. sativum*) cotyledons (Harris and Croy, 1985). The cDNA of this protein has recently been cloned and sequenced (Higgins et al., 1987).

Here, we present evidence that the chimeric *phalb* gene is expressed specifically in transgenic tobacco seeds. Its product, PHALB, enters the secretory pathway, is glycosylated in the ER, and some of its glycans are modified in the Golgi apparatus. Cell fractionation experiments show that the bulk of the PHALB protein leaves the Golgi apparatus, but does not accumulate in the protein bodies.

Materials and Methods

Construction of phalb Gene

A 1,251-bp Pst I–Eco RV fragment containing 1,188 bp of the 5′ upstream sequence and the signal peptide-coding region of the phytohemagglutinin (PHA) gene *dlec2* was obtained from pTV562 (pUC12–PHA in Fig. 1). This plasmid contains a 3.4-kb Pst I DNA fragment isolated from a genomic library of the common bean, *P. vulgaris*, cultivar Greensleeves (Hoffman and Donaldson, 1985; Voelker et al., 1987). The coding sequence of the pea albumin PA2 was obtained from the cDNA pPS15-21 clone (Higgins et al., 1987) after deletion of the upstream untranslated region by using the exonuclease III/mung bean nuclease system (Stratagene, La Jolla, CA). The precise end points of deletion derivatives were determined by dideoxy sequencing (Sanger et al., 1977) and one clone was selected for further gene construction. The pea albumin coding sequence was ligated in-frame to the Eco RV site of the PHA fragment. The conservation of the reading frame was checked by sequencing. The chimeric gene *phalb* was then inserted into the vector Bin 19 (Bevan, 1984) as an Eco RI–Pvu II fragment. Selection of the transconjugant *Agrobacterium tumefaciens* carrying the recombinant Bin 19 plasmid was performed after a triparental mating.

In Vitro Transcription

DNA from the appropriate plasmids was isolated by CsCl centrifugation and was transcribed in vitro using the T7 RNA polymerase and the cap analogue ^{7}mGpppG following established procedures described in the Promega Biotec (Madison, WI) manual.

Purification of the Pea Albumin PA2 and Preparation of Anti-PA2 Serum

The albumins were extracted from dry seeds of the pea, *P. sativum*, by homogenization with 5% potassium sulfate, 100 mM sodium phosphate buffer, pH 7.0. A PA2-enriched fraction was obtained by ion-exchange chromatography (DE52; Whatman Inc., Clifton, NJ) as described in Higgins et al. (1987). PA2 was then purified by electroelution after preparative SDS-PAGE. Antibodies to the purified PA2 protein were raised in a New Zealand white rabbit, and this antiserum was used for all the experiments described here with the exception of the immunocytochemical localization. For the localization experiments, a second antiserum was prepared. A rabbit was injected with partially purified PA2 that had been allowed to react with the fixative for 1 h and subsequently dialyzed overnight against distilled water. Purification was according to the protocol of Higgins et al. (1987) but included only the ammonium sulfate fractionation and ion-exchange chromatography on Whatman Inc. DE52. The fractions rich in PA2 were identified by SDS-PAGE.

In Vitro Translation

Cell-free translation of in vitro transcribed mRNA (see below) was performed using a rabbit reticulocyte cell-free system containing 500 μCi/ml [^{35}S]methionine (1,250 Ci/mmol, Amersham Corp., Arlington Heights, IL). Aliquots of the translation products were immunoprecipitated with the

1. *Abbreviations used in this paper*: DB, denaturing buffer; endo H, endo-β-*N*-acetylglucosaminidase H; NDB, nondenaturing buffer; PA2, cytosolic pea albumin; PHA, phytohemagglutinin; PHA-L, PHA leucoagglutinating; TFMS, trifluoromethane sulfonic acid.

anti-PA2 serum. Immunoprecipitates were absorbed on protein A-Sepharose, washed, and analyzed by SDS-PAGE and fluorography (Fluorohance; Research Products International Corp., Elk Grove Village, IL).

Transformation of Tobacco Leaf Discs

Leaf discs of *Nicotiana tabacum*, var. Xanthi, were incubated with transconjugant *Agrobacterium tumefaciens* (strain LBA4404) for 2 d (Horsch et al., 1985). Shoot and root induction were successively performed with modifications described in Voelker et al. (1987). The kanamycin-resistant plantlets were transferred to soil for growth to maturity in the greenhouse. Seeds were harvested and analyzed at different stages after self-pollination.

Genomic Analysis of Transgenic Plants

Total DNA of leaves was extracted as described by Dellaporta et al. (1985). DNA digested with Bam HI was subjected to electrophoresis in 0.8% Tris-Acetate-EDTA agarose gels, and blotted onto a nylon membrane according to Chromczynski and Qasba (1984). The filter was then hybridized to the probe labeled with the oligolabeling procedure described by Feinberg and Vogelstein (1983).

Immunoblot Analysis

Total proteins were extracted from tobacco seeds by grinding and subsequent boiling in sample denaturing buffer (DB; 20 mM Tris-HCl, pH 8.6, 1% SDS, 0.3% β-mercaptoethanol, and 10% glycerol). Alternatively, the seeds were extracted at 0°C in a low salt, nondenaturing buffer (NDB; 25 mM NaCl, 50 mM Tris-HCl, pH 7.5, 0.1% Triton X-100). After centrifugation at 1,000 *g* for 10 min, proteins were precipitated with trichloroacetic acid (7.5%) and redissolved in DB just before SDS-PAGE analysis (Laemmli and Favre, 1973). Proteins were separated on 15% acrylamide gels and transferred to nitrocellulose according to the procedure in the Bio-Rad Laboratories (Richmond, CA) manual. Anti-PA2 serum and goat anti-rabbit IgG coupled to horseradish peroxidase (Bio-Rad Laboratories) were both diluted to 1/1,000 for immunoblot analysis. The serum used to detect complex glycans was produced in rabbits by injecting purified cell wall β-fructosidase obtained from suspension-cultured carrot cells. This serum recognizes exclusively the complex glycans of plant glycoproteins (Faye and Chrispeels, 1988).

Protein Assay

Protein concentration was determined by the method of Lowry et al. (1951).

Endoglycosidase H and Trifluoromethane Sulfonic Acid (TFMS) Treatment

Chemical deglycosylation was performed on total tobacco proteins using anisole and TFMS (Aldrich Chemical Co., Milwaukee, WI) as described by Edge et al. (1981). Endo-β-*N*-acetylglucosaminidase H (endo H) (from *Streptomyces griseus*, Miles Laboratories, Inc., Elkhart, IN) digestion was performed by incubation at 37°C for 24 h in 100 mM sodium acetate, pH 5.8, with 0.01 U enzyme.

Germination of Seeds

Seeds derived from self-pollinated plants were sterilized for 30 min in 10% household bleach and germinated on Murashige-Skoog medium in the presence of 100 μg/ml of kanamycin (Lindsmaier and Skoog, 1965).

Preparation of Membrane Fractions

Seeds from transgenic plants were homogenized with a mortar and pestle in 12% sucrose, 50 mM sodium phosphate, pH 7.5, 1 mM EDTA at 4°C. This homogenization procedure disrupts the protein bodies whose stored proteins become part of the soluble fraction of the homogenate. The homogenate was centrifuged to remove cell walls and debris, and the supernatant fractionated on a Sepharose 4B column (Van der Wilden and Chrispeels, 1983). The organelle fraction, containing a mixture of microsomes and subcellular organelles, was excluded from the column and collected separately from the soluble fraction containing proteins that were included in the gel.

Isolation of Protein Bodies

Mature dessicated seeds were homogenized with a mortar and pestle in glycerol and the debris eliminated by centrifugation (10,000 *g* for 10 min) and filtration. The supernatant was layered onto a nonaqueous density gradient of potassium iodide in glycerol as described in Begbie (1979). The gradient consisted of two layers with densities of 1.3 g/cm^{-3} and 1.4 g/cm^{-3}. After centrifugation for 24 h at 50,000 *g*, three fractions were collected: the supernatant (the load portion of the gradient); the intermediate layer (the layer with a density of 1.3 g/cm^{-3}); and the organelles on top of the 1.4 g/cm^{-3} layer (protein bodies). The fractions were diluted tenfold in 10 mM Tris/HCl, pH 7.5, and analyzed for presence of PHALB by immunoblotting.

Enzyme Assays

Aryl α-mannosidase was measured as described by Van der Wilden et al. (1983) using *p*-nitrophenylphosphate as a standard, NADH-cytochrome *c* reductase was assayed as described by Donaldson et al. (1972), and glucan synthase I as described by Ray (1977).

Immunocytochemistry

Late maturation stage embryos were dissected and processed for electron microscopy as previously described for zein-containing transgenic tobacco seeds (Hoffman et al., 1987). Thin sections mounted on nickel grids were blocked in 5% (wt/vol) nonfat dry milk in TBST (50 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 0.5% vol/vol Tween-20) for 10 min at room temperature. The grids were then labeled with anti-PA2 rabbit serum (elicited against fixed antigen) diluted 1:200 in the blocking solution and incubated for 20 min at room temperature. Immunological controls were run as a parallel set of grids with identical conditions using normal rabbit serum. The grids were then briefly washed in TBST, indirectly labeled with 10 nM colloidal gold coupled to goat anti-rabbit IgG (Janssen Life Sciences Products, Piscataway, NJ) for 5 min at room temperature, washed in TBST followed by distilled water, and stained in 5% aqueous uranyl acetate for 20 min at room temperature. The grids were examined and photographed with Hitachi Ltd. (Tokyo) H300 and H500 electron microscopes.

Results

Construction of the Chimeric phalb Gene

Our previous results showed that introduction into tobacco of a 3.4-kb DNA fragment carrying the PHA-L gene *dlec2* results in the seed-specific expression of this gene in the transgenic plants (Voelker et al., 1987). Assuming that the 5′ sequence of *dlec2* provides the signals needed for seed-specific transcription, we decided to use the 1,251-bp Pst I–Eco RV subfragment containing the promoter and the signal peptide of PHA-L for the construction of the chimeric gene *phalb* as diagrammed in Fig. 1. We fused this PHA-L gene fragment to the coding area and the 3′ downstream sequence of the cDNA encoding the cytosolic pea albumin, PA2. We expected that after insertion into the tobacco genome, the signals provided by the PHA gene would initiate transcription correctly, and after proceeding through the reading frame of the chimeric *phalb* gene, transcription would be terminated and the mRNA polyadenylated by the signals provided by the PA2 3′ sequence. To remove the GC tail and the 5′ untranslated region of the 980-bp pea cDNA that encodes the major pea albumin PA2 (Higgins et al., 1987), it was cloned into the plasmid Bluescribe (Bluescribe-ALBT in Fig. 1), and unidirectional deletions carried out with exonuclease III and mung bean nuclease, as explained in Fig. 1. One deletion clone, with sequences removed up to nucleotide +3 relative to the start of translation (bp 38 of

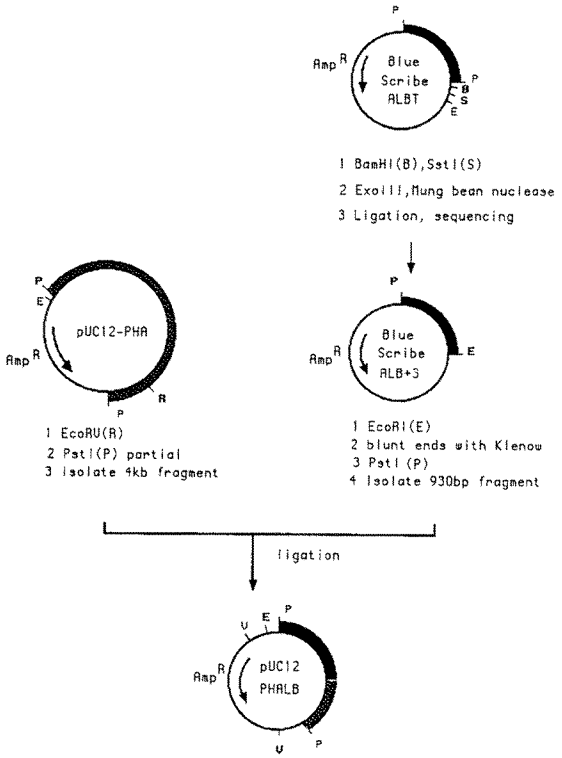

Figure 1. Construction of the chimeric PHA albumin (*phalb*) gene. Plasmids used for the construction of *phalb* are described in Materials and Methods. Blackened segments designate the albumin gene (■). Dotted segments designate the PHA gene fragment (□). The single line represents the vector. Location of ampicillin-resistance gene is indicated (*arrows*). The *phalb* construct was inserted into Bin 19 cut with Sma I–Eco RI as a Pvu II(V)–Eco RI piece cut out pUC12–PHALB.

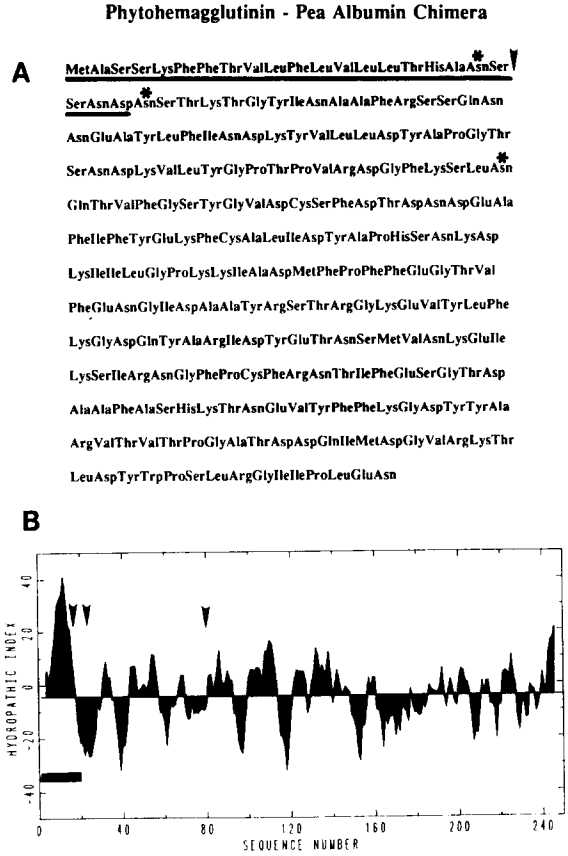

Figure 2. Characteristics of the chimeric protein PHALB. (*A*) Amino acid sequence of the chimeric protein PHALB deduced from *phalb* DNA sequence. *Arrowheads*, signal peptide cleavage site of PHA. *Asterisk*, glycosylation site. Underlined sequence indicates the amino acids derived from PHA. The adjacent two amino acids were created by the cloning procedure. The following threonine residue represents the second amino acid from the PA2 protein. (*B*) Hydropathy profile of the PHALB protein. The SOAP program used here (Kyte and Doolittle, 1982) evaluates the hydrophilic and hydrophobic tendency of a polypeptide chain by determining the average hydropathy of a moving segment as it advances through the sequence from the amino to the carboxy terminus. The horizontal bar indicates the hydrophobic signal peptide of PHA. The arrowheads point to the glycosylation sites indicated in *A*.

pPS15-21; Higgins et al., 1987), is referred to as ALB+3. It was ligated to the promoter and the signal sequence of PHA to create a translational fusion. We sequenced 150 bp from the subcloned Hind III fragment of *phalb* (see Fig. 4 *B* for the location of the Hind III sites) including the fusion area, and confirmed that the PHA and the albumin genes were in frame. Fig. 2 *A* shows the amino acid sequence of the chimeric protein deduced from the DNA sequences published by Hoffman and Donaldson (1985) and Higgins et al. (1987), and from our own sequencing in the translational fusion area. This sequence consists of 255 amino acid residues. The first 23 amino-terminal amino acids are derived from PHA-L (Fig. 2 *A*, *underlined*) and cover the signal peptide cleavage site (Fig. 2 *A*, *arrowhead*). Two amino acid residues (AsnSer) link the PHA domain with the 230 amino acid carboxy-terminal PA2 sequence. The insertion of these two amino acids resulted from the deletion/cloning strategy applied when 6 bp from the vector Bluescribe were ligated in front of the deletion endpoint of ALB+3 before fusing it to the PHA-L gene sequence. The sequence indicates three potential glycosylation sites exist in the PHALB protein. The first site overlaps the signal peptide cleavage site, and will therefore disappear during processing in the ER if the protein is processed correctly (Hoffman and Donaldson, 1985; Miller et al., 1975). We have shown that the signal peptide of PHA in transgenic tobacco is cleaved at the same point as in bean (Sturm et al., 1988). The second site was created during the cloning procedure. Six nucleotides coding for asparagine and serine were added in front of the deletion derivative (see above). Because the first amino acid encoded by ALB+3 is a threonine, this cloning step created a glycosylation site. The third site is located on amino acid 50 of PA2, and is not used when the protein is synthesized in pea cotyledons, because the protein has no signal peptide and remains cytosolic (Higgins et al., 1987). The hydropathy plot obtained from the amino acid sequence of PHALB shows no hydrophobic region, other than the signal peptide of PHA, long enough to be a membrane-spanning domain (Fig. 2 *B*). The absence of a hydrophobic domain, which could prevent the complete translocation of PHALB across the ER membrane, should result in the release of a soluble protein into the ER lumen.

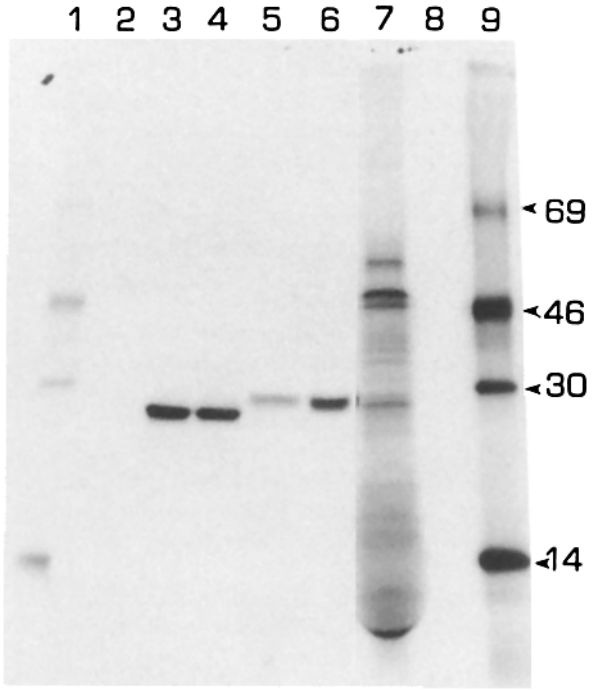

Figure 3. In vitro expression analysis of the *phalb* gene. Fluorograph of SDS-PAGE analysis of cell-free translation products of in vitro-transcribed RNA performed as described in Materials and Methods. After translation, aliquots were either analyzed directly by electrophoresis on 15% acrylamide-SDS gel (lanes *3*, *5*, and *7*) or immunoprecipitated with anti-pea albumin antibodies before loading (lanes *4*, *6*, and *8*). Lanes *1* and *9*, ^{14}C-labeled molecular weight standards. Numbers on the right indicate M_r values $\times 10^{-3}$. Lane *2*, Total translation products in absence of exogenous RNA. Lanes *3* and *4*, pea albumin RNA; lanes *5* and *6*, PHALB RNA; lanes *7* and *8*, total RNA extracted from developing cotyledons of the bean *Phaseolus vulgaris* (control).

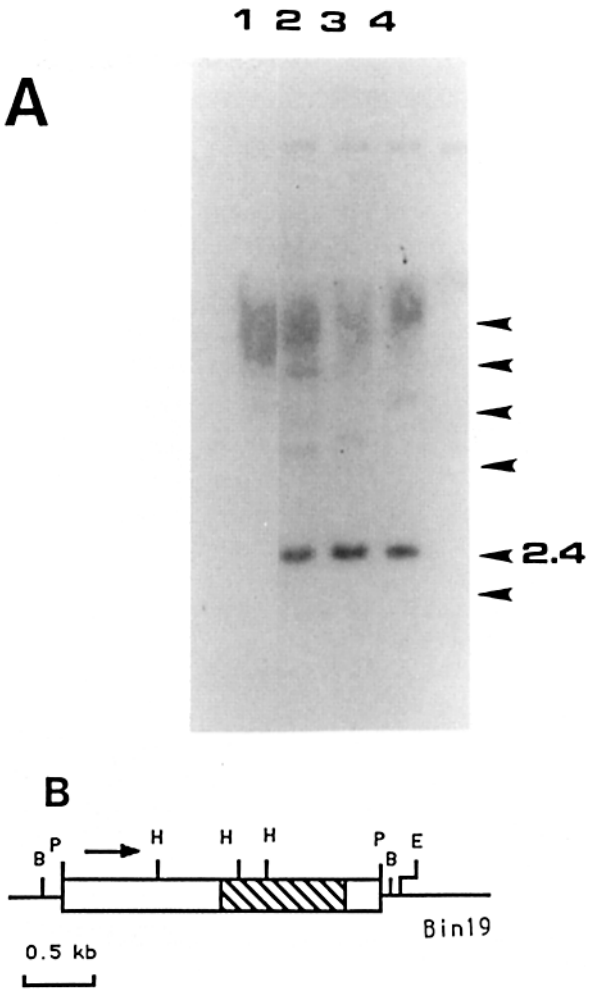

Figure 4. Southern blot analysis of DNA from transformed tobacco (3A) and restriction map of the integrated *phalb* gene (3B). (*A*) 5 μg of DNA isolated from tobacco leaves were digested with Bam HI, fractionated on 0.8% agarose gel, and blotted onto a nylon membrane. The filter was then probed with the Bam HI fragment of *phalb* (see *B*) and labeled by the ^{32}P-oligolabeling procedure. The specific activity of the probe was 4×10^8 cpm/μg. The final washing stringency was performed at 65°C in 0.1× SSC. Lane *1* represents DNA from untransformed tobacco plants. Lanes *2*, *3*, and *4* represent the DNA derived from three independently transformed tobacco plants. Arrowheads on the right indicate the position of the size markers (9.4, 6.5, 4.4, 3.5, and 2.0 kb). The figure shows the expected band of 2.4 kb in the three transformed plants. (*B*) The 24-kd Pst I fragment of *phalb* (*open bar*) cloned in Bin 19 (*single line*). The Eco RI–Pvu 2 fragment of Puc 12-*phalb* (see Fig. 1) was cloned in Bin 19 opened with Eco RI–Sma I. This cloning strategy allows one to cut out the *phalb* fragment with Bam HI because of the presence of multiple cloning sites. The arrow indicates the orientation of the *phalb* transcript. Hatched area indicates the coding region of *phalb*. *B*, Bam HI; *E*, Eco RI; *H*, Hind III; *P*, Pst I.

Expression of the phalb Construct In Vitro

We confirmed that our construct encodes a chimeric protein by immunoprecipitation with the anti-PA2 serum of the protein encoded in vitro by the mRNA obtained by in vitro transcription of the *phalb* construct (Fig. 3). In parallel, a deletion derivative of the PA2 cDNA (control) beginning at −12 bp from the translational start and an Sph I fragment of the *phalb* gene starting at −110 bp from the transcriptional start were subcloned into the expression vector Bluescribe (Stratagene) downstream of the plasmid T7 promoter. The RNAs obtained by in vitro runoff transcription with T7 polymerase were added to a rabbit reticulocyte lysate and the products analyzed by SDS-PAGE and fluorography, either directly or after immunoprecipitation with the anti-PA2 serum. These experiments demonstrate that the *phalb* construct (Fig. 3, lanes *5* and *6*) produces a protein immunologically related to PA2 (Fig. 3, lanes *3* and *4*) with a slightly lower electrophoretic mobility than PA2. The shift in mobility from M_r 26,000 to 29,000 is expected, since the *phalb* gene encodes an additional 25 amino acids at the NH_2 terminus (Fig. 2 *A*).

Transfer of the Chimeric phalb Gene to the Tobacco Genome

The tobacco transformation was performed with the binary Ti vector system of Bevan (1984), using *Agrobacterium tumefaciens* strain LBA4404 with Bin 19 as a cloning vector. After cocultivation of the transconjugates with leaf discs (Horsch et al., 1985), transformed shoots were selected for resistance to kanamycin. To monitor the cotransfer of the

phalb construct in transgenic tobacco plants, total leaf DNA was analyzed by Southern blotting. When using the *phalb* DNA fragment as a probe, a major single band with a mobility of 2.4 kb was detected in the Bam HI–digested DNA of transformed tobacco plants (Fig. 4 *A*, lanes *2*, *3*, and *4*), but was absent in digests from untransformed tobacco (Fig. 4 *A*, lane *1*). As shown in Fig. 4 *B*, a 2.4-kb fragment corresponding to the complete *phalb* construct is expected after Bam HI digestion. We conclude that the *phalb* constr ıct was inserted into the tobacco genome without major rearrangements. Germination of seeds from transformed plants in the presence of kanamycin demonstrated that the kanamycin-resistance marker is inherited in a Mendelian fashion.

Detection of PHALB Protein in Transgenic Tobacco Seeds

Total protein extracts of seeds from transformed and untransformed plants were analyzed by SDS-PAGE, either directly or after immunoblotting with the anti-pea albumin serum (Fig. 5 *A*). The extracts of seeds from transformed plants contain PA2-cross-reactive material that is absent from seeds of untransformed tobacco (Fig. 5 *A*, compare lanes *3* and *4*). The PHALB signal (Fig. 5, *diamonds*) is distorted and weakened by the presence of the storage protein (30,000–35,000 mol wt), as illustrated in Fig. 5 *A*, lanes *1* and *2*. Under these conditions of extraction, there is also a weakly cross-reacting polypeptide that is present in both the transformed and untransformed seeds (Fig. 5 *A*, open circle next to lane *3*). The tobacco storage protein is a salt-soluble globulin (Sano and Kawashima, 1983), and to avoid the extraction of this protein we used a low salt NDB for the homogenization of the seeds. Fig. 5 *B* (lane *6*) shows the effect of this mode of extraction. Fig. 5 *B*, lane *7* indicates the presence of several new polypeptides ranging from 29,000 to 32,000 mol wt in seeds from transformed tobacco on a gel stained with Coomassie brilliant blue. The immunoblot analysis of this gel indicates the presence of four PA2-related polypeptides in this molecular weight range (Fig. 5 *B*, lane 10).

Seeds from five of the six *phalb*-transformed plants investigated contained equivalent amounts of PHALB products as shown in Fig. 5. The amount of PHALB contained in 100 μg of protein (NDB extract) from seeds of one transformed tobacco plant was estimated on an immunoblot by comparison of its signal intensity with the signals obtained from increasing amounts of purified PA2 protein (0.1, 0.5, and 2.5 μg) mixed with 100 μg of untransformed tobacco seed protein (data not shown). This semiquantitative estimation showed that PHALB products represent ~2% of the protein in a low salt extract. Given that the NDB extracts ~1/3 of the total protein (data not shown), PHALB represents ~0.7% of the total protein in mature seeds.

PHALB Accumulates Specifically in the Developing Seeds of the Transformants and Is Rapidly Degraded After Germination

To determine whether PHALB protein accumulation is

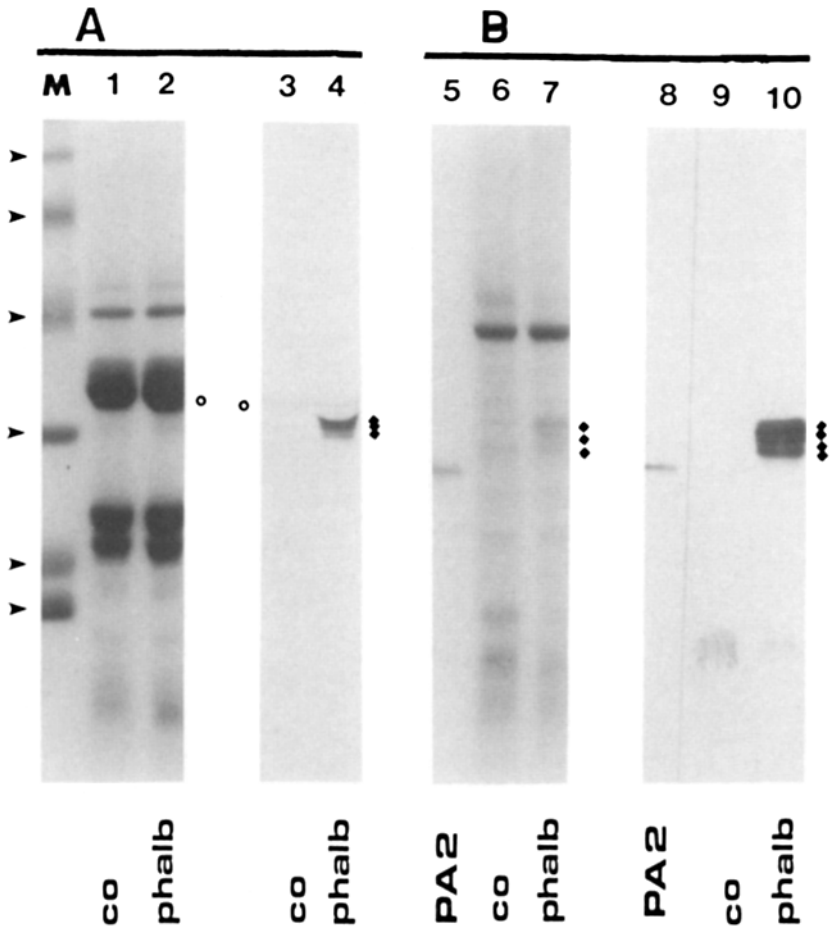

Figure 5. Detection of PHALB in transgenic tobacco seeds after separation by SDS-PAGE; comparison of two modes of protein extraction. (*A*) Tobacco seed protein extracted with SDS-containing buffer (DB). Lanes *1* and *2*, Coomassie blue staining. Lanes *3* and *4*, immunoblot where PHALB was visualized with antibodies against pea albumin (PA2) and peroxidase coupled to goat anti-rabbit IgG. Each lane contains 100 μg of total protein extract from transformed (lanes *2* and *4*) and untransformed (lanes *1* and *3*) tobacco. Circles indicate the location of the major bands of tobacco seed storage protein. Diamonds indicate PHALB products. Arrowheads indicate the position of the prestained protein standards (200, 98, 68, 43, 26, and 18 × 10^{-3} mol wt). (*B*) Tobacco seed protein extracted with a low salt buffer (NDB). (lanes *5*, *6*, and *7*) Coomassie blue staining; (lanes *8*, *9*, and *10*) immunoblot analysis as described above. Lanes *5* and *8*, 1 μg and 100 ng of purified PA2, respectively. In all other lanes, we loaded 100 μg of protein extracted in NDB. Lanes *6* and *9*, seeds from untransformed tobacco. Lanes *7* and *10*, seeds from transformed tobacco.

specific for the seeds, proteins were extracted both under denaturing and nondenaturing conditions from leaves, stems, roots, flowers, and seeds of a plant transformed with *phalb*. Extracts were analyzed for their PHALB content by the immunoblotting procedure. No signals were obtained for any organs other than seeds (data not shown). To determine the time of onset of PHALB accumulation in maturing seeds, we made protein extracts of seeds at different times after pollination. The analysis of these extracts by immunoblotting is shown in Fig. 6. PHALB polypeptides accumulate from day 15 after anthesis. At this same time, under our conditions of growth, the major tobacco storage protein starts to accumulate (Voelker et al., 1987).

PA2 belongs to the major albumin fraction located in the cytoplasm of parenchyma cells, which is not appreciably degraded during the time period that the major storage proteins are degraded (Harris and Croy, 1985; Murray, 1979). When tobacco seeds from a self-transformed plant were allowed to germinate, there was a rapid disappearance of PHALB leading to the total breakdown of the protein between 5 and 10 d after germination (Fig. 6, lanes *8* and *9*).

PHALB Products Enter the Secretory Pathway

Because entry into the secretory pathway is normally accompanied by the cotranslational removal of the signal peptide and the addition of N-linked glycans to available glycosylation sites, we were able to determine the intracellular fate of PHALB by biochemical analysis. In a first approach, we assayed for the presence of oligosaccharide sidechains in PHALB. Seed protein extracts were chemically deglycosylated with TFMS, which removes the oligosaccharides, and the effect on the PHALB polypeptides was analyzed by immunoblotting. Deglycosylation with TFMS gave rise to a single polypeptide of M_r 27,000 as shown in Fig. 7 *A*, lane *3*. Thus, the four polypeptides which cross-react with the anti-PA2 serum are glycoforms of the PHALB polypeptide with a slightly higher molecular weight than PA2 (Fig. 7 *A*, lane *4*). To monitor the removal of the PHA signal peptide from the chimeric protein PHALB, we compared the electrophoretic mobilities of the PHALB synthesized in vivo in tobacco, after chemical deglycosylation, with the unprocessed PHALB synthesized in vitro in a cell-free system. The samples were first analyzed by immunoblotting, then the filter was autoradiographed to reveal the position of the [^{35}S]methionine-labeled PHALB synthesized in vitro. Fig. 7 *B* shows the superimposition of the immunoblot and the autoradiograph. The comparison of the mobilities of deglycosylated, in vivo synthesized PHALB (Fig. 7 *B*, lane *6*) with in vitro synthesized PHALB (lane *7*) shows a difference in relative molecular weight of ~2,000. This indicates that in tobacco seed cells, the signal peptide was removed from the chimeric protein. Affinity chromatograph on Con A–Sepharose 4B was used as described in Faye et al. (1986) to demonstrate that most of the PHALB gene products bound to the lectin while a small proportion remained unbound (data not shown). These results indicate that most polypeptides have high mannose glycans. We conclude that in *phalb*-transformed tobacco, the PHA signal peptide of PHALB is removed efficiently, and the subsequent glycosylations produce a heterogenic population of PHALB glycoforms.

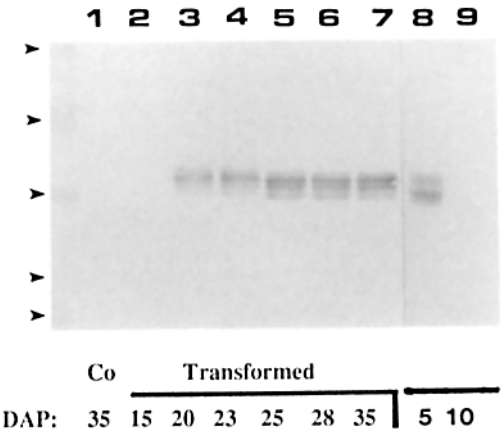

Figure 6. Accumulation and degradation of PHALB protein in seeds of transgenic tobacco plants. 100 μg of NDB-extracted protein of developing seeds (lanes *1–7*) and seedlings (lanes *8* and *9*) of transformed tobacco were subjected to the immunoblotting procedure after separation by SDS-PAGE. In lanes *1–7*, numbers at the bottom refer to days after pollination. Mature, dry seeds were allowed to germinate on Murashige-Skoog medium. Proteins were extracted in NDB after 5 (lane *8*) and 10 d (lane *9*). The amount of protein loaded corresponds to ~20 seedlings in lane *8* and ~50 seedlings in lane *9*. Arrowheads indicate the position of the molecular weight markers as described in Fig. 5.

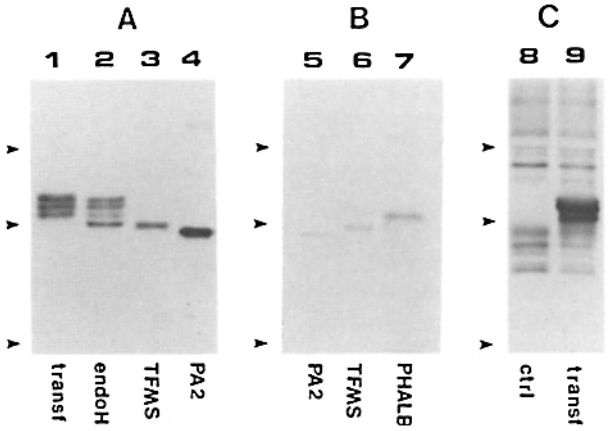

Figure 7. Glycosylation status of in vivo–synthesized PHALB protein. (*A*) Endo H and TFMS treatment. NDB extracts from mature PHALB transformed seeds treated with endo H (lane *2*) or TFMS (lane *3*) were analyzed by immunoblotting as described in Materials and Methods. Lane *1*, untreated extract. Lane *4*, 200 ng of purified PA2. (*B*) Comparison of deglycosylated PHALB from tobacco with unprocessed PHALB synthesized in vitro. TFMS-treated PHALB from mature seeds (lane *6*) was loaded on the same gel as ^{35}S-labeled PHALB synthesized in vitro as described in Materials and Methods (lane *7*). After immunoblot analysis, the filter was autoradiographed. We marked the filter with ^{14}C-ink to align the film and the immunoblot without ambiguity. The figure shows the superimposition of the blot and the autoradiograph. Lane *5* represents 100 μg of purified PA2. (*C*) Immunoblot of seed proteins of transformed (lane *9*) and untransformed (lane *8*) tobacco, extracted with NDB, and probed with an antiserum specific for the xylose-containing complex glycans of plant glycoproteins.

PHALB Protein Passed Through the Golgi Apparatus

The spatial separation of the attachment of N-linked oligosaccharides in the ER and their subsequent processing by the enzymes localized in the Golgi compartment (Sturm et al., 1987*a*) allowed us to determine if PHALB enters the Golgi apparatus. Seed protein extracts were treated with endo H, which cleaves high mannose N-linked oligosaccharide sidechains, and analyzed by immunoblotting (Fig. 7, lane *2*). The presence of an endo H–sensitive band (*upper band*, lane *2*) shows that some tobacco PHALB products contain high mannose glycans. The fact that three bands are endo H resistant but TFMS sensitive (Fig. 7, compare lanes *2* and *3*) indicates that these three forms contain complex glycans.

The complex glycans of plant glycoproteins have the typical structure $Man_3Xyl(GlcNAc)_2$ with the xylose residue bonding the core mannose in a β,1→2 linkage (for examples see Ashford et al., 1987; Fournet et al., 1987; Ishihara et al., 1979; Sturm et al., 1987*b*; and others). This glycan is very immunogenic and antisera against plant glycoproteins often show wide cross-reactivity with other glycoproteins. Using an antiserum directed exclusively against the complex glycans of the extracellular protein β-fructosidase (Faye and Chrispeels, 1988), we confirmed that complex glycans do reside on the PHALB polypeptides. Fig. 7 *C* shows an immunoblot of seed protein extracts of control and transformed plants probed with the antiglycan serum. The extract of the control tobacco seeds contains numerous glycoproteins that cross-react with this antiserum, but the extract from transgenic seeds contains an additional series of cross-reacting polypeptides with the mobility of PHALB. These results confirm the endo H–digestion result and show that the bulk of the PHALB products reached the Golgi apparatus.

We investigated the possibility of the retention of the PHALB products in the Golgi apparatus by subcellular fractionation. Homogenization of developing seeds in Tris-HCl buffer (pH 7.8) containing 12% sucrose keeps organelles such as mitochondria intact, while the ER and Golgi apparatus vesiculate, resulting in the formation of microsomes. Proteins which are contained in the cisternae of the ER and Golgi complex *in situ* generally remain within the vesicles. However, protein bodies (vacuoles) break and their contents mix with the cytosol. The homogenate will also contain the proteins that are in the periplasmic space or that are readily extractable from the cell wall. These proteins will be in the soluble fraction, after the organelles have been removed. Cell wall fragments were removed by centrifugation and the homogenate fractionated by gel-permeation chromatography on Sepharose 4B to separate the organelle fraction (microsomes, mitochondria, etc.) from the soluble portion of the homogenate (Van der Wilden and Chrispeels, 1983). To confirm that the ER and Golgi apparatus are in the excluded organelle fractions and that the vacuoles break and release their content, we measured the activities of the marker enzymes: NADH-dependent cytochrome *c* reductase (ER-marker enzyme), glucan synthase I (Golgi-marker enzyme in plant cells), and aryl α-mannosidase (vacuole or protein body marker enzyme). We observed >98% of the activities of NADH–cytochrome *c* reductase and glucan synthase I activities in the excluded organelle fraction and >98% of the aryl α-mannosidase activity in the soluble fraction (data not shown). Immunoblot analysis of three representative Sepharose column fractions is shown in Fig. 8 *A*. Only the slowest migrating form of PHALB is detected in the organelle fraction (Fig. 8 *A*, lane *1*). The cross-reacting polypeptide that is present in both transformed and untransformed seeds (see Fig. 5) is shown by the open circle. All the other PHALB products are present in the soluble fraction (Fig. 8 *A*, lane *3*). A semiquantitative estimation shows that >90% of the PHALB polypeptides are in the soluble fraction. This demonstrates that the majority of the protein passed through the Golgi apparatus, and is now in a compartment where the proteins are readily solubilized when the seeds are homogenized in an isotonic buffer. Such a compartment could be the vacuoles, the cytosol, or the periplasmic space.

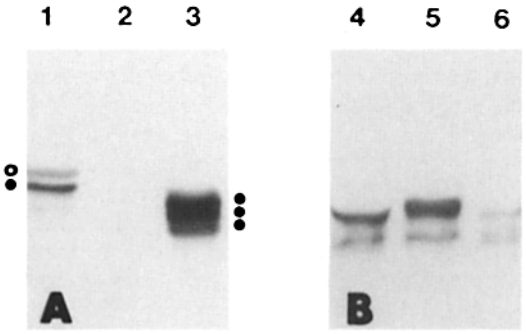

Figure 8. Localization of PHALB in tobacco seeds by subcellular fractionation. (*A*) Isolation of membranous organelles on Sepharose 4B column. Seeds were homogenized in 12% sucrose as described, and the homogenate separated into an organelle fraction and soluble proteins. Proteins from the different fractions were precipitated, redissolved, and analyzed by immunoblot with the antiserum against pea albumin. Lane *1*, organelles; lane *2*, intermediate fraction; lane *3*, soluble homogenate. The small, open circle marks a polypeptide which cross-reacts with the PA2 antiserum (see also Fig. 5, lane *3*). The solid circles mark the PHALB polypeptides. (*B*) Isolation of protein bodies on a glycerol/KI gradient. Analysis by SDS-PAGE and immunoblotting (lanes *4*, *5*, and *6*). Total glycerol extract (lane *4*), load fraction of the gradient (lane *5*), and protein storage vacuole fraction (lane *6*). The lanes were loaded with an equal proportion of the gradient fractions.

Only a Small Fraction of PHALB Accumulates in the Storage Vacuoles

To determine whether PHALB is transported to the protein storage vacuoles, we isolated the vacuoles from a glycerol extract of dry seeds. Protein storage vacuoles are large, fragile organelles which are disrupted during homogenization in aqueous buffers, even under isotonic conditions. In hydrophilic organic solvents, such as glycerol, the protein bodies remain intact and can be separated from other cellular organelles on potassium iodide–glycerol density gradients. After centrifugation and removal of the fat layer, we collected three gradient fractions: the load fraction of the gradient containing the original homogenate (fraction T1, Sturm et al., 1988), the intermediate layer with a density of 1.3 g/cm^{-3}, and the protein storage vacuole fraction (fraction T4) on top of the 1.4 g/cm^{-3} layer. SDS-PAGE analysis of these gradient fractions shows that T4 is greatly enriched in storage protein, as can be expected for a fraction that has the protein

storage vacuoles. This fraction also contains the vacuolar marker enzyme aryl α-mannosidase (Sturm et al., 1988). The entire glycerol extract and the fractions T1 and T4 were analyzed by SDS-PAGE and immunoblotting for PHALB polypeptides (Fig. 8 *B*). The immunoblot of the total glycerol extract (before centrifugation) shows the same distortion of the PHALB polypeptides observed earlier (Fig. 5), caused by the presence of the storage protein. After centrifugation, most of the PHALB remained in the T1 fraction (soluble proteins plus small organelles; Fig. 8 *B*, lane *5*) with a small amount in the T4 fraction (protein storage vacuoles, Fig. 8, lane *6*). Fig. 8 *B*, lanes *5* and *6*, were loaded with equal proportions of the total fraction so that the relative staining intensity of PHALB is a measure of the total amount in each compartment. No significant amounts of PHALB were present in the intermediate glycerol layer between the T1 and T4 fractions (see Sturm et al., 1988).

We conclude from these experiments that very little PHALB arrived in the protein storage vacuoles. Whether the small amount of PHALB which is associated with the protein storage vacuoles is really in the vacuoles or sticks to the outside could not be determined.

Immunocytochemistry

To determine the subcellular location of the PHALB protein, we labeled thin sections of transgenic tobacco seeds with the anti-PA2 rabbit serum followed by colloidal gold coupled to goat anti-rabbit IgG. Unlike in our other studies with transgenic tobacco (Greenwood and Chrispeels, 1985; Sturm et al., 1988), we were unable to obtain a pattern of gold particles which was specific for these transformed seeds. We used two different antisera for these experiments: a rabbit serum made against partially purified native PA2 protein, and a rabbit serum made against purified glutaraldehyde-fixed PA2 protein. These sera gave specific recognition of PHALB and pea albumin on immunoblots, but failed to give a specific reaction with tissue sections. We obtained labeling of the cell wall/periplasmic space area of the cells, but such a labeling pattern was also obtained with untransformed seeds. The preimmune serum did not label the sections at all. These anomalous results cannot be explained at the present time.

Discussion

The objective of this work was to test whether transport of proteins to vacuoles (including protein storage vacuoles or protein bodies) requires more targeting information than is contained in a hydrophobic signal peptide for entry into the lumen of the ER. Such second signals are present on lysosomal hydrolases (see von Figura and Hasilik, 1986; Kornfeld, 1987) and on enzymes targeted to yeast vacuoles (Johnson et al., 1987; Valls et al., 1987). A chimeric protein (PHALB), consisting of the signal peptide of a protein body protein (PHA-L) and a cytosolic polypeptide (PA2), was shown to enter the ER and to pass through the Golgi apparatus, without substantial accumulation in the protein storage vacuoles of transgenic tobacco seeds. We conclude that a signal peptide alone is not sufficient for targeting a protein to the protein storage vacuoles, and that the transport to this compartment is not by bulk flow.

The Expression Pattern of the PHA-regulatory Sequences Is Conserved in the Hybrid Gene

Introduction of the PHA-L gene into tobacco with its own regulatory sequences (1,188 bp upstream of the coding sequence) resulted in the seed-specific expression (Voelker et al., 1987) and accumulation of PHA-L polypeptides in protein bodies (Sturm et al., 1988). The expression pattern of the chimeric gene *phalb* driven by PHA-regulatory sequences was conserved; i.e., PHALB accumulation was found to be seed specific and restricted to the second half of seed development (15–30 d after anthesis). This indicates that the PA2 DNA fragment does not alter the specificity of the PHA promoter in a major way. The level of accumulation of PHALB (~0.7% of the total protein) was somewhat higher than the accumulation of PHA-L driven by the same promoter in tobacco (0.2%; Voelker et al., 1989). Since the proteins arrive in different subcellular compartments (see below), differential break-down may explain the difference in accumulation of the two proteins. The accumulation level of PHALB in transgenic seeds (~0.7% of the total protein) is similar to that found after transfer and integration in tobacco of exogenous seed storage protein genes such as β-phaseolin (1.2–1.5%; see Sengupta-Gopalan et al., 1985), β-conglycinin (1%; see Beachy et al., 1985), or soybean agglutinin (0.2%; see Okamuro et al., 1986).

PHALB Enters the ER and Is Modified in the Golgi Complex

Efficient removal of the signal peptide from a chimeric protein consisting of a secretory protein-signal peptide and a cytoplasmic protein can be achieved in vitro by microsomal membranes (hybrid β-lactamase-globin; see Lingappa et al., 1984; hybrid preproinsulin-chloramphenicol acetyltransferase, see Eskridge and Shields, 1986), or *in vivo* by heterologous systems (hybrids β-lactamase-globin and preprolactin-globin in *Xenopus* oocytes, see Simon et al., 1987). Our results show that the expression of *phalb* in tobacco resulted in the accumulation of four PHALB polypeptide classes (ranging in mol wt from 27,000 to 31,000) in the seeds. These polypeptides differ in molecular weight by ~1,000–1,200. When they are deglycosylated, there is only a single polypeptide slightly larger than PA2 (this increase in molecular weight is expected due to the five additional amino acids at the amino terminus of mature PHALB) and ~2,000 mol wt smaller than *in vitro*-synthesized PHALB (with signal peptide). We conclude from these results that synthesis of PHALB is accompanied by the efficient removal of the signal peptide, and that the four PHALB polypeptides found *in vivo* are different glycoforms of the protein.

The molecular weight range of the glycosylated polypeptides indicates that both glycosylation sites of the mature polypeptide are used, and experiments with endo H, Con A–Sepharose, and the serum directed at the complex glycans of plant glycoproteins (Faye and Chrispeels, 1988) show that in addition to high mannose glycans, complex glycans are also present on the PHALB polypeptides. Obviously, the glycosylated protein passed from the ER to the Golgi apparatus, indicating that it was properly folded for such transport. Improperly folded proteins are not efficiently transported out of the ER (Copeland et al., 1986; Gething et al., 1986). The present data do not allow us to construct a model or suggest

which type of glycan is present on each of the four polypeptide classes.

PHALB Does Not Accumulate in the Protein Storage Vacuoles

After homogenization of the tobacco seeds in an isotonic medium (12% sucrose), the microsomal fraction contained only one class of the PHALB glycoforms, while most of the polypeptides were in the soluble fraction. The microsomal fraction contains both the ER, as shown by the presence of NADH-dependent cytochrome *c* reductase activity, and the Golgi apparatus, as shown by the presence of glucan synthase I activity. Both enzyme activities were absent from the soluble fraction. These results parallel similar observations made with the microsomal fractions of bean seeds whose enzymatic activities have been extensively characterized. The results indicate that the bulk of the PHALB polypeptides were not retained in the ER/Golgi apparatus system and had been transported to another compartment. The presence of the largest and endo H-sensitive form of PHALB in the microsomal fraction (Fig. 8 *A*) may indicate that this is the biosynthetic precursor of the other forms; the precursor would be expected to have high mannose glycans which are larger than the small Golgi-modified glycans of plant glycoproteins. Sturm et al. (1988) observed the same phenomenon with PHA-L in transgenic tobacco. They found that PHA-L with two high mannose chains was associated with the microsomal fraction, while PHA-L with complex glycans had been transported to the protein bodies of the tobacco seeds.

Using fractionation of glycerol extracts of seeds on KI/glycerol gradients, Sturm et al. (1988) showed that PHA accumulates in protein bodies of tobacco when the gene is expressed in tobacco under its own promoter. In contrast, when we applied the same approach to the seeds of *phalb* transformants, we found very little PHALB in the protein body fraction. Thus, the information which is present in the PHA polypeptide to direct it to the protein bodies of tobacco seeds is apparently absent from the PHALB protein. Assuming that the cytoplasmic PA2 protein is free of targeting signals, we conclude that the transport from the Golgi complex to the protein bodies needs a second signal. Thus, even though in the developing seeds >50% of the protein produced is deposited in the protein bodies, the routing to this "protein sink" does not involve bulk flow. This finding parallels similar observations made with mammalian cells and with yeast cells where it has also been found that transport to vacuoles or lysosomes requires a second targeting signal while secretion follows a bulk-flow route (Johnson et al., 1987; Valls et al., 1987; Rothman, 1987; von Figura and Hasilik, 1986).

Where in the Cell Is PHALB?

The cell fractionation and glycan analysis studies clearly show that PHALB became glycosylated and passed through the Golgi apparatus where some of its glycans were modified. The protein did not remain stuck in the secretory system, since it was not associated with the organelle fraction rich in ER and Golgi vesicles. When developing seeds were homogenized in isotonic sucrose, PHALB was in the soluble fraction. This fraction is composed of cytosolic proteins, the contents of the vacuoles, and the proteins present in the periplasmic space and therefore readily extractable from the cell wall. The simplest interpretation of these results is that the protein had been secreted and was solubilized during homogenization. The alternate explanation, that PHALB was released in the cytosol after passing through the Golgi, seems less satisfactory. We cannot rule out the possibility that PHALB remained in large secretory vesicles that were fragile and broke during homogenization. However, Golgi-derived vesicles normally survive homogenization in isotonic sucrose (Chrispeels, 1983). It is unfortunate that our attempts to locate PHALB by immunocytochemical means were unsuccessful.

The Sorting Information of Vacuolar Protein Is in the Polypeptide Domain

Vacuoles contain many proteins that do not have covalently attached glycans, and tunicamycin does not inhibit the transport to the vacuole of proteins that are normally glycosylated; it is, therefore, unlikely that glycans contain vacuolar targeting information (Bollini et al., 1985; Chrispeels et al., 1987). We recently carried out site-directed mutagenesis on the gene for PHA-L to remove the glycosylation sites and showed that PHA-L without glycans is targeted to the protein storage vacuoles of transgenic tobacco seeds (Voelker et al., 1989). This result confirms that the targeting information of PHA-L must be in the polypeptide domain. Based on the analysis of the transport of invertase to the yeast vacuole, we have identified a region of the PHA-L polypeptide, between amino acids 15 and 40, that contains the vacuolar targeting domain of this protein (Tague and Chrispeels, 1988).

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