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. 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.
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 chimerism of yeast and plant cells. Vacuoles of plant cells have 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, 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 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, dec2 (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 was cloned into the plasmid pUC12 in frame with PHA-L. 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 a cosmid 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 byideoxy 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 R–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 7mGppG 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 on DEAE-cellulose (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 [35S]methionine (1,250 Ci/mmol, Amer sham Corp., Arlington Heights, IL). Aliquots of the translation products were immunoprecipitated with the

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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.

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anti-PA2 serum. Immunoprecipitates were absorbed on protein A-Sepha-
rose, 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 trans-
conjugant 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 subse-
quent 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 centrifuga-
tion 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)
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 glycanas was produced in rabbits by injecting purified cell
cell β-fructosidase obtained from suspension-cultured carrot cells. This se-
rum 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. (1980). Endo-β-N-acetylglucosaminidase H (endo H) (from
Straining greenrrae, 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 pres-
ence 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 homog-
enate 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 sub-
cellular 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 desiccated 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 gra-
dient 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 col-
lected: 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 immuno-
blotting.

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 gli
can 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
bloked in 5% (wt/vol) nontat 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
gcoupled to goat anti-rabbit IgG (Jansen Life Sciences Products, Piscata-
way, NT) for 5 min at room temperature, washed in TBST followed by dis-
tilled 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 dele2 results in the seed-specific expression of this gene in the
transgenic plants (Voelker et al., 1987). Assuming that the
5' sequence of dele2 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 sig-
nal 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 se-
quence of the cDNA encoding the cytosolic pea albumin,
PA2. We expected that after insertion into the tobacco ge-
nome, 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

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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 (o). 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.

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 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. 2A 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. 2A, underlined) and cover the signal peptide cleavage site (Fig. 2A, 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 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.

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. 2B). 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.
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 3 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, 26,000 to 29,000 is expected, since the phalb gene encodes an additional 25 amino acids at the NH2 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

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, 14C-labeled molecular weight standards. Numbers on the right indicate Mr values x 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 32P-oligolabeling procedure. The specific activity of the probe was 4 x 10^6 cpm/μg. The final washing stringency was performed at 65°C in 0.1x 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 II fragment of Cab 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.
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 construct 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
specific for the seeds, proteins were extracted both under de-
naturing and non denaturing conditions from leaves, stems, 
roots, flowers, and seeds of a plant transformed with phalb. 
Extracts were analyzed for their PHALB content by the im-
munoblotting procedure. No signals were obtained for any 
organs other than seeds (data not shown). To determine the
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Extracts were analyzed for their PHALB content by the im-
munoblotting 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 pollina-
tion. 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 pro-
teins are degraded (Harris and Croy, 1985; Murray, 1979).
When tobacco seeds from a self-transformed plant were al-
lowed to germinate, there was a rapid disappearance of
PHALB leading to the total breakdown of the protein be-
tween 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 accom-
panied by the cotranslational removal of the signal peptide
and the addition of N-linked glycans to available glycosyla-
tion sites, we were able to determine the intracellular fate of
PHALB by biochemical analysis. In a first approach, we as-
sayed for the presence of oligosaccharide sidechains in
PHALB. Seed protein extracts were chemically deglyco-
sylated 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 elec-
 trophoretic mobilities of the PHALB synthesized in vivo in
tobacco, after chemical deglycosylation, with the unpro-
cessed 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 au-
toradiograph. The comparison of the mobilities of deglyco-
sylated, in vivo synthesized PHALB (Fig. 7 B, lane 6) with
in vitro synthesized PHALB (lane 7) shows a difference in
relative molecular weight of \( \sim 2,000 \). This indicates that in
tobacco seed cells, the signal peptide was removed from the
chimeric protein. Affinity chromatograph on Con A–Sepha-
rose 4B was used as described in Faye et al. (1986) to demon-
strate 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 effi-
ciently, and the subsequent glycosylations produce a het-
ergenic population of PHALB glycoforms.

![Figure 6](image_url)

**Figure 6.** Accumulation and degradation of PHALB protein in seeds of transgenic tobacco plants. 100 \( \mu g \) of NDB-extracted pro-
tein of developing seeds (lanes 1–7) and seedlings (lanes 8 and 9) of transformed tobacco were subjected to the immunoblotting pro-
cedure after separation by SDS-PAGE. In lanes 1–7, numbers at the
bottom refer to days after pollination. Mature, dry seeds were al-
lowed 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 \( \sim 20 \) seedlings in lane 8 and \( \sim 50 \)
seedlings in lane 9. Arrowheads indicate the position of the molecu-
lar weight markers as described in Fig. 5.

![Figure 7](image_url)

**Figure 7.** Glycosylation status of in vivo-synthesized PHALB pro-
tein. (A) Endo H and TFMS treatment. NDB extracts from mature
PHALB transformed seeds treated with endo H (lane 2) or TFMS (lane 7) were analyzed by immunoblotting as described in Mate-
rials and Methods. Lane 1, untreated extract. Lane 4, 200 \( \mu g \) of puri-
fied 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 Mate-
rials 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 \( \mu g \) of purified PA2. (C) Immunoblot of seed pro-
teins 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., 1987a) 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 side-chains, 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$_x$Xyl(GlcNAc)$_2$, with the xylose residue bonding the core mannose in a $\beta$1→2 linkage (for examples see Ashford et al., 1987; Fournet et al., 1987; Ishihara et al., 1979; Sturm et al., 1987b; 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 $\beta$-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 $\alpha$-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 $\alpha$-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.

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; Vals 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 breakdown 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 Okamura 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 preprolac-tatin–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 accomplished 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. 8A) 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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