In Vitro Mutated Phytohemagglutinin Genes Expressed in Tobacco Seeds: Role of Glycans in Protein Targeting and Stability

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Phytohemagglutinin is a glycoprotein that accumulates in the protein storage vacuoles of bean seeds. The mature glycoprotein has a high-mannose and a complex glycan. We describe here the use of site-directed mutagenesis and expression of the mutated genes in transgenic tobacco to study the role of glycans in intracellular targeting. The reading frame for phytohemagglutinin-L was mutated so that either one or both of the glycosylation signals were disrupted to specifically prevent the attachment of asparagine-linked glycans. Expression of these genes with the β-phaseolin promoter in the seeds of transgenic tobacco plants showed that phytohemagglutinin-L with only one glycan or without glycans was correctly targeted to the protein storage vacuoles of the seeds. Furthermore, the absence of either the complex glycan or the high-mannose glycan did not alter the processing of the other glycan. On the basis of these results, we propose that the targeting signal of this vacuolar protein is contained in its polypeptide domain and not in its glycans.

INTRODUCTION

Eukaryotic cells contain numerous proteins that have short oligosaccharide sidechains (glycans) attached to specific asparagine residues. These glycoproteins may occur in membrane systems (endoplasmic reticulum, Golgi complex, plasma membrane, tonoplast), accumulate in vacuoles (or lysosomes), or be secreted from the cells. The glycans, which can represent up to 50% or more of the molecular mass of proteins, profoundly influence their physicochemical and biological properties. For example, nonglycosylated vesicular stomatitis virus (VSV) G protein has altered solubility properties (Leavitt et al., 1977). When VSV G protein is synthesized in vivo without glycans, it is not transported to its final destination, the plasma membrane (Machamer et al., 1985). Similarly, disruption of N-linked glycosylation by site-directed mutagenesis of certain mammalian serum proteins results in reduced levels of secretion (Dorner et al., 1987). Glycosylation can be important for oligomerization (Merlie et al., 1982), stabilization against proteolysis (for review, see Olden et al., 1985), or biological function of a glycoprotein (Prives and Bar-Sagi, 1983). For many other glycoproteins, however, gly-

cosylation seems not to be essential for biological function (see Olden et al., 1985, for a list of examples.)

We have studied the biosynthesis and intracellular transport of phytohemagglutinin (PHA) and are interested in the possible function of its glycans. Phytohemagglutinin, a tetrameric glycoprotein consisting of two closely related subunit polypeptides, accumulates in the protein storage vacuoles (protein bodies) of developing bean (*Phaseolus vulgaris*) embryos. Entry of the nascent polypeptides (E and L subunits) in the endoplasmic reticulum (ER) is accompanied by the cotranslational attachment of high-mannose glycans at asparagines 12 and 60. After transport to the Golgi, the glycan at asparagine 60 is processed by the removal of mannose residues and the addition of N-acetylglucosamine (GlcNAc), fucose, and xylose residues. After arrival in the protein storage vacuoles, this complex glycan is further modified by the removal of terminal N-acetylglucosamine residues (Vitale et al., 1984a, 1984b; Vitale and Chrispeels, 1984; Sturm and Chrispeels, 1986). The similarity between these processing steps and the formation of mannose 6-phosphate groups (i.e. the addition and subsequent removal of GlcNAc groups) led to the idea that the oligosaccharides of PHA might be involved in vacuolar targeting in a way analogous to the mannose 6-phosphate signal of mammalian lysosomal proteins (Sly

and Fischer, 1982). Targeting of lysosomal hydrolases in mammalian cells results from the action of two targeting signals that act in sequence. The signal peptide allows these proteins to enter the lumen of the ER, and the mannose 6-phosphate groups provide the positive sorting signal in the *trans*-Golgi network for transport to the lysosomes. The absence of the second signal causes lysosomal enzymes to be transported along the default pathway and secreted from the cells (reviewed by Kelly, 1985; Pfeffer and Rothman, 1987; Burgess and Kelly, 1987).

In yeast cells, targeting of glycoproteins (acid hydrolases) to vacuoles requires a second signal that is contained in the polypeptide portion of the protein (Johnson et al., 1987; Valls et al., 1987), rather than in the glycans (Schwaiger et al., 1982). Circumstantial evidence suggests that yeast and plant vacuoles are homologous and that the vacuolar targeting mechanisms are the same in plants and yeast. PHA-L, synthesized in yeast, is targeted to yeast vacuoles (Tague and Chrispeels, 1987) and can, as a fusion protein, reroute the normally secreted yeast invertase to the vacuolar compartment (Tague and Chrispeels, 1988). There is also evidence that, in plants, targeting to the vacuole requires positive sorting information (Dorel et al., 1988). For PHA, this information could be in the polypeptide domain or in the glycans. Bollini et al. (1985) studied the biosynthesis and transport of PHA in the presence of tunicamycin, an inhibitor of *N*-glycosylation. They found that unglycosylated PHA formed tetramers and was transported to the storage vacuoles. Because inhibition of glycosylation was only partial, it could not be ruled out that the cotransport of glycosylated PHA played a role, or that the antibiotic had some other effects on the system. To avoid these problems, we decided to examine the biosynthesis of unglycosylated PHA in transgenic tobacco after mutating the glycosylation sites. We have shown previously that PHA-L synthesized in the developing seeds of transgenic tobacco is processed and transported to the storage vacuoles in a manner indistinguishable from the beans (Sturm et al., 1988). The heterologous system obviously is able to interpret faithfully all processing and targeting signals of the protein. To study the contribution of the individual glycans in synthesis, assembly, processing, and targeting of PHA-L in vivo in the absence of interfering normal PHA and without the potential side effects of tunicamycin, we introduced mutated PHA-L genes into the tobacco genome and studied the parameters of PHA-L physiology in the developing seeds of transgenic plants. We disrupted the two carbohydrate attachment sites of the polypeptide (Asn-*X*-Thr/Ser) by site-directed mutagenesis of the PHA-L reading frame with oligonucleotides. After introduction into tobacco and expression of the modified PHA-L with a β-phaseolin promoter, we found that partially and totally unglycosylated PHA-L are able to assemble into tetramers and are transported to the protein storage vacuoles.

RESULTS

Elimination of Glycosylation Sites in the PHA-L Polypeptide by Site-Directed Mutagenesis and Assembly of the Hybrid Genes

There are three potential sites for the addition of asparagine-linked glycans (Asn-*X*-Ser/Thr) in the predicted amino acid sequence of PHA-L (Hoffman and Donaldson, 1985). The first one overlaps the signal peptide cleavage site, and its asparagine is in the signal peptide of PHA. This site is probably not glycosylated in vivo. The other two sites, Asn^{12}-Glu-Thr and Asn^{60}-Thr-Thr, are used, and mature PHA-L carries one high-mannose oligosaccharide at asparagine 12 and one complex oligosaccharide at asparagine 60 (Sturm and Chrispeels, 1986; the amino acid residue number refers to the mature protein). To obtain mutated PHA-L genes, synthetic 20-base oligonucleotides were synthesized and used as mutagenic primers hybridizing to the antisense DNA of the PHA-L coding sequence at the appropriate positions (Figure 1). One oligonucleotide
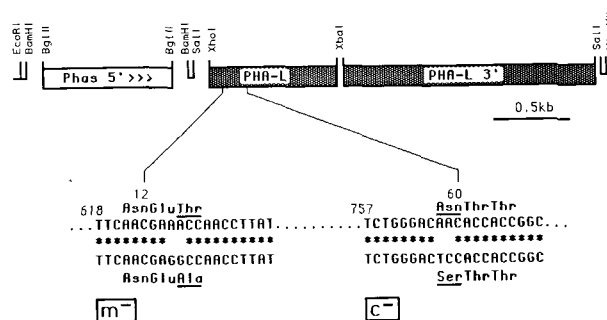

Figure 1. Assembly of the Hybrid Gene and Site-Directed Mutagenesis.

The upper bar represents the final hybrid gene construct. The assembly is shown as cloned into the multiple cloning site of the plant transformation vector Bin19. For plasmid sources and cloning strategy, see "Methods." The 850-bp BglII β-phaseolin upstream fragment is linked to the induced XhoI site 5' of the PHA-L reading frame via a 12-bp BamHI/SalI fragment from the multiple cloning site of pUC9. The oligonucleotide site-directed mutagenesis in the frame of PHA-L is indicated below. Twenty base sections of the coding sequence are shown (numbers refer to Hoffman and Donaldson, 1985) with the glycosylation amino acid signals added above (numbers refer to the glycosylated asparagines of mature PHA-L) (Hoffman and Donaldson, 1985; Sturm and Chrispeels, 1986). Below the gene sequence, the sequences of both oligonucleotides used for mutagenesis are shown aligned to their target gene sequences. Mismatches are indicated by the absence of stars. Affected amino acids and the substituted residues are underlined.

eliminates the glycan attachment site at Asn[12] by changing the codon for threonine into one for alanine. This mutant, called m[-], yielded PHA that has no glycan in the position of the high-mannose glycan. The other oligonucleotide eliminated the glycan attachment site at Ans[60] by changing the codon for asparagine into one for serine. This mutant, called c[-], resulted in PHA that has no glycan in the position of the complex glycan.

The original PHA-L coding sequence, the mutations m[-] and c[-], and the double mutant m[-]c[-] were cloned behind the 5′ upstream sequence of the β-phaseolin gene. This upstream sequence results in high expression of transgenes in the seeds of transgenic tobacco (Sengupta-Go-palan et al., 1985; Hoffman et al., 1987); the original 3′ region of the PHA-L gene was added to reassemble a functional gene (see Figure 1 for a map and the principle of the mutagenesis strategy and gene assembly). The completed chimeric genes were cloned into the plant transformation vector Bin19 (Bevan, 1984) and via *Agrobacterium* cocultivation of leaf discs (Horsch et al., 1985), the constructs were transferred to *Nicotiana tabacum* cv Xanthi (Voelker et al., 1987). For each of the four constructs, we regenerated three or more plants and analyzed the seeds by immunoblotting for PHA-L antigen.

Expression of PHA-L Using the β-Phaseolin Promoter

In our previous study (Voelker et al., 1987), we used the entire PHA-L gene (dlec2) to transform tobacco, and the levels of PHA we obtained after immunoprecipitation of the protein were quite low (0.01% to 0.02% of total seed protein). Using the β-phaseolin promoter, Sengupta-Go-palan et al. (1985) and Hoffman et al. (1987) found much higher levels with greater variability of phaseolin (0.5% to 2.0%) and zein (0.01% to 2.0%), respectively, in the seeds of transgenic tobacco. We, therefore, decided to drive the expression of PHA-L and the three mutants with the β-phaseolin promoter. The levels of PHA-L were measured in the mature seeds of 14 transformed plants. PHA-L antigen was quantified with a novel procedure (see "Methods") by making an immunoblot of the total low-salt extract of the seeds after SDS-PAGE, cutting out the bands, dissolving the colored peroxidase reaction product in acetone, and measuring the absorbance at 580 nm. The levels of PHA obtained with unmodified PHA-L (plants 810, A, B, C) and the m[-], c[-], and m[-]c[-] mutants in independently derived tobacco transformants varied from 0.02% to 1.7% of total seed protein (Figure 2). Interestingly, the five m[-]c[-] plants showed consistently the lowest level of PHA (0.02% to 0.04%). When we used this procedure on the plants transformed with dlec2 (PHA-L promoter, coding region, and 3′ region), we found PHA levels of 0.05% to 0.1%, about 5 times higher than those observed in our earlier work (Voelker et al., 1987). These plants are shown as
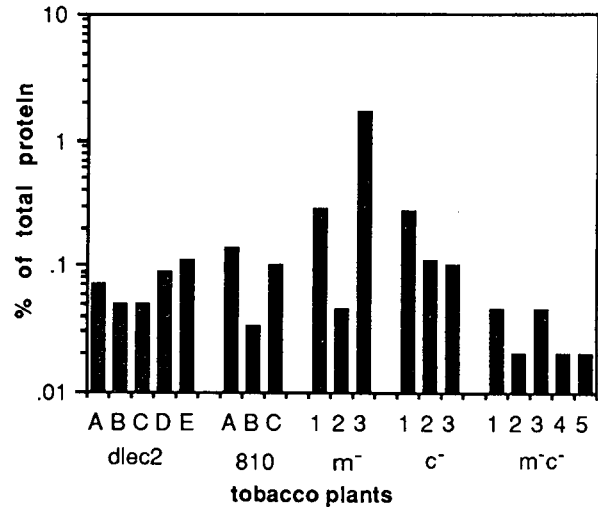

Figure 2. Survey of the PHA-L Contents of Seeds from Independently Regenerated Tobacco Plants.

Seeds from tobacco plants transformed with the cognate PHA-L gene dlec2 (columns dlec2, A to E, obtained earlier, Voelker et al., 1987) and from all plants generated in the course of this work were analyzed. PHA-L was quantitated after low-salt protein extraction at 5°C, and the value corrected for total protein extractable in SDS buffer (8 mg of protein/50 mg of mature seeds). The low-salt medium extracts only 20% of the total protein. The graph shows the amount of PHA-L protein of mature seeds relative to the total SDS buffer extractable protein. Mature cotyledons from the bean cultivar Greensleeves gave a value of 6.8% for total PHA with this method.

dlec2, A to D in Figure 2. In our previous study, our measurements of PHA-L were lower because the protein structure was disrupted first by solubilization in SDS, and PHA immunoprecipitated subsequently before immunoblot analysis. This procedure eliminates the proteolytic processing products that in mature seeds are more abundant than the full-length polypeptides (Figure 3). In general, comparing the β-phaseolin and the PHA-L proteins in tobacco, we observed that, on the average, both promoters allow for the accumulation of similar amounts of PHA in the seeds. There is very little variability in PHA-L levels between independent transformations with the PHA-L promoter, but the β-phaseolin promoter shows a 50-fold variability.

Accumulation of PHA-L during Tobacco Seed Development

Figure 3 shows the accumulation of PHA-L driven by the β-phaseolin promoter during tobacco seed development.
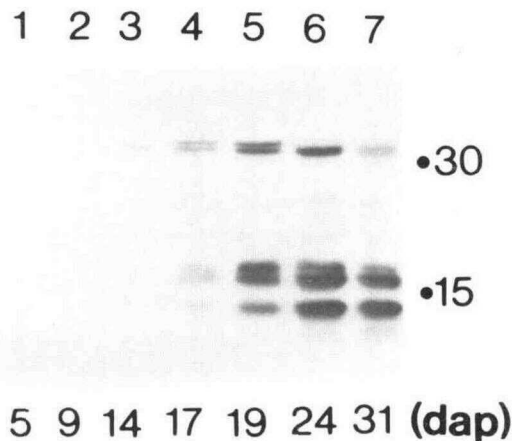
Figure 3. Time Course of PHA-L Accumulation in Seeds of Transformed Tobacco.

Seeds were collected at the indicated days after pollination (dap) and extracts from 20 seeds from a plant transformed with the intact gene (plant 810A) were subjected to SDS-PAGE and immunoblotted. PHA antigen was detected with PHA antiserum as described. Molecular mass markers are shown in kilodaltons.

The PHA-antiserum recognizes polypeptides of the expected size (mol wt 32,000) as well as smaller polypeptides (mol wt 18,000 to 13,000). That these smaller polypeptides are not the result of hydrolysis during isolation was demonstrated by adding a small amount of pure PHA obtained from beans to the seeds of untransformed tobacco plants. When PHA was isolated from this mixture, there were no smaller PHA polypeptides and there was no diminution of the PHA signal on the immunoblot. The faster migrating forms of PHA-L found in tobacco seeds probably are the products of proteolytic processing of the PHA-L polypeptides after their arrival in the protein storage vacuoles. With the β-phaseolin promoter, most of the PHA accumulated between 15 and 20 days after pollination (DAP), and there was some decrease in the level of PHA after day 24. We obtained similar kinetics of PHA-L accumulation with the other three β-phaseolin PHA-L constructs (m⁻, c⁻, and m⁻c⁻) generated in this study (data not shown).

Verification of the Molecular Weight of the Mutant Glycoforms of PHA-L and of the Nature of the Glycans

The expression of the mutated PHA-L genes (m⁻, c⁻, and m⁻c⁻) should result in glycoforms with different molecular weight values depending on the number of glycans that are present. These glycans could be of the high-mannose or the complex type, and this can be ascertained by treatment with endoglycosidase H (endo H), an enzyme that cleaves high-mannose glycans without removing complex glycans from the protein. Extracts of mature tobacco seeds were fractionated by SDS-PAGE either before or after treatment with endo H, and the PHA antigens detected by immunoblotting (Figure 4). Lane 1 of Figure 4 shows the products resulting from the intact PHA-L gene driven by the β-phaseolin promoter, whereas lane 2 shows the same products after endo H treatment. Endo H treatment resulted in a decrease in the molecular weight of PHA-L from 32,000 to 30,000 because of the removal of the high-mannose glycan (Vitale et al., 1984b). The smaller processing products shown in lanes 1 and 2 have the same molecular weights, indicating that they have no high-mannose glycans. The products of the m⁻ and c⁻ mutants (lanes 3 and 5, respectively) have molecular weight values of 30,000 and comigrate with endo H-treated PHA from the control gene. This is consistent with the interpretation that the m⁻ and c⁻ products each lack one glycan. The product of the double mutant m⁻c⁻ (lane 7) is again somewhat smaller because of the absence of both glycans. The m⁻ form of PHA-L (lane 3) is resistant to endo H (lane 4), indicating that its single oligosaccharide at Asn^{60} is of the complex form. In contrast, treating PHA-L c⁻ (lane 5) with endo H (lane 6) removes the high-mannose residue at Asn^{12}, and the product comigrates with unglycosylated PHA-L (lane 7). These results indicate that the absence of one oligosaccharide does not influence the processing or lack of processing of the other one. The glycan-free c⁻m⁻ form is, of course, unaffected by endo H (compare lanes
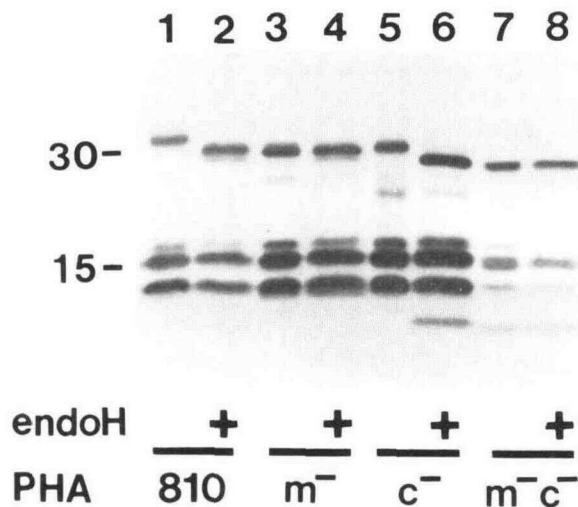

Figure 4. Analysis of the Glycoforms of Tobacco PHA-L.

Mature tobacco seeds were extracted with a low-salt buffer, and proteins were separated by SDS-PAGE and immunoblotted for PHA detection. Extracts from each construct (indicated below bars) appear twice: before and after (+) endoglycosidase H (endo H) treatment. Numbers on the left indicate the position of molecular mass markers in kilodaltons.

Figure 5. Velocity Gradient Centrifugation of Tobacco m⁻ PHA-L.

Immunoblot analysis of the fractions of a linear sucrose gradient centrifuged at 150,000g for 38 h (lanes 2 to 12). The analysis of m⁻ seeds is shown (total extract in lane 1 as a reference). The position of bean PHA-L (run separately) in the gradient is shown (+). All other glycoforms of tobacco PHA-L sedimented with the same velocity (not shown).

7 and 8) due to absence of any glycosylation site in the mature PHA-L polypeptide translated from the c⁻m⁻ frame.

In summary, in all four constructs, the β-phaseolin promoter caused the production of PHA-L in the seeds of transgenic tobacco. PHA-L was found to be doubly glycosylated with one processed sidechain (Sturm et al., 1988). Eliminating the glycosylation site at Asn[12] leaves one complex glycan on the m⁻ polypeptide at Ans[60]. In contrast, one high-mannose sidechain is attached to PHA-L at Asn[12] after substitution of the asparagine 60. Clearly, the mutated genes yielded PHA products of the expected size, and the specificity of sidechain modification was conserved in tobacco even in singly glycosylated forms.

All Glycoforms and Unglycosylated PHA-L Exist as Tetramers

Native PHA isolated from beans has been shown to exist as a tetramer with a sedimentation coefficient of 6.4S (Bollini and Chrispeels, 1978). To test whether the lack of one or both glycans might affect oligomer formation, we subjected low-salt extracts of tobacco seeds to velocity gradient centrifugation and compared the sedimentation behavior of tobacco PHA-L to bean PHA-L. As an example, the immunoblot analysis of the fractions of a sucrose gradient of m⁻ PHA-L is shown in Figure 5. Lane 1 is a control showing the PHA antigen pattern of complete m⁻ seed protein extract before centrifugation. Lanes 2 to 12 represent equal proportions of the fractions of a sucrose gradient. Almost all of the tobacco m⁻ PHA-L co-sedimented with bean PHA-L. (Its position in the gradient was also assayed by immunoblot and is indicated by crosses below the blot.) The same results were observed with c⁻ PHA-L and m⁻c⁻ PHA-L (not shown). We conclude that

tetramer formation is not affected by the modification or absence of the oligosaccharides. In addition, the processing products, migrating at mol wt 13,000 to 18,000, cosediment with the full-length polypeptides, demonstrating that these polypeptides are not released from the tetramers, but are products of a specific posttranslational processing of the native PHA-L molecule.

The Products of All Mutated Genes Are Targeted to the Protein Storage Vacuoles

Sturm et al. (1988) have shown that fully glycosylated PHA-L can be detected in the matrix of the protein storage vacuoles of seeds from tobacco transformed with the PHA-L gene, indicating that the vacuolar targeting signals carried by the PHA-L molecule are recognized by the tobacco cells. To determine whether the elimination of one or both oligosaccharides from PHA-L affected its intracellular targeting, we analyzed the location of mutant PHA-L forms by immunocytochemistry with the electron microscope. Figure 6 shows the immunodetection with anti-PHA IgGs (Sturm et al., 1988) on thin sections from seeds of tobacco transformed with the m⁻ mutant (A) and the m⁻c⁻ mutant (B). In both cases, the matrix of the protein storage vacuoles was found to be labeled by gold particles. No significant labeling of other organelles could be observed. Eliminating the high-mannose or both oligosaccharide attachment sites of the PHA-L polypeptide does not affect the intracellular targeting of the protein.

All PHA-L Forms Appear To Be Proteolytically Processed after Arrival in the Protein Storage Vacuoles

To complement and extend the information obtained by immunocytochemistry, we examined the PHA content of protein storage vacuoles isolated from homogenates of mature dry seeds. In hydrophilic organic solvents, such as glycerol, the storage vacuoles of mature dry seeds remain intact and can be separated from other cell structures on potassium iodide-glycerol density gradients. We applied a modified step gradient version of this procedure as developed by Sturm et al. (1988) to tobacco seeds transformed with unmodified and all three mutant PHA-L genes and analyzed the PHA in different gradient fractions (Figure 7). Sets of three lanes are shown for each construct. The left lane of each set shows the total seed extract, the middle lane, the load portion from the glycerol gradient, and the right lane, the organelles at the 1.3/1.4 g/ml density interface. This fraction contains the protein storage vacuoles as shown by the presence of the vacuolar marker enzyme, α-mannosidase, and the tobacco seed storage proteins (Sturm et al., 1988). The other organelles do not enter the gradient and remain in the load fraction. Lanes 2 to 4 show the analysis of the seeds with the unaltered PHA-L frame.
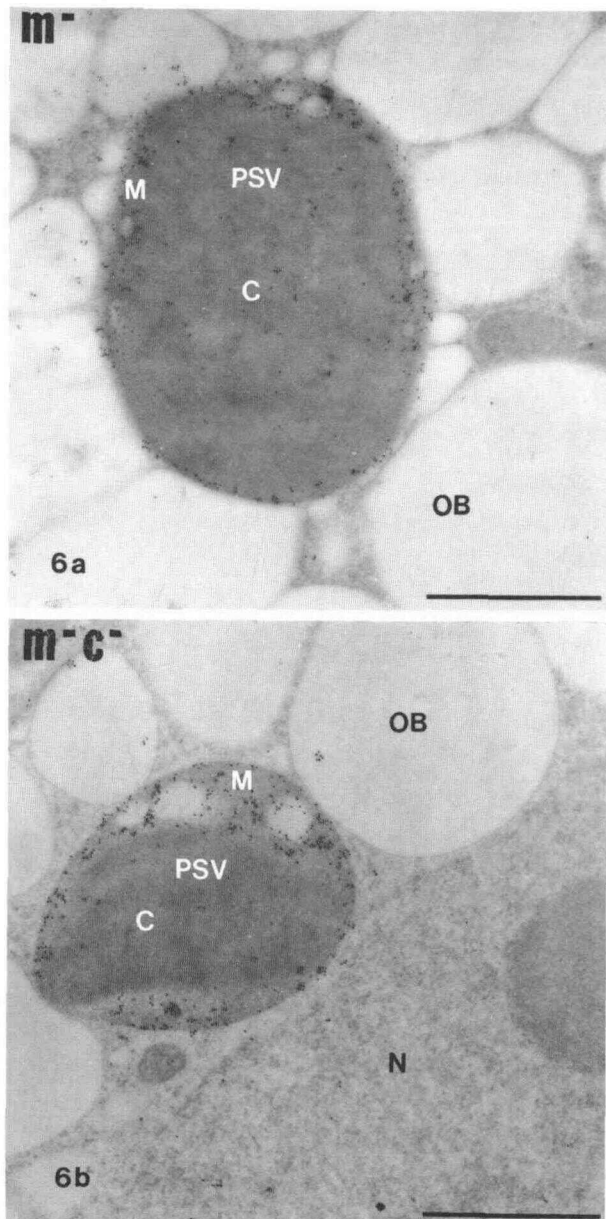
The 32,000 mol wt band of full-length PHA-L from tobacco forms a doublet (lane 2) with the lower polypeptide (major) comigrating with authentic bean PHA-L (lane 1). The upper band is primarily in the load fraction (lane 3), whereas the lower band is in the vacuole (protein body) fraction (lane 4). These results confirm our earlier findings (Sturm et al., 1988), in which we showed that the lower band has a high-mannose and a complex glycan, whereas the upper band has two high-mannose glycans (Sturm et al., 1988). The results in lane 4 show that the proteolytic processing products of PHA-L appear mainly in the vacuole fraction, and this is presumed to be the site of this processing. Similar results were obtained with the gradient fractions of the m⁻ seed extracts (lanes 5 to 7) and c⁻ seed extracts (lanes 8 to 10). Again, note the appearance of processing products around 15,000 mol wt in the vacuolar fractions (lanes 7 and 10). There is a doublet at 30,000 mol wt in the total extract lanes (lanes 5 and 8); the upper polypeptide is in the load fraction (lanes 6 and 9) and lower polypeptides in the vacuole fractions (lanes 7 and 10). The results are similar for m⁻c⁻ (lanes 11 to 13), except that there is much less PHA, especially in the vacuole fraction (lane 13).

Taking all analyses together, we conclude that all PHA-L glycoforms arrive in the protein storage vacuoles. After arrival in this compartment, a large fraction of molecules becomes proteolytically cleaved at specific site(s) to yield polypeptide fragments ranging from 13,000 to 18,000 mol wt. Some PHA-L never reaches the storage vacuoles; this PHA-L probably remains in the endoplasmic reticulum (Sturm et al., 1988).

DISCUSSION

We describe here the use of site-directed mutagenesis and subsequent gene expression in transgenic tobacco for studying the role of the two glycan sidechains in the assembly, stability, and intracellular targeting of PHA-L, a bean seed vacuolar protein. This approach allowed us to assess the importance of individual carbohydrate side-chains of the glycoprotein without the complication of using an inhibitor of glycosylation. In the experiments reported here, the expression of PHA-L was driven by the β-phaseolin promoter rather than the PHA-L promoter used in our previous studies (Voelker et al., 1987).

Differences between β-Phaseolin and PHA-L Promoters for the Onset of Activity during Development Are Conserved in Transgenic Tobacco

After insertion of a chimeric gene into the tobacco genome, the β-phaseolin promoter yields expression levels of up to 2% of total seed protein, with the onset of protein accumulation occurring around 16 DAP (Sengupta-Gopalan et al., 1985; Hoffman et al., 1987). We find this behavior conserved in our phaseolin/PHA-L hybrid gene and differ-

Figure 6. Electron Microscopic Localization of PHA Glycoforms in the Storage Vacuoles of the Embryos of Transgenic Tobacco.

(a) m⁻ seeds.
(b) m⁻c⁻ seeds.
Developing tobacco seeds were fixed at 20 to 23 DAP and embedded as described. Thin sections were labelled with affinity-purified rabbit anti-PHA IgG (0.25 μg/μl), followed by goat anti-rabbit IgG coupled to 10 nm colloidal gold. C, crystalloid; M, matrix; OB, oil body; N, nucleus.

1   2   3   4   5   6   7   8   9   10   11   12   13

30−

15−

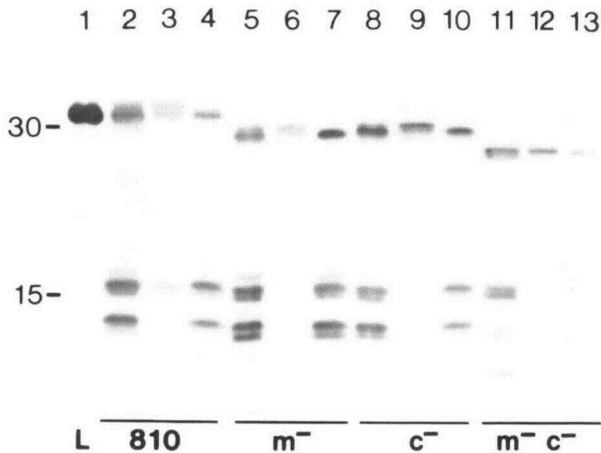L   810   m⁻   c⁻   m⁻ c⁻

Figure 7. Detection of PHA-L in the Storage Vacuoles after Cell Fractionation.

Immunoblot analysis as described. Lane 1 contains bean PHA-L as a size marker for the full-length polypeptide. Lanes 2 to 13, analysis of proteins from all four constructs after the glycerol step gradient fractionation. Three lanes are shown per construct (bar). Left lane of each set: total glycerol seed extract; middle lane of each set: fraction T1 (load volume after centrifugation); right lane of each set: fraction T4 at the 1.3/1.4 g·cm⁻³ interface after centrifugation, containing the protein storage vacuoles. Positions of size markers are indicated in kilodaltons.

ent from the PHA-L promoter, which results in the accumulation of gene product after 20 DAP in tobacco (Voelker et al., 1987). This temporal difference resembles the situation observed in the bean. In bean cotyledons, phaseolin and PHA-E are expressed early in the maturation process (Hall et al., 1978; Chappell and Chrispeels, 1986), whereas PHA-L starts to accumulate later (Staswick and Chrispeels, 1984). Clearly, tobacco recognizes the temporal gene expression signals of the bean promoters. In quantitative terms, we find that, on the average, both promoters cause the accumulation of similar levels of gene products in tobacco seeds (0.1% to 0.2% of total protein). In contrast to the lack of variability in PHA-L levels obtained with the PHA-L promoter (the twofold variation described here was observed with many more transgenic plants), the phaseolin promoter yields a much greater plant-to-plant variation (40-fold) in transgenic tobacco (see also Hoffman et al., 1987). The cause of this striking difference is unknown.

Oligosaccharide Sidechains of PHA-L Are not Necessary for Oligomerization or Intracellular Targeting

Elimination of the glycosylation signals of the PHA-L polypeptide due to amino acid substitution caused by site-

directed mutagenesis yielded the predicted glycoform when the protein was synthesized in transgenic tobacco seeds. The sidechain of Asn^{12} keeps its high-mannose character after deleting the glycosylation site at Asn^{60}, and the sidechain at Asn^{60} keeps its complex character when the site at Asn^{12} is eliminated. The absence of one of the two high-mannose sidechains attached to the nascent PHA-L polypeptide in the ER does not influence the post-translational processing steps of the other sidechain in the Golgi. Earlier work in this laboratory (Faye et al., 1986) has shown that whether a high-mannose sidechain is modified or not depends on its accessibility to glycan processing enzymes in the Golgi apparatus. The absence of one of the two glycan sidechains also does not alter the folding of the PHA-L polypeptide in a way that changes the accessibility of the residual glycan to the processing enzymes. This is in contrast to our results obtained with phaseolin, in which absence of a specific glycan sidechain changes the processing of an oligosaccharide attached at another site of the polypeptide (Sturm et al., 1987). Phytohemagglutinin-L with one or two glycans formed tetramers in vivo. This observation is in accordance with results of Bollini et al. (1985), showing the oligomerization of unglycosylated PHA in tunicamycin-treated cotyledon tissues (Bollini et al., 1985) and the observation that signal peptide-containing unglycosylated PHA-L synthesized by *Escherichia coli* forms oligomers (Hoffman and Donaldson, 1987).

Immunocytochemistry with the electron microscope and cell fractionation using nonaqueous glycerol gradients demonstrate that partially or totally unglycosylated PHA-L is targeted properly to the protein storage vacuoles of the seeds of tobacco. These results show unequivocally that the glycans of PHA-L are not essential for the intracellular targeting process, and confirm circumstantial evidence (reviewed in Chrispeels et al., 1987) that glycans are not necessary for vacuolar targeting in plant cells. The cell fractionation experiments showed that PHA-L polypeptides become proteolytically processed after arrival in the storage vacuoles. Cleavage of the full-length polypeptide into several specific fragments does not lead to the release of these fragments from the tetrameric assembly. Phaseolin (Sengupta-Gopalan et al., 1985) and β-conglycinin (Beachy et al., 1985), proteins that are not proteolytically processed in their normal environment, are also proteolytically processed when their genes are expressed in tobacco. This processing phenomenon resembles the post-translational processing in the protein storage vacuoles of other seed storage proteins and lectins (e.g. legumin, glycinin, pea lectin) first described for pea legumin by Chrispeels et al. (1982). Since tobacco seeds accumulate a seed-storage protein of the legumin type that is proteolytically processed (Sano and Kawashima, 1983), it must possess a processing protease in the vacuoles. Beans, on the other hand, do not have a major processed protein and may lack such an enzyme.

What Is the Role of the Glycans on PHA?

Although we observed wide variations in the amount of PHA per plant (0.01% to 1.7%), we consistently found less PHA in the five independently derived m⁻c⁻ plants. Assuming that these plants contain, on the average, similar amounts of PHA-L mRNA due to the identical promoter used for all constructs, it could reflect accelerated breakdown of the m⁻c⁻ polypeptides. There is indeed much circumstantial evidence that glycans are involved in protein stability and protect some proteins from proteolytic degradation (see Olden et al., 1985). In analyzing the subcellular location of the m⁻c⁻ PHA-L, we observed more PHA-L in the load fraction of the glycerol gradients (containing ER and Golgi) than in the protein storage vacuole fraction. The vacuole fraction, furthermore, contained only a small amount of processing products. This raises the question of whether processing stops when polypeptides of 13,000 to 18,000 mol wt have been formed; it is possible that a proportion of these smaller polypeptides are broken down totally, especially in the m⁻c⁻ seeds. In addition, there may be less efficient transport of the vacuoles when the glycans are absent. More detailed questions about transport and stability can be answered only by pulse-chase experiments, and tobacco seeds do not lend themselves well to such experiments. To answer such questions, we are now developing a bean transformation system.

METHODS

Bacterial Strains and Vectors

The F⁺dut⁻ung⁻ E. coli strain RZ1032, used for generation of uracil containing bacteriophage M13 DNA, was obtained from L. Wickner (University of California, Los Angeles). The vector, Bin19, was obtained from M. Bevan (Bevan, 1984).

Plant Genomic DNA Fragments

The 850-bp BglII β-phaseolin 5′ upstream fragment was derived from the plasmid p8.8pro obtained from L. Hoffman (Hoffman et al., 1987). The source for the PHA-L reading frame was a genomic dlec2 gene fragment isolated from the bean cultivar Greensleeves in our laboratory. The 5′ region had been deleted up to position −6 relative to the reading frame by the nuclease Bal31 and a XhoI linker was added (M142, Voelker et al., 1986). The XbaI site at the 3′ end of this fragment contains the translational stop codon of PHA-L (DNA sequence, see Hoffman and Donaldson, 1985). The 1500-bp XbaI/SalI 3′ fragment of the same gene dlec2 (from pTV562, Voelker et al., 1987) was added to the hybrid gene to supply the original transcriptional termination signals. The pUC12 plasmid containing the PHA-L reading frame and 3′ fragment was labelled pTV783.

Assembly of the Hybrid Gene

The 850-bp BglII β-phaseolin 5′ region of p8.8pro was cloned into the BamHI site of pUC9, and DNA of an isolate (pTV794) in the proper orientation opened at the multiple cloning site with SalI. Subsequently, the combined coding sequence and 3′ fragment of dlec2 (from pTV783) were inserted as a XhoI/SalI fragment into the SalI site of the phaseolin construct pTV794. The assembled gene (pTV800) is shown in Figure 1, and the final gene was transferred to the plant vector Bin19 as an HindIII/EcoRI fragment.

Site-Directed Mutagenesis

For site-directed mutagenesis according to Kunkel (1985), the 1700-bp phaseolin/PHA-L EcoRI/XbaI fragment of pTV800 was cloned into the bacteriophage M13mp19 and single-strand DNA isolated from bacteriophages grown on a dut⁻ung⁻ host. The single-strand DNA had the PHA-L antisense orientation. Synthetic 20-base oligonucleotides (see Figure 1 for sequence) were hybridized singly or together as primers followed by in vitro extension with the Klenow fragment of E. coli DNA polymerase I at room temperature. A dut⁺ung⁺ E. coli strain was transported with the reaction products, and replicative forms of the bacteriophages obtained were screened for mutations by restriction enzymes (the 5′ mutation creates a new HaeIII site, and the 3′ mutation creates a new HinfI site). After the final selection, the mutations were verified by DNA sequencing (dideoxy chain termination) (Sanger et al., 1977). The mutated fragments were excised from mp19 by EcoRI and XbaI and added to the 1500-bp 3′ dlec2 XbaI/SalI fragments, which had been cloned into the plant vector Bin19. For the final constructs, changes in the DNA sequence, and amino acid substitution, see Figure 1.

Protein, Protein Extraction, and Immunoblotting

Purified PHA-L protein was purchased from Sigma. For the protein extraction of tobacco seeds, fresh material was ground at 0°C with a buffer containing 50 mM Tris, pH 8, 30 mM NaCl, 0.1% Triton X-100, and 1% β-mercaptoethanol. The supernatant of the subsequent centrifugation (14,000g for 5 min) was termed the low-salt extract. For immunoblotting, appropriate quantities of protein (determined according to Lowry et al., 1951) were fractionated by SDS-PAGE, the proteins transferred to nitrocellulose, and PHA detected using a rabbit anti-PHA-L antibody generated as described in Voelker et al. (1987). We used goat anti-rabbit horseradish peroxidase coupled IgGs (Bio-Rad) as secondary antibody and 4-chloro-1-napthol as a peroxidase substrate.

Endo H Digestion, Glycerol Gradient and Velocity Gradient Centrifugations, and Immunocytochemistry

Endo H digestions of low-salt extracts were carried out according to Trimble and Maley (1984). Glycerol gradient centrifugation followed the method of Begbie (1979) as modified by Sturm et al. (1988). After centrifugation, the fractions T1 and T4, labeled according to Sturm et al. (1988), were harvested, diluted 10 times with low-salt buffer, and dialyzed extensively against the same

buffer to remove glycerol and KI. After dialysis, the fractions were centrifuged at 20,000g for 15 min to sediment low-salt insoluble proteins, and the supernatant was concentrated by acetone precipitation. Equal portions of the gradient fractions were loaded for immunoblotting. The velocity gradient centrifugation of low-salt extracts was executed as described (Bollini and Chrispeels, 1978). Fractions were precipitated with acetone after dialysis against the low-salt buffer and equal proportions loaded for immunloblotting. Immunocytochemistry at the electron microscope level was carried out as described in Sturm et al. (1988).

Quantitation of Horseradish Peroxidase Immunoblotting Signals

This procedure, developed in the course of this work, is based on the principle of dissolving the precipitated color product of the reaction of peroxidase with its substrate 4-chloro-1-napthol and measuring the absorbance of the solution. In practice, equal-sized areas of nitrocellulose containing the signal (dot blot or immunoblot band) were cut out, and the colored precipitate was dissolved in 1 ml of acetone in an Eppendorf tube. (This solvent only swells the nitrocellulose.) After several minutes, when all traces of color were dissolved, a quick centrifugation sedimented the nitrocellulose, and the absorbance of the supernatant could be measured at 580 nm, the absorption peak of the color product in the visible spectrum. Including a background standard and a dilution series of antigen of known concentrations from the same blot allowed us to establish a standard curve for the quantification of unknown samples. Separation of the proteins by SDS-PAGE before blotting increases the sensitivity of about 10-fold over the use of dot blots.

To quantitate the PHA-L levels in the low-salt extracts from the seeds of transformed tobacco, we used a two-step reference strategy. First, a dilution series with known amounts of PHA-L was mixed with low-salt extracts of seeds from untransformed tobacco to obtain equal micrograms of total protein in each sample. Equal amounts of total protein in equal volumes were dotted on nitrocellulose, air-dried, fixed with a 5% methanol solution, and used for immunodetection of PHA. Squares of equal size were cut out of the filters, and after dissolving the color product in acetone, the absorption at 580 nm was measured and a standard curve obtained. The PHA-L concentration of the seed extracts from the high-expressing plant m⁻3 (Figure 2) was determined by comparison with the PHA-L dot standard curve obtained from the same blot. Subsequently, this m⁻3 extract was used as a new standard (in dilutions) after SDS-PAGE, which increased the sensitivity enough to measure the PHA-L contents of low-salt extracts of all regenerated plants.

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