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Expression of Mutant Patatin Protein in Transgenic Tobacco Plants: Role of Glycans and Intracellular Location

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The influence of N-glycosylation and subcellular compartmentation on various characteristics of a vacuolar glycoprotein is described. One member of the patatin gene family was investigated as a model system. Different glycosylation mutants obtained by destroying the consensus site Asn-X-Ser/Thr by oligonucleotide-directed mutagenesis were expressed in leaves of transgenic tobacco plants under the control of a light-inducible promoter. The various patatin glycomutants retained their properties in comparison with the wild-type protein with respect to protein stability, subcellular compartmentation, enzymatic activity, and various physicochemical properties studied showing the N-glycosylation not to be essential for any of these characteristics. To test the importance of the co-translational transport and the subcellular (vacuolar) location for the properties of the patatin protein, another mutant was constructed in which the signal peptide was deleted, leading to its synthesis and accumulation in the cytosol. Biochemical analysis of this protein in comparison with its vacuolar form again revealed no significant differences with respect to its enzymatic activity or its stability in normal vegetative cells. During seed development, however, the cytoplasmic form was more stable than the vacuolar form, indicating the appearance of proteases specific for the protein bodies of developing seeds.

INTRODUCTION

Plant cells contain numerous distinct compartments, such as the nucleus, endoplasmic reticulum, mitochondria, chloroplasts, microbodies, and vacuoles, that are separated by intracellular membranes. These organelles fulfill specialized functions within the cell and are characterized by a specific protein composition. To maintain this compartmentation, mechanisms must exist to direct newly synthesized proteins to their proper destination. Secreted or vacuolar proteins are synthesized as pre-proteins with a hydrophobic amino-terminal signal peptide. These proteins are co-translationally translocated across the membrane of the endoplasmic reticulum (ER) by a receptor-mediated process (Blobel, 1980). This process is accompanied by the co-translational cleavage of the signal peptide, and, in many cases, the attachment of glycan moieties at asparagine residues, provided that this amino acid is present within the consensus sequence Asn-X-Ser/Thr. These proteins are then transported from the ER to the Golgi complex, where the glycans can be modified further to complex glycans. Targeting to the vacuole requires additional signals (Dorel et al., 1988), whereas secreted proteins are believed to be transported along the default pathway (Burgess and Kelly, 1987; Pfeffer and Rothman, 1987).

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Although N-glycosylation of proteins represents one very abundant modification of at least those proteins following the ER-Golgi pathway, its role is not well understood. Studies performed mainly in mammalian systems suggest the absence of any general role for the glycosylation; rather, glycosylation can be important for a variety of different functions. Thus, glycan moieties modulate the physicochemical properties of certain polypeptides (Hickman et al., 1977; Chu et al., 1978; Matzuk and Boime, 1988), they may protect polypeptides against uncontrolled proteolysis (Olden et al., 1978), and, in a few cases, they may be involved in the biological activity of the proteins (Dubé et al., 1988). In mammalian cells, for example, mannose 6-phosphate groups act as sorting signals for the transport of glycosylated enzymes to the lysosomes (Sly and Fischer, 1982), whereas, in yeast (Johnson et al., 1987) and plant cells (Voelker et al., 1989), the sorting signal is believed to be part of the polypeptide portion of vacuolar proteins.

Most of the experiments have been based on the use of inhibitors of the glycosylation process (such as tunicamycin). Although these experiments have undoubtedly allowed us to study the glycosylation process, they are hampered by the fact that these inhibitors generally inhibit N-glycosylation of all proteins within the treated tissue,

thus making the exact assignment of the role of glycosylation for a single protein nearly impossible.

Therefore, we decided to use a genetic approach to analyze the contribution of the glycosylation on various characteristics of a well-defined protein in higher plants. As a model system, we chose a 40-kD vacuolar glycoprotein from potato tubers that has been given the trivial name patatin. In potato tubers, patatin is represented by a family of isoproteins with lipid acyl hydrolase activity that accounts for 40% of the total soluble protein.

In previous experiments, we have shown that one member of this protein family is correctly targeted and processed in leaves of transgenic tobacco plants (Sonnewald et al., 1989b). The protein becomes N-glycosylated at Asn^{60} and Asn^{90}, and both glycans are modified to small, complex glycans. To assay the role of glycosylation for the patatin protein, we expressed different patatin glycomutants in transgenic tobacco under the control of a light-inducible promoter (Eckes et al., 1986). In the different mutants, either the first, the second, or both glycosylation sites had been eliminated.

In most cases, glycosylation of a protein is connected to its subcellular compartmentation following the ER-Golgi pathway. To dissect the possible importance of N-glycosylation from the importance of the subcellular compartmentation, a patatin mutant was designed in which the signal peptide was deleted, resulting in the synthesis and accumulation of the patatin protein in the cytosol. The cytosolic patatin was expressed under the control of a seed-specific promoter to avoid a possible negative influence of the lipid acyl hydrolase activity in the cytosol on the viability of the transgenic tobacco plants.

RESULTS

Construction of a Mutant Patatin Protein Devoid of N-Glycosylation Sites and Its Transfer and Expression in Transgenic Tobacco Plants

To analyze the influence of glycan residues on various characteristics of a patatin protein, we used the coding region of the patatin gene pgT5 (Rosahl et al., 1986). The amino acid sequence deduced from the DNA sequence of this patatin gene contains a 23-amino acid amino-terminal signal peptide and three potential N-glycosylation sites at positions Asn^{60}, Asn^{90}, and Asn^{202} (Rosahl et al., 1986). Because only the first two sites are glycosylated in vivo (Sonnewald et al., 1989b), three different mutants were constructed in which either the first, the second, or both consensus sites were destroyed, as shown in Figure 1.

To minimize protein structure disturbance, mutagenesis was performed in both cases in such a way that the threonine residue was replaced by an alanine residue. Subclones of the pgT5 gene were mutagenized using a synthetic oligonucleotide 30 nucleotides long (cf. Figure 1) and then exchanged against the corresponding part in the wild-type gene of pgT5, resulting in mutants that lost the glycosylation site at positions Asn^{60} (P-Pat-1), Asn^{90} (P-Pat-2), or Asn^{60} and Asn^{90} (P-Pat-1-2). Subsequently, the mutated genes were placed behind the promoter of the ST-LS1 gene and transferred to tobacco via the *Agrobacterium tumefaciens* system (see Methods). Transgenic tobacco plants arising after kanamycin selection were probed for the presence of the different chimeric constructs by DNA gel blotting (data not shown). Only plants containing intact nonrearranged copies of the different genes were used for further analysis.

Glycosylation of the Patatin Protein Has no Significant Influence on Protein Stability in Vivo and in Vitro

Experiments performed mainly in mammalian systems indicated that glycosylation does not fulfill a unique role but rather serves different functions, one of which is the stability of the protein. Because wild-type patatin protein accumulates to high levels in leaves of a foreign host (amounting to between 0.1% and 0.5% of the total protein) (cf. Sonnewald et al., 1989b), which is different from other proteins when expressed in a foreign organ in a foreign host (Beachy et al. 1986), we wanted to know whether this high stability might be due to its extensive glycosylation. Total protein was isolated from leaves of 10 independent transgenic tobacco plants for each of the constructs P-Pat-1, P-Pat-2, P-Pat-1-2, and compared with the wild-type protein by protein gel blotting experiments. Although substantial variation was observed between independent transformants with respect to the amount of patatin protein or patatin-specific RNA accumulating, no

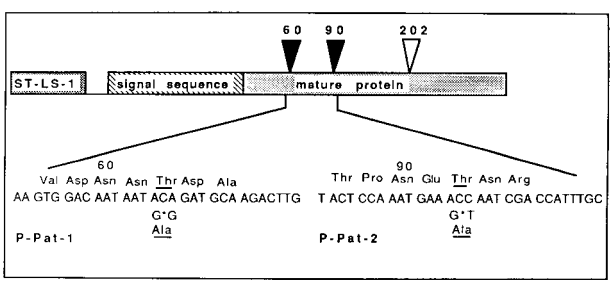

Figure 1. Construction of the Glycosylation Mutants.

The potential N-glycosylation sites are marked by arrows. The numbers given above represent the amino acid positions of the acceptor asparagine residues. The in vivo glycosylated asparagine residues are marked by black arrows. Thirty-base oligonucleotides were used to replace the threonine codons by alanine codons, thereby eliminating the glycosylation consensus sequence Asn-X-Ser/Thr.

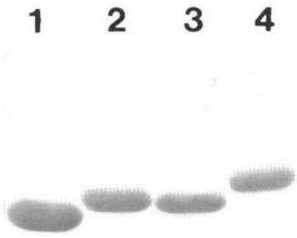

Figure 2. Protein Gel Blot Analysis of the Glycomutants.

Protein extracts (30 μg) isolated from transgenic tobacco leaves were separated with 12.5% SDS-PAGE, and the patatin protein was identified using anti-patatin, protein-specific antibodies. Lane 1, P-Pat-1-2; lane 2, glycomutant P-Pat-2; lane 3, P-Pat-1; lane 4, wild-type patatin (D8).

significant differences in protein accumulation were observed for the different constructs, again varying between 0.1% and 0.5% of the total protein (data not shown).

A representative result is shown in Figure 2. Obviously, the molecular weight of the mutant proteins P-Pat-1 and P-Pat-2 is reduced as compared with the wild-type protein (Figure 2, lane 4), with the reduction of about 1.5 kD being equivalent to the loss of 1 glycan residue. The amount of protein detectable by the antibody is, however, about the same for these two mutants missing the first or the second glycosylation site when compared with the wild-type protein.

The same holds true for the double mutant P-Pat-1-2. The further reduction in size observed for this double mutant is equivalent to the loss of 2 glycan residues (roughly 3 kD). It is important to note that the double mutant P-Pat-1-2 showed the same electrophoretic mobility in SDS-PAGE as the wild-type patatin after chemical deglycosylation with trifluoromethanesulfonic acid, indicating that the third glycosylation site, although present in the double mutant, was not used in vivo (data not shown). The smaller molecular weight proteins seen in lanes 2 and 3 of Figure 2 were not specific for these glycosylation mutants but were sometimes also seen in wild-type patatin protein containing extracts and probably represent specific degradation products.

Because we did not detect any significant influence of the glycosylation on the amount of protein accumulating in vivo, we decided to test the resistance of the different proteins against protease digestion in vitro. Protein extracts were treated with trypsin, and aliquots were taken after different time intervals and analyzed by SDS-PAGE and protein gel blotting.

As shown in Figure 3, elimination of the glycan moieties did not alter the stability of the protein with respect to protease digestion. In all cases, a peptide accumulated that seemed resistant to trypsin digestion. The mobility of this peptide in SDS-PAGE was dependent on the state of glycosylation, indicating that it comprises the residues Asn^{60} and Asn^{90}.

Lipid-Acyl Hydrolase Activity of the Patatin Glycomutants Does not Differ from the Wild-Type Patatin

Patatin isolated from potato tubers or transgenic tobacco leaves possesses a lipid acyl hydrolase activity that is detectable in SDS-PAGE under semi-native conditions using α-naphthyl acetate as substrate. To test whether the glycan moieties attached to asparagine residues 60 or 90 would influence the esterase activity, purified patatin from the different glycosylation mutants was separated by SDS-PAGE and visualized either by α-naphthyl acetate (Figure 4A) or Coomassie Blue staining (Figure 4B). Staining of the proteins with Coomassie Blue revealed two distinct forms of patatin, monomeric and oligomeric. Of these two forms, only the oligomeric possessed esterase activity.

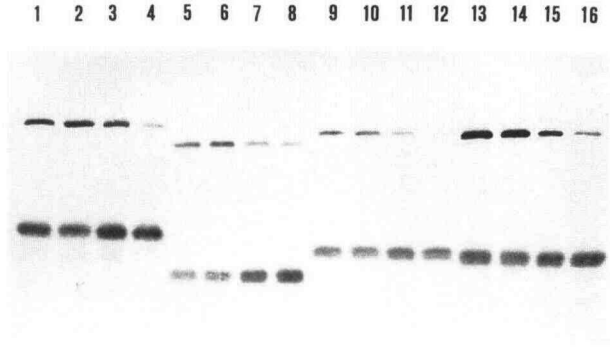

Figure 3. Trypsin Digestion and Immunodetection of the Glycomutants.

Protein extracts (100 μg) isolated from transgenic tobacco leaves were incubated with 50 μg of trypsin on ice. Twenty-five-microgram aliquots were taken after 7.5 min (lanes 1, 5, 9, and 13), 15 min (lanes 2, 6, 10, and 14), 30 min (lanes 3, 7, 11, and 15), and 60 min (lanes 4, 8, 12, and 16). The proteins were separated with 12.5% SDS-PAGE, and patatin was visualized using anti-patatin, protein-specific antibodies. Lanes 1 to 4, wild-type patatin (D8); lanes 5 to 8, P-Pat-1-2; lanes 9 to 12, P-Pat-2; lanes 13 to 16, P-Pat-1.

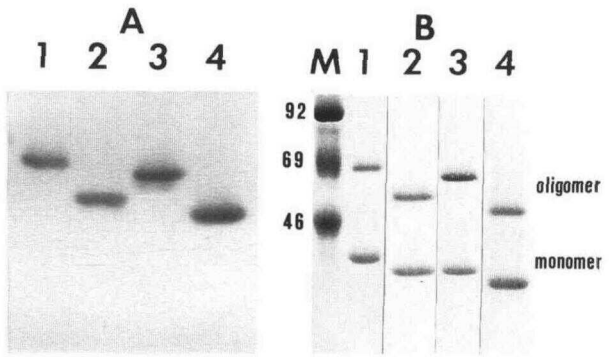

Figure 4. Monomeric Patatin Does not Show Esterase Activity Detectable in Situ.

(A) In situ esterase staining. Purified patatin protein from the different glycomutants was separated with 12.5% SDS-PAGE under semi-native conditions. The esterase activity was subsequently visualized by incubating the gel in a solution containing α-naphthyl acetate and fast blue RR. Lane 1, wild-type patatin (D8); lane 2, P-Pat-1; lane 3, P-Pat-2; lane 4, P-Pat-1-2.

(B) Purified patatin protein (5 μg) was separated as in **(A)**, and the proteins were visualized by Coomassie Blue staining. Lane 1, D8; lane 2, P-Pat-1; lane 3, P-Pat-2; lane 4, P-Pat-1-2.

This activity was not influenced by the presence or absence of the glycan moieties. The molecular weights of the active complexes were estimated to run between 50 kD and 70 kD, which could be explained by a lowered SDS content of the possible dimers. The ratio between the monomeric and the oligomeric form varied between 1:2 and 2:1, depending on the extract.

To analyze the esterase activities further, we compared the substrate specificity of the different glycoforms with that of the unmodified patatin. As substrates, *p*-nitrophenyl fatty acid esters with chain lengths ranging from C-2 to C-18 were tested. Untransformed tobacco contained a short-chain esterase activity that decreased with increasing carbon chain length, as shown in Figure 5A. This activity copurified in all enzyme preparations. The unmodified as well as the different glycoforms showed the highest activities with 10-carbon chain and 16-carbon chain acyl esters (Figure 5A). We concluded that the glycosylation had no influence on the esterase activity of patatin with respect to substrate specificity. In addition, no significant differences were observed between wild-type patatin and the glycomutants with respect to specific activities and pH dependence (data not shown).

Effect of the Glycan Moieties on the Physicochemical Properties of Patatin

The presence of glycan moieties is known to influence the solubility and hydrophobicity of the different glycoproteins. The solubility of the different glycoforms of patatin was compared with that of the unmodified protein by adding increasing amounts of ammonium sulfate to protein extracts from transgenic tobacco leaves. The precipitation of patatin was followed by assaying for esterase activity of the different fractions. As shown in Figure 5C, patatin was soluble until 45% ammonium sulfate saturation. At 55% saturation, the proteins precipitated independent of the presence or absence of the glycan moieties. Furthermore, hydrophobicity, measured by binding of the proteins to phenyl-Sepharose, for all different glycoforms was similar to that of the unmodified protein (data not shown).

As a further criterion for changes in the physiochemical

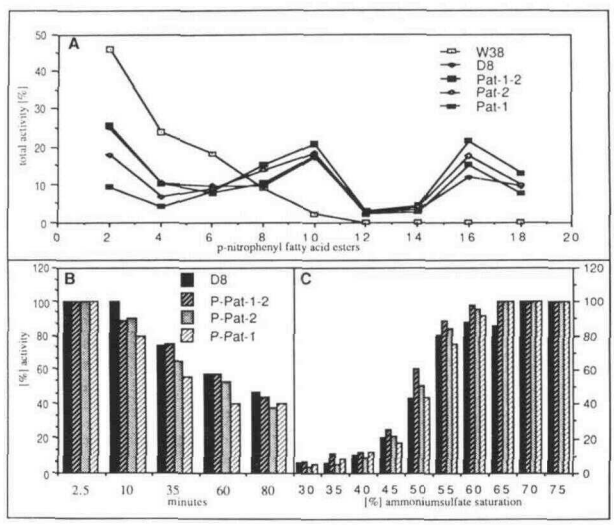

Figure 5. Glycosylation Is of no Detectable Influence on Either the Enzymatic Activity or Several Physicochemical Properties of the Patatin Protein.

(A) Substrate specificity of the different glycomutants. The different patatin forms are marked as in Figure 4B. Patatin protein was isolated from transgenic tobacco leaves as described in Methods. The substrate specificity of the esterase was determined using *p*-nitrophenyl fatty acid esters with carbon chain lengths of 2 to 18 as substrates. The reaction was followed at 405 nm and the activity was calculated as [ΔA_{405}/10 min]. The relative activity (for one substrate) is given as percent of the total activity. W38, untransformed control.

(B) Inactivation of the different glycomutants by guanidine hydrochloride. Enzyme solutions were incubated in 1.6 M guanidine hydrochloride on ice. Aliquots were taken after 2.5 min, 10 min, 35 min, 60 min, and 80 min, and the esterase activity was determined with *p*-nitrophenyl caprate as substrate.

(C) Solubility of the different glycomutants in ammonium sulfate. Protein extracts from transgenic tobacco leaves were incubated with increasing amounts of ammonium sulfate for 2 hr at 4°C. The proteins were precipitated by centrifugation, and the esterase activity of the different fractions was determined with *p*-nitrophenyl caprate as substrate.

properties of the different glycoforms, we tested their stability with respect to repeated freezing and thawing cycles and determined the response of the enzymes to guanidine hydrochloride denaturation. Multiple freeze-thaw cycles did not result in a significant decrease of enzyme activity (data not shown), but exposure of the enzymes to 1.6 M guanidine hydrochloride for 80 min on ice reduced the activity of the unmodified protein to about 50% (Figure 5B). The mutated proteins were reduced in their activity to about 40%, which could indicate that they are more susceptible to denaturation. The glycoform in which both glycans were missing (P-Pat-1-2) showed in several independent experiments a slightly higher stability than the other mutants (Figure 5B).

Different Glycomutants Are Faithfully Targeted to the Leaf Vacuole

The signals responsible for the subcellular targeting of proteins following the ER-Golgi pathway in higher plants have not yet been identified. Glycoresidues represent potential candidates for these signals, especially when taking into account that in mammalian systems mannose 6-phosphate moieties direct glycosylated proteins into lysosomes (Sly and Fischer, 1982). Wild-type patatin has been shown previously to be compartmented in vacuoles in both potato tubers and transgenic tobacco (Sonnewald et al., 1989a, 1989b). To answer the question of whether the glycan moieties attached to Asn^{60} and/or Asn^{90} are needed for the correct targeting of patatin to the vacuole, we analyzed the subcellular localization of the different glycoforms by immunocytochemical methods. Leaves of the transgenic tobacco were fixed by high-pressure freezing, subsequently freeze substituted, and embedded in LR-White resin. Thin sections were labeled with anti-patatin protein-specific antibodies, followed by goat anti-rabbit immunoglobulin G coupled to 15-nm colloidal gold. As a control, we used untransformed tobacco leaves. Figure 6 shows that, in all cases, the only organelle showing specific labeling was the vacuole, indicating that the proteins were transported correctly to vacuoles in a glycan-independent manner. No labeling was obtained with thin sections of leaves from nontransformed control tobacco showing the specificity of the reaction (cf. Figure 6a). The vacuolar localization of the different glycoforms was also independently demonstrated by the biochemical isolation of tobacco leaf vacuoles (data not shown).

Construction of a Patatin Mutant Protein Missing the Signal Peptide and Its Transfer and Expression in Seeds of Transgenic Tobacco Plants

The set of experiments described above was performed to test the role of N-glycosylation with respect to various characteristics of the patatin protein. No significant change in the parameters tested was observed. One result of these experiments was that the subcellular location of the various patatin forms, i.e., the vacuole, was not dependent upon the glycosylation status of the protein. One possible explanation for the failure to observe any change in the various patatin glycomutants could be that not so much the glycosylation, but rather its final subcellular location (i.e., the vacuole, which is characterized by an acidic pH) and/or the way the patatin protein is synthesized (i.e., being imported into the lumen of the ER in a co-translational way) would influence protein folding and, thus, the various characteristics tested.

To answer these questions, a mutant of the patatin protein was constructed in which the signal sequence was deleted using a synthetic 40-base oligonucleotide (cf. Figure 7 and Methods). The mutagenesis changed the amino terminus of the mature protein from NH_2-Thr-Leu-Gly to NH_2-Met-Ala-Leu-Gly. Because the patatin protein contains a lipid acyl hydrolase activity that might be deleterious for normal plant cell development due to a possible destruction of internal membranes, the coding region of the mutated (P-ΔPat) as well as the wild-type (P-Pat) gene was placed behind the 5′-upstream sequence of the β-phaseolin promoter (Hoffman et al., 1987), which should result in the expression of these chimeric genes only in seeds of transgenic tobacco.

After mobilization into *A. tumefaciens*, the constructs were used to transform tobacco (cf. Methods). Regenerated plants were tested for the presence of the chimeric genes by DNA gel blotting, and only those containing intact, nonrearranged copies of the P-Pat and P-ΔPat constructs were used for further analysis.

Deletion of the Signal Peptide Leads to the Accumulation of Large Amounts of Patatin Protein in the Cytosol

Transgenic tobacco plants harboring either the wild-type patatin gene or the mutant gene devoid of the region encoding the signal peptide developed normally and did not differ from wild-type during the seed-setting phase. With respect to the fact that patatin encodes a lipid acyl hydrolase activity, this could mean either that the activity of this protein while expressed in the cytoplasm is not deleterious to the various membranes of the plant cell, or that no protein accumulates in the cytosol, and/or that this protein is inactive.

To test these possibilities, seed extracts from transgenic tobacco plants were analyzed by protein gel blotting experiments. A representative result is shown in Figure 8. No significant difference in the amount of patatin protein accumulating in the cytosol (lane 4) or in the seed vacuoles (lanes 1 to 3) was detectable, although, again, a substantial variation in the amount of protein accumulating in different

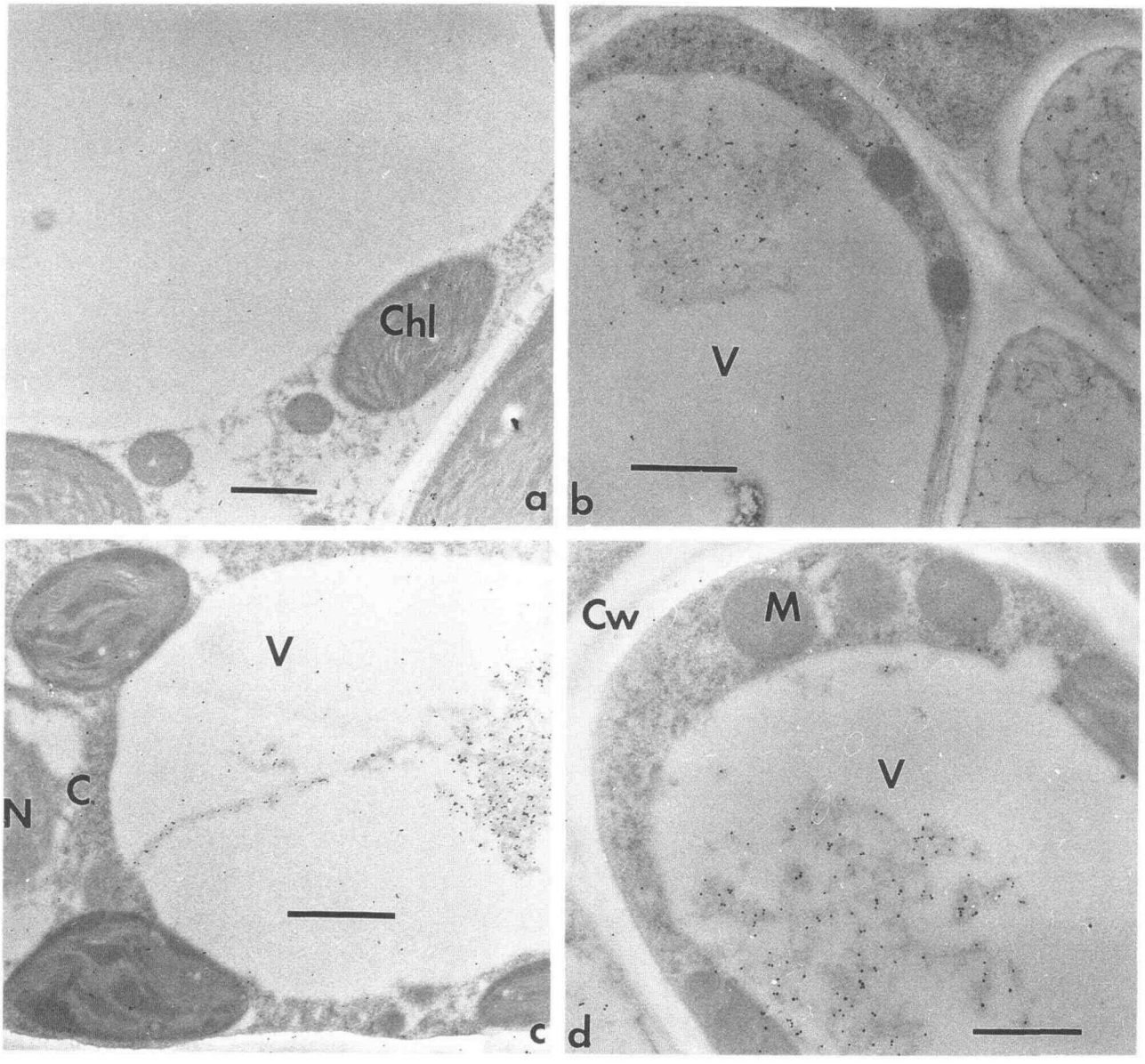

Figure 6. Immunocytochemical Localization of the Different Glycomutants in Vacuoles of Transgenic Tobacco Leaves.

(a) Thin sections from untransformed tobacco were incubated with anti-patatin, protein-specific antibodies, followed by goat anti-rabbit immunoglobulin G coupled to 15 nm colloidal gold.
(b) Same as **(a)** but for a P-Pat-1 expressing transgenic tobacco plants.
(c) Same as **(a)** but for a P-Pat-2 expressing transgenic tobacco plants.
(d) Same as **(a)** but for a P-Pat-1-2 expressing transgenic tobacco plants.
C, cytosol; Chl, chloroplast; Cw, cell wall; M, mitochondria; N, nucleus; V, vacuole. Bars = 1 μm. Magnification: **(a)** ×10,680; **(b)** to **(d)** ×13,350.

independent transformants of each of the constructs was observed. Figure 8 also shows that the P-ΔPat-derived protein displayed a lower molecular weight in SDS-PAGE when compared with the wild-type P-Pat-derived protein, which is in accordance with the assumption that the P-ΔPat protein remains in the cytosol of developing seeds, thus being inaccessible for N-glycosylation. In contrast, the wild-type protein lost its signal peptide but gained two glycans, thus resulting in an increase in molecular weight.

To verify this indirect conclusion concerning the subcel-

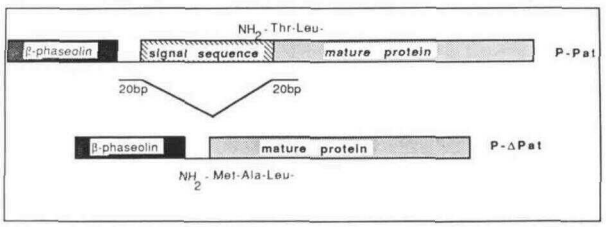

Figure 7. Construction of a Patatin Mutant Devoid of the Signal Sequence.

A 40-base oligonucleotide, overlapping 20 bases on each site of the signal sequence, was used to delete the signal sequence. As a result of the mutagenesis, the amino terminus of the mature protein was changed from NH_2-Thr-Leu to NH_2-Met-Ala-Leu. β-Phaseolin, promoter of the β-phaseolin gene.

lular location of the proteins, protein bodies were isolated from seeds of transgenic tobacco plants and assayed for the presence of the patatin protein. The purity of the protein bodies was determined by measuring the α-mannosidase activity (data not shown). Whereas the wild-type patatin protein accumulated to high levels in protein bodies (cf. Figure 4, lanes 1 to 4), the cytosolic form P-ΔPat was not detectable in protein bodies (cf. Figure 9, lanes 5 to 8). Lane 8 of Figure 9 appears to contain less patatin than lane 6 of Figure 9, which seemingly contradicts our interpretation that the wild-type patatin was enriched in the protein bodies. We believe this was due to a lower amount of total protein loaded [standardized according to Bradford (1976)]. Most importantly, however, in the extract from the protein bodies (Figure 9, lane 8), the relative amount of the wild-type patatin (P-Pat) and the mutant patatin devoid of the signal peptide (P-ΔPat) was inverse to lane 6 of Figure 9, proving the enrichment of the wild-type patatin in protein bodies.

These results, therefore, demonstrate that the patatin protein accumulates to the same level in the cytosol of transgenic seeds as compared with the protein bodies or vacuoles. A close inspection of Figure 8 shows that, in the case of the wild-type patatin present in seeds, numerous additional bands are detectable by the anti-patatin, protein-specific antibody that are missing in the case of the cytoplasmic form and that have also not been detected in the various vacuolar forms (cf. Figure 2).

As described above, seed setting of the tobacco plants expressing the cytoplasmic form of patatin was indistinguishable from those plants harboring the vacuolar form. Similarly, no change with regard to germination frequencies was observed (data not shown).

Concerning the lipid acyl hydrolase activity of the patatin protein, these results are unexpected and could most easily be explained by assuming that the cytoplasmic form of the patatin protein had lost its enzymatic activity and/or displayed a changed activity profile. To this end, cytosolic and vacuolar patatin forms were isolated from transgenic tobacco seeds and their activity on various *p*-nitrophenyl fatty acid esters was determined. No change in the substrate specificity was observed (data not shown), meaning that both forms are active on 10-carbon chain and 16-carbon chain acyl esters.

DISCUSSION

The gene of one member of the patatin protein family was modified by oligonucleotide-directed mutagenesis with respect to its potential N-glycosylation sites, thus allowing us to study the function of individual glycan moieties in a "wild-type" background with respect to the cellular environment.

Elimination of the first, the second, or both glycosylation sites resulted in the synthesis of the expected glycoforms when the mutated genes were expressed in transgenic tobacco leaves. Furthermore, deglycosylation experiments with either endoglycosidase H or trifluoromethanesulfonic acid showed that, in the absence of one glycan moiety, the remaining glycan was further modified to a complex glycan (data not shown). Thus, the structure of the glycan

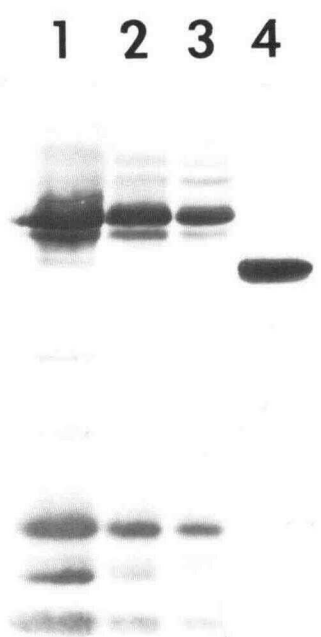

Figure 8. Immunodetection of the Vacuolar P-Pat (Lanes 1 to 3) and the Cytosolic Form P-ΔPat (Lane 4) of Patatin in Seeds of Transgenic Tobacco Leaves.

Protein extracts (50 μg) isolated from transgenic tobacco seeds were separated with 12.5% SDS-PAGE, and patatin was visualized using anti-patatin, protein-specific antibodies.

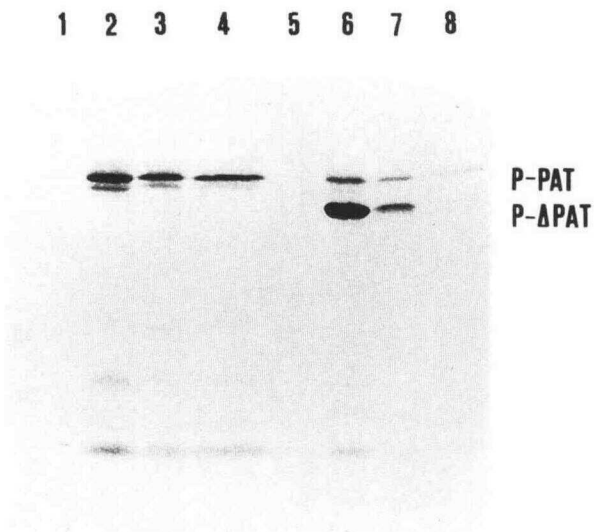

Figure 9. Immunodetection of the Vacuolar (P-Pat) and Cytosolic (P-ΔPat) Forms of Patatin in Protein Bodies Isolated from Transgenic Tobacco Seeds.

Protein bodies were isolated from transgenic tobacco [P-Pat, lanes 1 to 4; P-Pat/P-ΔPat (transgenic plant containing both the cytosolic and vacuolar form), lanes 5 to 8] seeds on a glycerol/potassium iodide gradient according to Begbie (1979). The protein body-containing fraction was determined by measuring the α-mannosidase activity. Fifty micrograms of protein of each fraction were separated with 12.5% SDS-PAGE, and patatin was visualized using anti-patatin, protein-specific antibodies. Lanes 1 and 5, lipid fraction; lanes 2 and 6, loaded fraction; lanes 3 and 7, interphase between the loading fraction and solution A; lanes 4 and 8, interphase between solution A and B containing the protein bodies.

residue attached was not changed in the different mutants. No evidence for the use of the third potential N-glycosylation site of the patatin protein has been obtained. Similar experiments performed in mammalian systems also showed no "alternative" N-glycosylation in the case of mutants in which some of the sites had been removed.

One characteristic change in the properties of certain viral (Machamer et al., 1985) or mammalian (Matzuk and Boime, 1988) proteins containing fewer glycan moieties was an increased susceptibility to degradation by proteases. Analysis of the different patatin glycoforms demonstrated that elimination of N-glycosylation did not increase the sensitivity of patatin to degradation by proteases either in vivo or in vitro. These results imply that not the glycosylation status but rather the folding of the patatin polypeptide was responsible for its stability. Besides the protection of glycoproteins from proteolytic breakdown, glycan moieties often directly influence the hydrophobicity and solubility of proteins and are involved in stabilizing the active conformation of the polypeptide. Thus, the solubility of IgA and IgE is highly reduced after inhibition of N-glycosylation with tunicamycin, leading to aggregation and accumulation of the proteins in the endoplasmic reticulum (Hickman et al., 1977). Along the same line, yeast invertase was shown to be more sensitive to multiple freeze-thawing cycles and guanidine hydrochloride treatment after deglycosylation (Chu et al., 1978), indicating that the glycans stabilize the active conformation of yeast invertase. In the case of patatin, however, neither the hydrophobicity, solubility, nor the sensitivity to multiple freeze-thawing cycles and guanidine hydrochloride treatment is significantly changed in the different glycomutants.

Patatin displays a lipid acyl hydrolase activity that allowed us to test the possible importance of glycan residues in this activity. As outlined in Results, no change with respect to either the substrate specificity, the specific activity, or the pH dependence of the lipid acyl hydrolase was observed for the glycomutants.

The glycan residues are also not necessary for targeting the patatin protein to its intracellular destination, i.e., the vacuole. Thus, although plant vacuoles and mammalian lysosomes are similar in their protein content (Van der Wilden et al., 1980), glycosylation does not seem to be required for the targeting of glycoproteins to plant vacuoles.

As a conclusion with regard to the importance of N-glycosylation for the patatin protein, our experiments did not allow us to detect significant changes in connection with any of the parameters tested. Although it is difficult to imagine that this energy-consuming modification is performed by the plant cell without any obvious influence on the phenotype of the protein, this finding is supported by the result of a similar study performed with a gene from bean encoding phytohemagglutinin (Voelker et al. 1989).

Compartmentation of different proteins and enzymatic activities into different subcellular organelles is a means for the cell to separate catabolic from biosynthetic pathways and to protect itself against deleterious activities, such as degrading enzymes mainly located in the vacuole. Because patatin displays a lipid acyl hydrolase activity that would confer to the definition of a deleterious enzyme, it was of interest to express the patatin protein in the cytosol. Furthermore, because the cytosol differs from the vacuole with respect to different parameters, such as pH and proteases, this experiment was also expected to allow a further insight into parameters important for the stability and activity of the patatin protein.

By deleting the signal peptide, a patatin mutant was constructed that no longer could be synthesized on membrane-bound ribosomes connected to its co-translational import into the endoplasmic reticulum but that had to be synthesized on "free" ribosomes. Again, no significant differences between this cytoplasmic protein and the wild-type form were observed. This observation allows several conclusions. First, because N-glycosylation was strictly connected to transport of the protein into the ER, the

cytoplasmic patatin represented another glycan-less mutant. As in case of the mutants transported in the ER, where one or two amino acids had to be changed at the N-glycosylation sites, one could argue that the different mutants are not comparable. This argument is weakened by the fact that the glycan-free cytoplasmic form of patatin also did not differ from the wild-type protein, although, in this case, the amino acids at the potential N-glycosylation sites were left unchanged.

The second conclusion arising from the analysis of the cytoplasmic patatin is that the co-translational translocation of patatin across the membrane of the endoplasmic reticulum was not required for its correct folding, oligomerization, and enzymatic activity. Although we were able to demonstrate that the protein was active in the cytosol, we were not able to find any deleterious effect exerted on the transgenic plants. The same held true when the cytosolic patatin was expressed under the control of the constitutive cauliflower mosaic virus 35S promoter. In all tested organs (flowers, leaves, stems, and roots), the protein was found to accumulate in the cytosol, but no phenotypical change of the transgenic plant was observed (U. Sonnewald, unpublished observation), a finding that is of interest with respect to the cellular role of patatin. Although the total amount of protein immunologically cross-reacting with patatin antibody was similar for both the cytoplasmic and the vacuolar (protein body) form, the extract isolated from protein bodies showed numerous bands of lower molecular weight that were not detected in either the cytoplasmic or the vacuolar form present in leaf vacuoles (cf. Figure 2 and Figure 7). Obviously, the appearance of these bands signalled the appearance of new proteolytic activities specific for the seed protein bodies.

METHODS

Plant Material

Tobacco (*Nicotiana tabacum* cv Wisconsin 38) plants were grown in tissue culture on Murashige and Skoog (1962) medium. Transformation of tobacco was done using the *Agrobacterium tumefaciens* system (Otten and Schilperoort, 1978).

Oligonucleotide-Directed Mutagenesis

Mutagenesis was performed by following the protocol of the manufacturer (Amersham, Buckinghamshire, U.K.) according to the Eckstein method (Taylor et al., 1985; Nakamaye and Eckstein, 1986). Synthetic oligonucleotides (30-mer and 40-mer) were synthesized on an Applied Biosystems DNA Synthesizer (380A). To confirm that the constructions were correct, all mutants were sequenced by the dideoxynucleotide chain-termination method (Sanger et al., 1977).

Protein Purification

Patatin protein was isolated from transgenic tobacco essentially as described by Racusen and Foote (1980), with the modifications of Sonnewald et al. (1989a). Protein extraction from tobacco seeds was done according to Voelker et al. (1989). The protein concentrations were determined by the method of Bradford (1976), with BSA as a standard.

Immunoblot Analysis

Proteins were separated on 12.5% SDS-polyacrylamide slab gels (Laemmli, 1970) and subsequently transferred to nitrocellulose using a semi-dry electroblotting apparatus (Multiphor II; LKB, Bromma, Sweden). Immunoblotting was done according to Rosahl et al. (1987).

Tryptic Digestion

One hundred micrograms of protein from tobacco leaves were incubated with 50 μg of trypsin (treated with *N*-tosyl-L-phenylalanine chloromethyl ketone) in 25 mM Tris-HCl, pH 7.5, 0.1% SDS (w/v) for the indicated time on ice. The digestion was terminated by the addition of 2 × sample buffer [20% glycerol (v/v), 10% 2-mercaptoethanol (v/v), 125 mM Tris-HCl, pH 6.8, 4% SDS (w/v), 0.002% bromphenol blue (w/v)] containing 4 mM phenylmethylsulfonyl fluoride and subsequent heating for 3 min at 95°C. The degradation of patatin was visualized by immunoblot analysis.

Enzyme Assay

Esterase activity was determined according to Andrews et al. (1988). As substrates, *p*-nitrophenyl fatty acid esters having carbon chain lengths from 2 to 18 (Sigma) were used. The reaction was followed at 405 nm and the activity calculated as [ΔA_{405}/10 min]. The relative activity of patatin on a specific substrate was determined as described by Höfgen and Willmitzer (1989). The patatin content was determined by scanning Coomassie Blue-stained SDS-polyacrylamide gels using a laser densitometer (LKB, Ultroscan XL).

In Situ Esterase Staining

Proteins were separated on a 12.5% SDS-polyacrylamide gel under nondenaturing conditions, and the esterase activity was determined by the subsequent addition of α-naphthyl acetate (in ethanol) and fast blue RR (Sigma) according to Rosahl et al. (1987).

Isolation of Protein Bodies

Protein bodies were isolated from transgenic seeds following the method of Begbie (1979) with the modifications of Sturm et al. (1988). The purity of the protein bodies was determined by measuring the α-mannosidase activity according to Van der Wilden et al. (1980).

Immunocytochemistry

Cryofixation by high-pressure freezing and freeze substitution was performed according to Studer et al. (1989). Embedding of the fixed material in LR-White resin (PolyScience Corp.) and immunogold staining of silver-colored thin sections were done as described previously (Sonnewald et al., 1989).

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