Sorting of Phaseolin to the Vacuole Is Saturable and Requires a Short C-Terminal Peptide

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Phaseolin, one of the major legume proteins for human nutrition, is a trimeric glycoprotein of the 7S class that accumulates in the protein storage vacuoles of common bean. Phaseolin is cotranslationally introduced into the lumen of the endoplasmic reticulum; from there, it is transported through the Golgi complex to the storage vacuoles. Phaseolin is also transported to the vacuole in vegetative tissues of transgenic plants. By transient and permanent expression in tobacco leaf cells, we show here that vacuolar sorting of phaseolin is saturable and that saturation leads to Golgi-mediated secretion from the cell. A mutated phaseolin, in which the four C-terminal residues (Ala, Phe, Val, and Tyr) were deleted, efficiently formed trimers but was secreted entirely outside of the cells in transgenic tobacco leaves, indicating that the deleted sequence contains information necessary for interactions with the saturable vacuolar sorting machinery. In the apoplast, the secreted phaseolin remained intact; this is similar to what occurs to wild-type phaseolin in bean storage vacuoles, whereas in vegetative vacuoles of transgenic plants, the storage protein is fragmented.

INTRODUCTION

Phaseolin is the major storage protein of common bean. Phaseolin is a member of the 7S vicilin class and one of the most important legume proteins for human nutrition; a number of efforts have been made to improve its nutritional value (Hoffman et al., 1988; Dyer et al., 1995). The structure, genetic makeup, cotranslational and post-translational modifications, and intracellular transport of phaseolin have been elucidated largely by numerous investigators (Bollini et al., 1982; Slightom et al., 1985; Sturm et al., 1987; Lawrence et al., 1994), but the mechanisms that allow correct intracellular targeting of phaseolin and the other 7S storage proteins have not been fully characterized.

Phaseolin is a homotrimeric soluble protein that accumulates in the protein storage vacuoles of cotyledonary cells. Its synthesis, maturation, and intracellular targeting are mediated by the secretory pathway, which delivers proteins into the endoplasmic reticulum (ER) and from there to the cell surface or the vacuoles (Okita and Rogers, 1996). The Golgi complex as well as other intermediate compartments mediate this traffic.

Protein constructs that have a transient signal peptide for cotranslational insertion into the ER, but no other specific sorting signal, are secreted from plant cells (Denecke et al., 1990; Hunt and Chrispeels, 1991). Soluble proteins destined for the different vacuoles are sorted from the proteins destined for the apoplast, probably at the exit of the Golgi complex (Ahmed et al., 1997; Paris et al., 1997). Sorting occurs because vacuolar proteins have structures, often identified as short stretches of amino acids in propeptides, that are not present in apoplastic proteins. The signals are variable, and different vacuolar sorting mechanisms must exist (Matsuoka et al., 1995; Kirsch et al., 1996). A putative integral membrane receptor that recognizes some but not all of these stretches in vitro has been identified (Kirsch et al., 1996; Ahmed et al., 1997).

By expressing different phaseolin constructs, we show here that although phaseolin synthesized in cells of transgenic tobacco leaves is exclusively vacuolar, vacuolar targeting of this protein can be saturated at high transient expression levels in the same cell types, resulting in the Golgi-mediated secretion of the protein. We also show that removal of the four C-terminal residues (Ala, Phe, Val, and Tyr) allows correct trimer formation but causes phaseolin to be entirely secreted outside of the cells in transgenic tobacco leaves and that this C-terminal sequence is most probably exposed on the surface of wild-type phaseolin trimers. Wild-type phaseolin is fragmented in vegetative vacuoles of transgenic plants (Murai et al., 1983; Sengupta-Gopalan et al., 1985; Bagga et al., 1992; Pedrazzini et al., 1997), whereas the mutated construct secreted into the

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The apoplast is not subjected to fragmentation. Our results are consistent with the presence of a saturable vacuolar sorting mechanism that recognizes a discrete signal located at the C terminus of phaseolin. In transgenic tobacco leaves that express wild-type phaseolin, this mechanism operates close to its saturation limit.

RESULTS

Assembly-Competent Phaseolin Is Partially Secreted from Transiently Transformed Protoplasts

This study was performed using the mutated phaseolin T343F and other mutants constructed in a T343F background. Figure 1 shows the sequence of wild-type β-phaseolin and of the mutated constructs used in this work (from amino acid 341 to the C terminus); we have not introduced any mutation in the preceding sequence. In T343F, we inactivated the second of the two N-glycosylation sites of a wild-type β-phaseolin sequence; this mutated phaseolin is therefore glycosylated with a single glycan (Pedrazzini et al., 1997). The mutation has no negative effect on the assembly and intracellular transport of phaseolin (Pedrazzini et al., 1997), and indeed in wild-type phaseolin, the second glycosylation site is a “weak” site, which is glycosylated only in some of the molecules. The advantage of using T343F rather than wild-type phaseolin resides in the fact that modifications of the single glycan by enzymes located in the Golgi complex can be used to trace the intracellular transport of the protein (Sturm et al., 1987).

While examining the intracellular transport of mutated phaseolin polypeptides, which we produced to study the relationships between trimerization and intracellular traffic, we observed that assembly-competent polypeptides, but not assembly-defective ones, could be detected in part in the incubation medium of transiently transformed tobacco protoplasts. This is shown in Figure 2A. Tobacco mesophyll protoplasts were transiently transformed with plasmid without inserts, as a control, or with plasmid carrying the sequence encoding T343F or T343F Δ360 (hereafter referred to as Δ360). The latter is an assembly-defective deletion mutant of phaseolin (Figure 1; Pedrazzini et al., 1997). Protoplasts were subjected to pulse–chase labeling with 35S-labeled methionine and cysteine. Finally, we analyzed phaseolin by SDS-PAGE and fluorography after immunoprecipitation from cell homogenates or incubation media.

The polypeptides detected when the control plasmid without inserts was used represent nonspecific contaminants recognized by the antiserum. Of these, the major immunoprecipitable contaminant is a polypeptide of 40 kD (Figure 2A, lanes 1 and 2); the synthesis of this polypeptide must be induced largely by the stress imposed by transient transformation, because there was almost no material at 40 kD when immunoprecipitations were performed using untreated protoplasts from transgenic leaves (Figure 2B).

After a 1-hr pulse, T343F synthesized during transient expression was detectable as an abundant band of 46 kD and less abundant fragments of 20 to 25 kD (Figure 2A, lane 3). After a 5-hr chase, a relevant proportion of intact phaseolin was converted into the smaller fragments (Figure 2A, cf. lanes 3 and 4). Post-translational fragmentation is a characteristic of phaseolin expressed in heterologous plants (Bagga et al., 1992), although in bean cotyledons, the storage protein does not undergo such fragmentation. Fragmentation is a result of transport to the heterologous vacuoles (Pedrazzini et al., 1997). A relevant amount of T343F phaseolin was also recovered unfragmented from the incubation medium, where it accumulated during the chase (Figure 2A, lanes 9 and 10). After a 5-hr chase, secreted T343F represented ~25% of the amount synthesized during the pulse and almost no intracellular intact phaseolin remained, suggesting that the rest had been targeted to the vacuole. The overall amount of T343F recovered after the chase in this and similar experiments was lower than that recovered after the pulse, most probably because of full degradation of a proportion of phaseolin in the vacuole.

Assembly-defective forms of phaseolin that remain monomeric are unable to be transported along the secretory pathway in transgenic plants (Pedrazzini et al., 1997). During transient expression, the assembly-defective construct Δ360,
Symbols are as given in parentheses; the open arrowhead indicates intact T343F; the filled arrowhead indicates phaseolin antiserum, and analyzed by SDS-PAGE and fluorography.

We have produced transgenic plants constitutively expressing T343F (Pedrazzini et al., 1997). As shown in Figure 2B, mesophyll protoplasts isolated from these plants do not secrete phaseolin to detectable amounts, suggesting fully efficient vacuolar sorting.

To confirm that in intact mesophyll tissue the location of T343F is exclusively vacuolar and not extracellular, we analyzed leaf tissue by immunoelectron microscopy. T343F was detected exclusively in the vacuolar lumen in large electron-dense protein bodies (Figure 3D) that could be decorated by the anti-phaseolin antiserum (Figures 3A and 3B) but not by the preimmune serum (Figure 3C). Similar protein bodies were not found in cells of tobacco transformed with the plasmid without an insert (data not shown). No labeling above the background was detectable in the cell wall or the intercellular spaces (Figures 3A and 3B). From the results shown in Figures 2B and 3, we conclude that in transgenic leaves, T343F phaseolin is efficiently targeted to the vacuoles, where it is fragmented and forms protein bodies and is not secreted at detectable levels.
Figure 3. In Transgenic Tobacco Leaf Cells, T343F Is Located in Vacuoles.

(A) to (C) Thin sections of transgenic tobacco leaves expressing T343F were treated with anti-phaseolin antiserum ([A] and [B]) or preimmune serum (C), and the bound antibodies were visualized by using goat anti-rabbit 15-nm gold complex.

(D) A lower magnification of an untreated sample.

CH, chloroplast; CW, cell wall; IS, intercellular space; PB, protein bodies formed by phaseolin; V, vacuole. Bars = 1 μm.
To determine whether any of the fragments contain the glycan, we performed radioactive labeling of protoplasts in the presence of tunicamycin, a fungal inhibitor of N-glycosylation. In the presence of the inhibitor, there was a marked decrease in the apparent molecular mass of the faster migrating phaseolin fragment, indicating that in normal conditions, this fragment is glycosylated, whereas the two slower migrating ones are not (Figure 4, cf. lanes 7 and 8). Therefore, in tobacco, as in bean cotyledons, the glycan of singly glycosylated phaseolin acquires a complex structure along the route to the vacuole. Secreted T343F was almost completely resistant to endoglycosidase H (Figure 4, cf. lanes 1 and 2). The proportion of resistant polypeptides was much higher in secreted T343F than in the intracellular T343F and was followed by 5 hr of chase. At the end of the chase, phaseolin was immunoselected from the cell homogenates and analyzed by SDS-PAGE and fluorography. Only the portion of the gel relative to the phaseolin fragments is shown.

**Phaseolin Is Secreted Only When Expressed at High Levels**

If secretion of phaseolin results from saturation of the vacuolar targeting mechanism, we expected a reduction in secretion when the level of expression was lowered. As a test, we transformed protoplasts with different amounts of plasmid (Figure 5). In our standard transformation protocol, we use 40 μg of plasmid to transform 10^6 protoplasts. This leads to secretion levels of T343F that vary from 20 to 50% after 5 hr of chase, using different preparations of protoplasts. Figure 5 shows that when 2 μg was used, secretion was below our limit of detection, although phaseolin was still sorted to the vacuole, as indicated by the accumulation of fragmentation products during the chase. When 20 μg of plasmid was used to transform the same preparation of protoplasts, expression of T343F was six times higher and part of the protein was secreted (Figure 5).

Therefore, the secretion of phaseolin is dose dependent, and it occurs when protoplasts are transiently transformed, but it does not occur when protoplasts are isolated from transgenic plants and are not subjected to transient transformation. This could indicate two possibilities: (1) the stress imposed on the cells by the transient transformation procedure has the side effect of altering the capacity of the vacuolar sorting mechanism; and (2) secretion is truly caused by the sudden burst of phaseolin expression, which saturates...
the normal vacuolar sorting capacity of the cells. To distinguish between these possibilities, we supertransformed protoplasts isolated from transgenic plants expressing T343F with the plasmid containing the same construct or with the plasmid without insert as a control. Transfection with the control plasmid did not change the destiny of phaseolin, whereas the overexpression of phaseolin resulted in secretion (Figure 6, lanes 7 and 8; cf. with lanes 5 and 6). The very small amount of intact phaseolin recovered in the medium after the pulse shown in lane 5 represents contamination from a small proportion of broken cells; consistently, there is no secreted phaseolin after the chase shown in lane 6.

The average overexpression of T343F as a result of the supertransformation was ~2.5-fold in the experiment shown in Figure 6. However, overexpression is probably much higher in the cells receiving the transfected DNA, because these are usually a small proportion of the total (Denecke et al., 1989). Secretion of the extra amount of phaseolin synthesized because of the transient transformation was almost quantitative. The amount of fragmentation products accumulated intracellularly after the chase was very similar in control-transformed and supertransformed protoplasts (Figure 6, cf. lanes 2 and 4). These results indicate that secretion is the result of saturation of the sorting machinery and that the mechanism that targets phaseolin to the vacuole is very close to its saturation in the transgenic plants.

**Deletion of the C-Terminal Sequence Ala–Phe–Val–Tyr Causes Complete Secretion of Phaseolin in Transgenic Tobacco Leaves**

The results presented above suggest that the vacuolar sorting of phaseolin occurs because of recognition events that are saturable. This is consistent with the presence of a receptor mechanism that recognizes specific structural features of phaseolin. Signals for sorting to the plant vacuoles have been identified on several proteins. There is not a unique consensus; however, a high percentage of hydrophobic residues is a characteristic of some of the known C-terminal vacuolar sorting signals (Bednarek et al., 1990; Neuhaus et al., 1991; Saalbach et al., 1996). When analyzing the phaseolin sequence, we observed that there was an enrichment in hydrophobic amino acids at its C terminus: Lys is followed by the sequence Gly–Ala–Phe–Val–Tyr (Figure 1). Therefore, we tested whether the hydrophobic C-terminal end of phaseolin contains information necessary for vacuolar targeting. We introduced a stop codon after residue 417 of T343F to generate T343F Δ418 (hereafter referred to as Δ418; Figure 1). Thus, Δ418 lacks the last four amino acid residues, Ala–Phe–Val–Tyr, which form a highly hydrophobic sequence. We produced transgenic tobacco plants expressing Δ418 under the control of the cauliflower mosaic virus 35S promoter.

We isolated total proteins from transgenic leaves and analyzed them by SDS-PAGE, which was followed by immuno-blot analysis with the anti-phaseolin antiserum. The results are shown in Figure 7A. Plants expressing Δ418 accumulated a major phaseolin polypeptide with an M, of ~45,000 and a minor one with a slightly lower relative molecular mass. These polypeptides are of the relative mass expected for intact phaseolin and may represent the glycosylated and unglycosylated forms of the protein. Immunoblot analysis of an extract from tobacco leaves expressing T343F is also shown in Figure 7A for comparison. Clearly, the typical phaseolin fragmentation products present when the protein is synthesized in transgenic plants are absent in Δ418-expressing plants.

Because the fragments in the 20- to 25-kD range are formed when phaseolin is targeted to the tobacco vacuoles, we investigated whether Δ418 accumulates in the apoplast instead of being delivered to vacuoles. We were able to visualize Δ418 and T343F on silver-stained SDS-PAGE gels, after immunoprecipitation with the anti-phaseolin antiserum (Figure 7B). When phaseolin was immunoprecipitated from total leaf homogenates, Δ418 and the fragmentation products of T343F were clearly detectable (Figure 7B, lanes 1...
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and 2; there was insufficient intact T343F to be detected, indicating that its proportion with respect to the T343F fragments was overestimated by immunoblot analysis. However, when phaseolin was immunoprecipitated from leaf protoplast preparations, Δ418 was absent, whereas the vacuolar fragments of T343F were, as expected, still present (Figure 7B, lanes 3 and 4). This indicates that Δ418 accumulates in the apoplast.

To study the trafficking of Δ418, we subjected protoplasts isolated from Δ418 transgenic leaves, or from T343F transgenic leaves as a control, to pulse-chase analysis, followed by immunoprecipitation with the anti-phaseolin antisemur, SDS-PAGE, and fluorography (Figure 7C). During the chase, Δ418 was secreted from the cells and accumulated in the extracellular medium; no fragmentation products were detectable. A very small amount of fragmentation products became visible in the intracellular Δ418 immunoprecipitates upon extremely long film exposures, indicating that a very small proportion of the mutated phaseolin can reach the vacuoles and that the protein can be fragmented. This proportion of mutated phaseolin, however, constitutes much less than 1% of total immunoprecipitable phaseolin (data not shown).

The C Terminus of Phaseolin Is Exposed on the Surface of the Protein

The fact that Δ418 is secreted could be due to different causes. (1) The last four amino acids are an essential part of the vacuolar sorting signal for phaseolin, and their deletion directly abolishes recognition by the sorting machinery. And (2), removal of the four residues causes defects to the overall structure of phaseolin, and secretion could then be due to a wrong conformation of the phaseolin molecules; these could be no longer recognized by the vacuolar sorting machinery, because of an altered surface exposure of a sorting signal constituted by amino acids other than the deleted ones. The three-dimensional structure of phaseolin has been established in large part (Lawrence et al., 1990, 1994); however, no information is available for the last 22 amino acids at the C terminus or for a few other segments of the protein.

We devised experiments to assess the oligomerization state of Δ418 phaseolin and the exposure of the C terminus of T343F phaseolin, thereby attempting to distinguish between the two possibilities mentioned above. We transiently transformed protoplasts with plasmids encoding T343F, Δ418, or Δ360 and subjected them to 1 hr of pulse-labeling. We then loaded the cell homogenates onto a 5 to 25% sucrose sedimentation velocity gradient. Phaseolin was then immunoprecipitated from each gradient fraction (Figure 8). The position of Δ418 along the gradient matched the position of the trimeric, transport-competent T343F exactly. In Figure 8, the assembly-defective mutant Δ360 (Pedrazzini et al., 1997) marked the position of phaseolin monomers along
The gradient. We thus conclude that Δ418 is competent for rapid and efficient trimerization and indistinguishable by velocity centrifugation from normal T343F phaseolin. Therefore, the absence of the four C-terminal residues does not alter the oligomeric state of phaseolin.

Secretion of T343F during transient expression is accompanied by a late and slight decrease in molecular weight. This can barely be seen when comparing lanes 9 and 10 of Figure 2A or lanes 5 and 6 of Figure 5, but it becomes evident when SDS-PAGE is performed for longer periods of time, allowing more pronounced separation of the different polypeptides, as shown in Figure 9, lanes 1 and 2. When synthesis was allowed to occur in the presence of the inhibitor of N-glycosylation tunicamycin, the time-dependent decrease in the molecular weight of secreted T343F was still evident, indicating that it is not due to glycan trimming (Figure 9, lanes 3 and 4). However, secreted Δ418 did not undergo any decrease in molecular weight during the chase (Figure 9, cf. lanes 5 and 6, and 7 and 8). This strongly suggests that secreted T343F undergoes removal of a few amino acids at the C terminus and that therefore the C-terminal tetrapeptide required for vacuolar sorting is exposed on the surface of the assembled protein. By using SDS-PAGE analysis, we could not establish whether intracellular T343F deposited into the vacuoles also undergoes this processing, because of the major fragmentation event that accompanies vacuolar deposition.

The results presented in Figures 8 and 9 suggest the existence of a direct interaction between the C terminus of phaseolin and the vacuolar sorting mechanism.

**DISCUSSION**

We have shown here that vacuolar sorting of phaseolin in tobacco leaf cells is very efficient in transgenic plants but can be saturated when high levels of phaseolin are expressed in transiently transformed protoplasts. Saturation leads to Golgi-mediated secretion of the excess of protein. We have also shown that deletion of the last four C-terminal amino acid residues abolishes sorting of phaseolin to the vacuole and leads to its complete secretion. Therefore, vacuolar sorting of phaseolin, a storage protein of the 7S (vicilin) class, is most likely mediated by a saturable receptor system that recognizes a signal located at the C terminus of its ligand protein.

**Vacuolar Sorting of Phaseolin**

In the transgenic tobacco plants that we produced, T343F phaseolin was not secreted to detectable levels but almost saturated the vacuolar sorting mechanism: no additional vacuolar phaseolin was detectable upon transient overexpression, and excess phaseolin was secreted. It is indeed possible that these transgenic plants have already slowly adapted to handle the excess of vacuolar protein that they are synthesizing. A further increase in the synthesis of phaseolin could require further adaptation that is not achieved during the short transient expression experiments. This would be a possible explanation for the otherwise fortunate coincidence between the level of phaseolin synthesis and the capacity of its sorting mechanism in transgenic tobacco leaves.

A saturable targeting mechanism is consistent with it being receptor mediated. In yeast, the receptor-mediated vacuolar targeting of proteinase A and carboxypeptidase Y is saturable: when expressed at artificially high levels, these two proteins are secreted (Rothman et al., 1986; Stevens et al., 1986). A putative receptor that recognizes some but not all plant vacuolar targeting signals in vitro has been identified in pea, but it is not known whether this receptor recognizes phaseolin (Kirsch et al., 1996; Paris et al., 1997).

**Figure 8.** Δ418 Is Trimeric.

Tobacco protoplasts were transiently transfected with plasmids encoding T343F, Δ418, or Δ360 and pulse-labeled for 1 hr with 35S-methionine and 35S-cysteine. Cell homogenates were subjected to sedimentation velocity fractionation on a continuous 5 to 25% (w/v) sucrose gradient. Gradient fractions were subjected to immunoprecipitation with the anti-phaseolin antiserum and analyzed by SDS-PAGE and fluorography. The top of the gradients is at left. Only the portions of the gels containing phaseolin are shown.

**Figure 9.** Secreted T343F, but Not Δ418, Undergoes Post-Translational Proteolytic Processing.

Tobacco protoplasts were transiently transfected with plasmids encoding T343F or Δ418 and subjected to pulse-chase in the presence (+) of tunicamycin (Tm) or in the absence (−) of the inhibitor. Pulse was for 1 hr with 35S-methionine and 35S-cysteine, and chase was for 0 or 5 hr. The incubation media were then immunoprecipitated with the anti-phaseolin antiserum and analyzed by SDS-PAGE and fluorography. Note the increase in mobility of T343F, but not of Δ418, between the pulse and the chase.
Transient expression in tobacco protoplasts has been used to study vacuolar sorting of other plant proteins. Targeting of the wild-type forms of barley lectin (Bednarek et al., 1990) and Brazil nut 2S albumin (Saalbach et al., 1996) was not saturated, but recombinant vacuolar chitinase of tobacco showed partial secretion that was directly proportional to its level of expression (Neuhaus et al., 1994). Because more than one targeting mechanism exists for plant vacuoles (Matsuoka et al., 1995), it is possible that not all of them are saturated at similar levels of protein synthesis; the different plasmids used for transfection may also lead to different expression levels, but saturability is clearly not unique to phaseolin.

The saturation of the vacuolar targeting of phaseolin suggests either that a component of the sorting machinery is not sufficiently abundant to handle the number of phaseolin molecules synthesized or that above a certain concentration, phaseolin undergoes a change in conformation that inhibits recognition by the sorting machinery. Electron microscopy shows that in tobacco leaf vacuoles, as in bean storage vacuoles, phaseolin forms protein bodies, probably because of the acidic environment. Currently, however, we have no evidence that upon transient expression, similar structures or aggregates of phaseolin are formed during its transport before being deposited into the vacuole: we were unable to detect any phaseolin aggregates by velocity gradient centrifugation, and the proteolytic trimming of secreted T343F phaseolin strongly suggests that the sequence necessary for its vacuolar sorting remains available for interactions with the sorting machinery.

Further support of this hypothesis comes from digestion of secreted T343F phaseolin with endoglycosidase H, which allowed us to determine that in plant cells, secretion of an overexpressed vacuolar protein occurs via the Golgi-mediated pathway and not via an alternative route. The fact that the vast majority of secreted T343F phaseolin molecules are resistant to endoglycosidase H digestion also indicates that their glycan is accessible to Golgi-processing enzymes: this confirms that at the time of passage through at least some of the Golgi stacks, most of the phaseolin that will be secreted is not aggregated. Therefore, if secretion is due to aggregation, this is a late and transient event during the trafficking of phaseolin, and the aggregates are easily disrupted during analysis. Therefore, we suggest that secretion is instead due to insufficient abundance of the sorting machinery.

The fact that the glycan of T343F acquires a complex structure both in secreted phaseolin and in phaseolin deposited into the vacuole has implications for the definition of the routes that lead to the two different locations as well. Analogous to the targeting to the lytic compartments of other eukaryotes, it is assumed that sorting of vacuolar proteins from secretion occurs in the trans-Golgi network, but this has not been proven directly. Because in plant cells N-linked glycans acquire complex structures in the medial and trans-Golgi complex (Fitchette-Lainé et al., 1994), our data indicate that the two routes do not fork before at least the cis- and medial Golgi complex stacks have been visited. This confirms the previous observation that complex glycans are present both on secreted and on vacuolar plant proteins (Faye et al., 1989; Vitale and Bollini, 1995) and extends it to the synthesis of an individual protein in cells from a single tissue.

### The Sorting Signal

The sequence Ala–Phe–Val–Tyr is uncharged and mainly hydrophobic. Phaseolin is the product of a small gene family, and the tetrapeptide is conserved in both α and β polypeptides (Slightom et al., 1985). Short propeptides at the C terminus are known to determine the vacuolar sorting of tobacco chitinase (seven amino acids; Neuhaus et al., 1991) and Brazil nut 2S albumin (four amino acids; Saalbach et al., 1996). A longer C-terminal propeptide (15 amino acids) contains the vacuolar sorting signal of barley lectin (Bednarek et al., 1990). A vacuolar sorting consensus sequence cannot be derived, but these propeptides are also rich in hydrophobic amino acids. Mutational analysis revealed the importance of hydrophobic residues in barley lectin (Dombrowski et al., 1993) but not in chitinase (Neuhaus et al., 1994). It should also be considered that in the native plants, the 7S and 25S storage proteins and barley lectin accumulate in storage vacuoles, whereas chitinase is located in vegetative vacuoles. Transport from the Golgi complex to the two types of vacuoles involves different structures (Chrispeels, 1983; Hohl et al., 1996). Eventually, in mature mesophyll cells, the two types of vacuoles probably merge (Schroeder et al., 1993; Paris et al., 1996), but it is possible that the mechanisms recognizing the C-terminal vacuolar sorting signals are more than one and are distinct for vegetative and storage proteins.

When T343F is secreted during transient expression, the C terminus is most probably trimmed by hydrolases that also are secreted by the protoplasts, indicating that it is exposed on the surface of phaseolin trimers. Whether this trimming also occurs upon delivery to the vacuole cannot be established by SDS-PAGE analysis, because vacuolar phaseolin is fragmented in tobacco cells. However, phaseolin accumulated in the storage vacuoles of bean cotyledonary cells lacks a few amino acids with respect to the ER-associated precursor (Bollini et al., 1982; D’Amico et al., 1992). The N-terminal sequence of phaseolin purified from bean cotyledons is the one predicted after signal peptide removal, indicating that trimming must be at the C terminus (Paaren et al., 1987). Therefore, the Ala–Phe–Val–Tyr sequence probably constitutes or is part of a short transient peptide and is most likely exposed on the surface of phaseolin trimers. This makes the sequence a good candidate for direct interactions with the vacuolar sorting machinery and does not favor the alternative hypothesis that deletion of the sequence affects the overall conformation of phaseolin and inhibits recognition by the sorting machinery only as an indirect effect.
Secretion is a safe haven for phaseolin: indeed, Δ418 accumulates in its intact form. When wild-type phaseolin is expressed in transgenic plants, the default targeting to the vacuole leads to its proteolytic cleavage in all tissues tested, with the only exception being rice endosperm (Murai et al., 1983; Sengupta-Gopalan et al., 1985; Bagga et al., 1992; Zheng et al., 1995; Pedrazzini et al., 1997). Comparisons of the amounts of T343F and Δ418 accumulated in leaves are beyond the scope of this work, but our data suggest that secretion could be a very effective means to preserve phaseolin from degradation. This could be considered as a more general, useful strategy when planning the expression of heterologous proteins of biotechnological interest in the plant secretory pathway. A strategy that has been successful in enhancing the stability of foreign vacuolar proteins is their accumulation in the ER via the KDEL/HDEL system normally used by soluble ER residents (Wandelt et al., 1992; Tabe et al., 1995; Khan et al., 1996). It would be interesting to compare accumulation in the ER versus the apoplast by using the same passenger proteins.

Vacuolar Sorting and ER Quality Control

The fact that Δ418 phaseolin is not sorted to the vacuole but is secreted means that the assembly-defective phaseolin constructs Δ363 and Δ360 (Pedrazzini et al., 1997), which are the results of more extensive C-terminal deletions, do not possess the vacuolar targeting signal of trimeric phaseolin as well. On the contrary, HiMet phaseolin, a trimeric but unstable mutated phaseolin that is transported to the vacuole and rapidly degraded there, still bears the sequence Ala–Phe–Val–Tyr (Hoffman et al., 1988; Pueyo et al., 1995). Assembly-defective phaseolin is subjected to ER quality control: it is extensively retained in the ER, where it shows prolonged interactions with the chaperone BiP and then is slowly degraded (Pedrazzini et al., 1997). The location where assembly-defective phaseolin is degraded is not known. Possible candidates are a subcompartment of the ER or the cytosol after dislocation from the ER; another candidate is the vacuole, which would be reached by a Golgi-independent pathway (Pedrazzini et al., 1997). The results we have presented here indicate that in whichever compartment degradation occurs, targeting of assembly-defective phaseolin to degradation by quality control does not depend on the signal that normally sorts phaseolin to the hydrolytic compartments of the plant cell.

METHODS

Recombinant DNA

The strategies to construct T343F and Δ360 phaseolin have been described previously (Pedrazzini et al., 1997). To construct Δ418, the antisense oligonucleotide 5′-CTGAGTCACCGTCTTTCTTTCCC-3′ was used in polymerase chain reaction to introduce a stop codon (underlined) after residue 417 in the T343F phaseolin coding sequence. The construct thus lacks the last four residues of wild-type phaseolin (Ala, Phe, Val, and Tyr). Δ418 was introduced into the vector pDHA (Tabe et al., 1995) for transient expression experiments. For constitutive expression in transgenic tobacco, pDHA containing Δ418 was inserted into the HindIII site of the binary vector pGAC70 (An et al., 1985), which was then used to transform Agrobacterium tumefaciens LBA4404 by electroporation.

Production of Transgenic Tobacco Plants and Transient Transformation of Leaf Protoplasts

The transformed agrobacterium was used to produce transgenic tobacco (Nicotiana tabacum cv Petit Avana SR1) plants, as described by Pedrazzini et al. (1997).

Protoplasts were prepared from axenic leaves (4 to 7 cm long) of untransformed tobacco or from transgenic tobacco expressing T343F or Δ418 phaseolin. Protoplasts were subjected to polyethylene glycol-mediated transfection, as described previously (Pedrazzini et al., 1997). Vector pDHA without inserts was used as a negative control for transfection. Unless otherwise stated, 40 μg of plasmid was used to transform 10⁶ protoplasts in 1 mL. After transfection, protoplasts were allowed to recover overnight in the dark at 25°C in K3 medium (Pedrazzini et al., 1997), at a concentration of 10⁶ cells/mL, before performing pulse–chase experiments.

Pulse–Chase Labeling of Protoplasts and Analysis of Phaseolin

Pulse-chase labeling of protoplasts using Pro-Mix (a mixture of 35S-methionine and 35S-cysteine; Amersham) was performed as described previously (Pedrazzini et al., 1997). In some experiments, before radioactive labeling, protoplasts were incubated for 45 min at 25°C in K3 medium supplemented with 50 μg/mL tunicamycin (stock solution 5 mg/mL in 10 mM NaOH, stored at 4°C; Boehringer Mannheim) and maintained in the presence of the inhibitor for the entire experiment. At the desired pulse–chase time points, 3 volumes of W5 medium (154 mM NaCl, 5 mM KCl, 125 mM CaCl₂·2H₂O, and 5 mM glucose) was added, and the samples were centrifuged for 5 min at 50g. The supernatant, containing the proteins secreted into the incubation medium, was removed, leaving 100 μL to cover the protoplasts. The supernatant and protoplasts were frozen separately in liquid nitrogen and stored at −80°C. Homogenization of the frozen samples and immunoprecipitation of phaseolin using rabbit antiserum raised against phaseolin purified from bean cotyledons were performed as described previously (Pedrazzini et al., 1997). Unless otherwise stated, for each pulse–chase time point, immunoprecipitation was performed using cell homogenate and incubation medium from the same amount of protoplasts. Digestion of immunoprecipitated proteins with endoglycosidase H (recombinant; Boehringer Mannheim) was performed as described previously (Ceriotti et al., 1991).

For the analysis of phaseolin assembly by sedimentation velocity, after radioactive labeling and homogenization, homogenates were brought to 8 mM MgCl₂ and 3 mM ATP and loaded on top of a continuous 5 to 25% [w/v] linear sucrose gradient made in 150 mM NaCl, 1 mM EDTA, 0.1% Triton X-100, and 50 mM Tris–Cl, pH 7.5. Samples were centrifuged at 39,000 rpm in an SW40 Ti rotor (Beckman Instruments, Inc., Fullerton, CA) for 20 hr at 20°C. Phaseolin was then immunoselected from each gradient fraction.
Vacuolar Sorting of Phaseolin

SDS-PAGE analysis was always performed on 15% acrylamide gels. Rainbow 14C-methylated proteins (Amersham) were used as molecular weight markers. Gels were treated with 2,5-diphenyloxazole dissolved in dimethyl sulfoxide and radioactive polypeptides revealed by fluorography. Quantification of the relative intensities of bands in the fluorographs was performed by microdensitometry, using a Camag TLC Scanner II (Camag, Muttenz, Switzerland). Care was taken to use film exposures that were in the linear range of film darkening.

Total Leaf Protein Extraction and Protein Gel Blot Analysis

For analysis of total proteins, leaves from transformed plants were homogenized in an ice-cold mortar with 0.2 M NaCl, 1 mM EDTA, 2% β-mercaptoethanol, 0.2% Triton X-100, and 0.1 M Tris-Cl, pH 7.8, supplemented with Complete (Boehringer Mannheim) protease inhibitor cocktail. The buffer/tissue ratio was 6:1 (milliliters per gram fresh weight of tissue). The homogenate was centrifuged at 1500 g for 4°C for 10 min, and the supernatant was separated by SDS-PAGE. Immunodetection was performed using the enhanced chemiluminescence (ECL) system (Amersham), following the manufacturer’s protocol. Proteins were detected using the anti-phaseolin antisera.

To determine whether Δ418 phaseolin is located intracellularly or extracellularly, we immunoselected protoplasts or leaf extracts from transgenic plants with the anti-phaseolin antisera, and the selected material was analyzed by SDS-PAGE followed by silver staining.

Immunomicroscopy

Leaf fragments (1 to 2 mm) were fixed in 0.8% glutaraldehyde and 3.3% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, at 4°C for 2 hr and in 1% OsO4 in the same buffer for 2 hr. They were then dehydrated in an ethanol series and embedded in Spurr’s resin (Spurr, 1969). Thin sections were etched with 1% aqueous periodic acid and hydrated in an ethanol series and embedded in Spurr’s resin (Spurr, 1969). Thin sections were etched with 1% aqueous periodic acid and hydrated in an ethanol series and embedded in Spurr’s resin (Spurr, 1969). Thin sections were etched with 1% aqueous periodic acid and hydrated in an ethanol series and embedded in Spurr’s resin (Spurr, 1969). Thin sections were etched with 1% aqueous periodic acid and hydrated in an ethanol series and embedded in Spurr’s resin (Spurr, 1969).

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REFERENCES


Sorting of Phaseolin to the Vacuole Is Saturable and Requires a Short C-Terminal Peptide
Lorenzo Frigerio, Maddalena de Virgilio, Alessandra Prada, Franco Faoro and Alessandro Vitale
Plant Cell 1998;10:1031-1042
DOI 10.1105/tpc.10.6.1031

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References
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