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Different Legumin Protein Domains Act as Vacuolar Targeting Signals

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Legumin subunits are synthesized as precursor polypeptides and are transported into protein storage vacuoles in field bean cotyledons. We expressed a legumin subunit in yeast and found that in these cells it is also transported into the vacuoles. To elucidate vacuolar targeting information, we constructed gene fusions of different legumin propolypeptide segments with either yeast invertase or chloramphenicol acetyltransferase as reporters for analysis in yeast or plant cells, respectively. In yeast, increasing the length of the amino-terminal segment increased the portion of invertase directed to the vacuole. Only the complete legumin α chain (281 amino acids) directed over 90% to the vacuole. A short carboxy-terminal legumin segment (76 amino acids) fused to the carboxy terminus of invertase also efficiently targeted this fusion product to yeast vacuoles. With amino-terminal legumin-chloramphenicol acetyltransferase fusions expressed in tobacco seeds, efficient vacuolar targeting was obtained only with the complete α chain. We conclude that legumin contains multiple targeting information, probably formed by higher structures of relatively long peptide sequences.

INTRODUCTION

The secretory system is used to transport proteins via the endoplasmic reticulum (ER) and the Golgi apparatus to the cell surface or to the vacuole/lysosome (Schekman, 1985; Jones and Robinson, 1989; Chrispeels, 1991). Translocation across the ER membrane is mediated by an amino-terminal signal sequence (Perara and Lingappa, 1988). Secretion occurs by default, whereas positive sorting information is required for transport to the vacuole (Kelly, 1985; Pfeffer and Rothman, 1987; Wieland et al., 1987; Dorel et al., 1989).

In mammalian cells, transport to the lysosomes is mediated by mannose-6-phosphate (von Figura and Hasilik, 1986), but in yeast and plant systems, glycans do not act as vacuolar sorting signals (Schwaiger et al., 1982; Voelker et al., 1989). In the yeast hydrolases carboxypeptidase Y (CPY) (Johnson et al., 1987; Valls et al., 1987) and proteinase A (Klionsky et al., 1988), vacuolar sorting information resides in short amino acid sequences at the amino terminus. In plants, Tague et al. (1990) found that the 43 amino-terminal amino acids of mature phytohemagglutinin (PHA) from the common bean are also sufficient to sort invertase to the yeast vacuole. A set of four contiguous amino acids (QRPL) in the CPY signal was identified as critical for vacuolar localization (Valls et al., 1990). For PHA, the sequence LQR was also found to be important (Tague et al., 1990). However, the proteinase A sorting signal does not resemble the CPY targeting element and the PHA LQR sequence is only partially conserved among lectins. Changes in this region in native PHA do not result in higher levels of secretion, indicating the presence of multiple targeting information in PHA.

The study of PHA-invertase fusions in *Arabidopsis* showed that the PHA segments that target invertase to yeast vacuoles are not sufficient for transport to plant vacuoles, indicating that differences exist between yeast and plant vacuolar sorting processes (Chrispeels, 1991). In a different lectin from barley, a carboxy-terminal propeptide domain of 15 amino acids is necessary for targeting to the vacuoles of tobacco cells (Bednarek et al., 1990).

Here we report vacuolar sorting results obtained from the analysis of the 11S globulin legumin, the major field bean seed protein that accumulates in cotyledon cell storage vacuoles. This protein contains six similar subunits, each composed of two disulfide-linked chains arising from a common precursor (Bassüner et al., 1983; Horstmann, 1983). An amino-terminal signal sequence mediates the cotranslational insertion into the lumen of the ER (Bassüner et al., 1984) and the propolypeptide is then transported by way of the Golgi apparatus (Zur Nieden et al., 1984) into the storage vacuoles. In the ER, 11S propolypeptides are assembled into trimers, whereas hexamers are formed only in the storage vacuoles (for review, see Akazawa and Hara-Nishimura, 1985). The 11S globulins do not contain any sites for *N*-linked glycosylation (Bäumlein et al., 1986;

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Schlesier et al., 1990) and no other form of glycosylation could be detected (Croy et al., 1979; Hurkman and Beevers, 1980).

We expressed in yeast a native legumin propolypeptide gene and gene fusions between different amino-terminal and carboxy-terminal segments of that gene (Bäumlein et al., 1986) and the *SUC2* yeast invertase gene (Taussig and Carlson, 1983). We demonstrated that legumin is transported to the yeast vacuoles and that vacuolar targeting information resides both in long amino-terminal and short carboxy-terminal segments. In addition, fusions of amino-terminal legumin segments with chloramphenicol acetyltransferase (CAT) were expressed in tobacco seeds. In plant cells, long amino-terminal segments are also necessary for targeting to vacuoles. However, our results also show that multiple targeting information occurs in the legumin protein and that this information is contained in complex structures rather than in distinct short amino acid sequences.

RESULTS

Expression of Legumin in Yeast

Restriction fragments of a genomic legumin clone *LeB4* (Bäumlein et al., 1986) and a corresponding cDNA clone B273 (G. Saalbach, unpublished results) encoding a legumin subunit of the B type (Horstmann, 1983) were combined to obtain a complete legumin gene without introns. A SalI restriction site was generated by in vitro mutagenesis 6 bp in front of the ATG start codon, and a SalI-SphI fragment comprising the legumin coding sequence plus 6 bp 5′-untranslated and 143 bp 3′-untranslated sequence was inserted into the yeast plasmid pAAH5 (Ammerer, 1983) where the gene is under the control of alcohol dehydrogenase 1 (*ADH1*) regulatory sequences.

In addition to the original legumin gene, a legumin gene modified by a frameshift starting 36 codons upstream of the stop codon (Saalbach et al., 1988) was used. In the corresponding polypeptide, the 36 carboxy-terminal amino acids would be substituted by a different piece of 52 amino acids.

The plasmids were transformed into yeast, and SDS extracts from these strains were analyzed in protein gel blotting experiments. Figure 1 shows that both the original and the modified proteins could be detected in yeast cells. The intact legumin accumulated to a level of approximately 0.5% of total yeast proteins, but the amount of the modified protein was reduced. Only very low levels of legumin could be detected in the periplasmic space and the culture medium. Estimates from protein gel blots revealed that less than 1% of the expressed legumin was secreted from the yeast cells (data not shown). In yeast, the legumin precursor is not cleaved into the α and β chains.

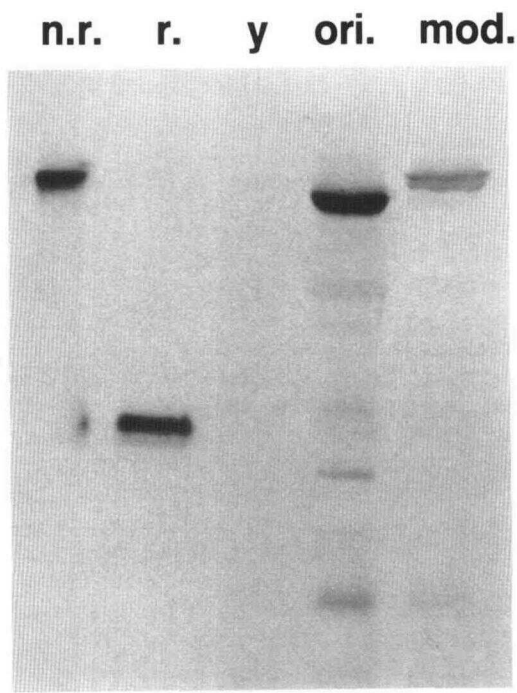

Figure 1. Protein Gel Blot Showing the Expression of Legumin in Yeast.

Lanes 1 and 2, native legumin as marker, nonreduced (n.r.), reduced (r.; the band in this lane represents the α chain, the β chain is not recognized by the antibodies used); lane 3 (y), extract from wild-type yeast as control; lanes 4 and 5, extracts from transformed yeast strains harboring the original (ori.) and the modified (mod.) legumin genes, respectively.

Legumin Is Transported to Yeast Vacuoles

Immunocytochemistry and vacuole isolation procedures were used to detect the intracellular localization of legumin in yeast. Thin sections for electron microscopy were treated with legumin antibodies (gift of R. Manteuffel, Gatersleben) and protein A-gold. The very clear labeling of vacuoles, as shown in Figure 2, indicates efficient targeting of legumin to yeast vacuoles.

The modified legumin could be detected in vacuoles isolated on a discontinuous Ficoll gradient (Stevens et al., 1982). The vacuole fraction and the corresponding total lysate were assayed for α-mannosidase activity (vacuolar marker) and both samples were adjusted to equal α-mannosidase concentration. The modified legumin was detected in these samples by protein gel blotting. Equal legumin band intensities were observed on the blot shown in Figure 3, allowing the conclusion that this modified legumin was also efficiently transported to the yeast vacuole.

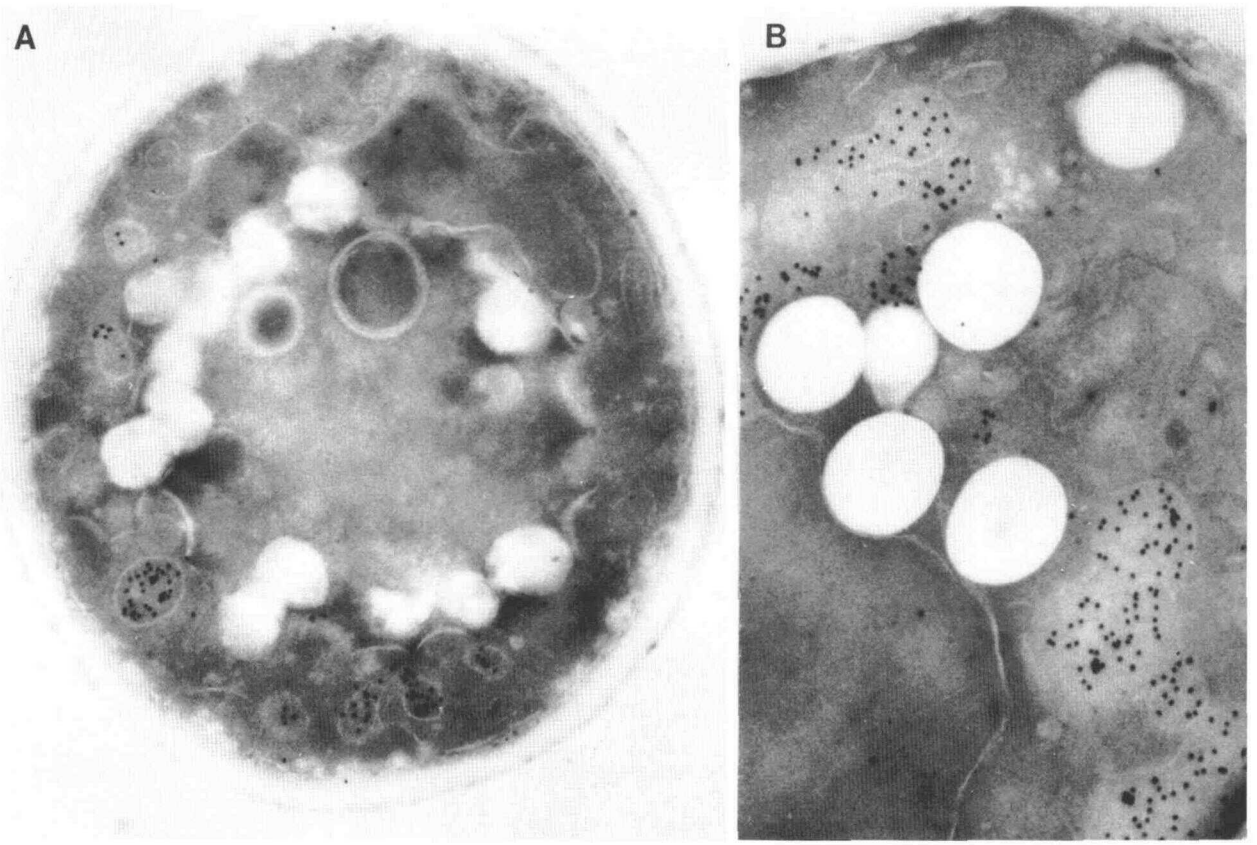

Figure 2. Immunocytochemical Localization of Legumin in Vacuoles of Transgenic Yeast.

Gold particles label the vacuoles very clearly and are completely absent from all other cell compartments.
(A) Magnification ×19,200.
(B) Magnification ×24,400.

Construction of Legumin-Invertase, Invertase-Legumin, and Legumin- *CAT* Fusion Genes

To determine which sequences in the legumin subunit contain vacuolar targeting information, a series of gene fusions of both amino-terminal and carboxy-terminal segments of legumin with yeast invertase or bacterial *CAT* were constructed, as shown in Figure 4. For the amino-terminal fusions, the intron-free legumin gene was cloned in front of a *SUC2* (invertase) or a *CAT* gene to create legumin-invertase or legumin-*CAT* fusions. Carboxy-terminal portions of legumin were removed by deletion mutagenesis using oligonucleotides 24 to 36 bases long comprising the junction sequences. For the carboxy-terminal fusions, a restriction fragment encoding the carboxy-terminal 76 amino acids of legumin was cloned behind the *SUC2* gene. Sequences from the amino terminus of the legumin fragment were removed by deletion mutagenesis. By using oligonucleotide-directed mutagenesis, every desired fusion could be generated exactly. All fusion sequences were verified by sequence analysis.

Seven carboxy-terminal legumin segments ranging from 13 to 76 amino acids in length were fused to the complete invertase, carrying its own signal sequence (Figure 4C). In the invertase-legumin (Inv-Le-C76-38) fusion, 38 amino acids were deleted from the carboxy-terminal end of Inv-Le-C76.

For expression in yeast, appropriate restriction fragments with only 6-bp untranslated legumin 5′ sequence (see above) or 26-bp untranslated *SUC2* 5′ sequences plus 15-bp linker were inserted into pAAH5, as described above, and transformed into the strain SEY6210 (*suc2-Δ9*).

Legumin-*CAT* fusions were expressed under the control of the strong seed-specific legumin promoter (Bäumlein et al., 1987, 1988) in transgenic tobacco. In addition, several

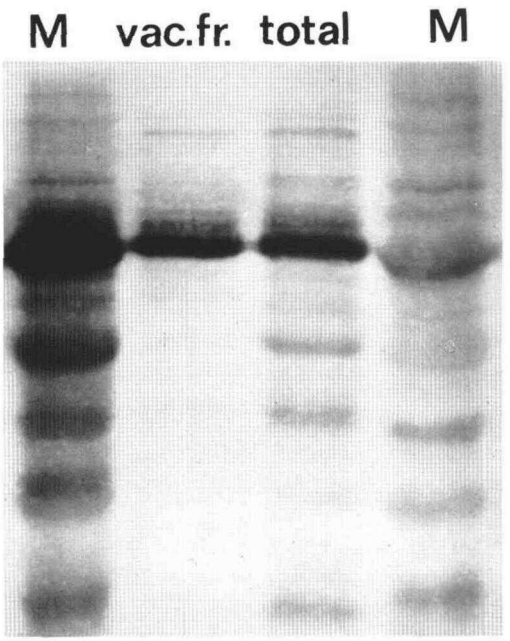

Figure 3. Detection by Immunoblotting of the Modified Legumin in Isolated Yeast Vacuoles.

Left lane (M), extract from yeast cells bearing the modified legumin; right lane (M), extract from untransformed yeast; two middle lanes, samples prepared from isolated vacuoles (vac.fr.) (see Methods) and total spheroplast lysate (total) of yeast cells bearing the modified legumin.

of these fusions were also expressed with the cauliflower mosaic virus (CaMV) 35S promoter in tobacco, and the Le-CAT-462 fusion was expressed in yeast, as described above, to compare the fate of the fusion proteins in the unicellular eukaryote and in different tissues of a higher plant.

Long Amino-Terminal Segments of Legumin Are Necessary To Direct Legumin-Invertase Fusions to Yeast Vacuoles

Results from yeast strains bearing amino-terminal legumin-invertase fusions are summarized in Table 1. The legumin amino acid sequence is shown in Figure 5. We found that efficient secretion occurred when the invertase signal sequence was replaced exactly by the legumin signal sequence. With up to 28 additional amino acids, absolutely no reduction of secretion was observed. Up to 62 additional amino acids retained only low and variable amounts of invertase inside the cells. The segment with an additional 86 amino acids retained 33% of the protein inside the cells. The same result (33% inside the cells) was obtained with

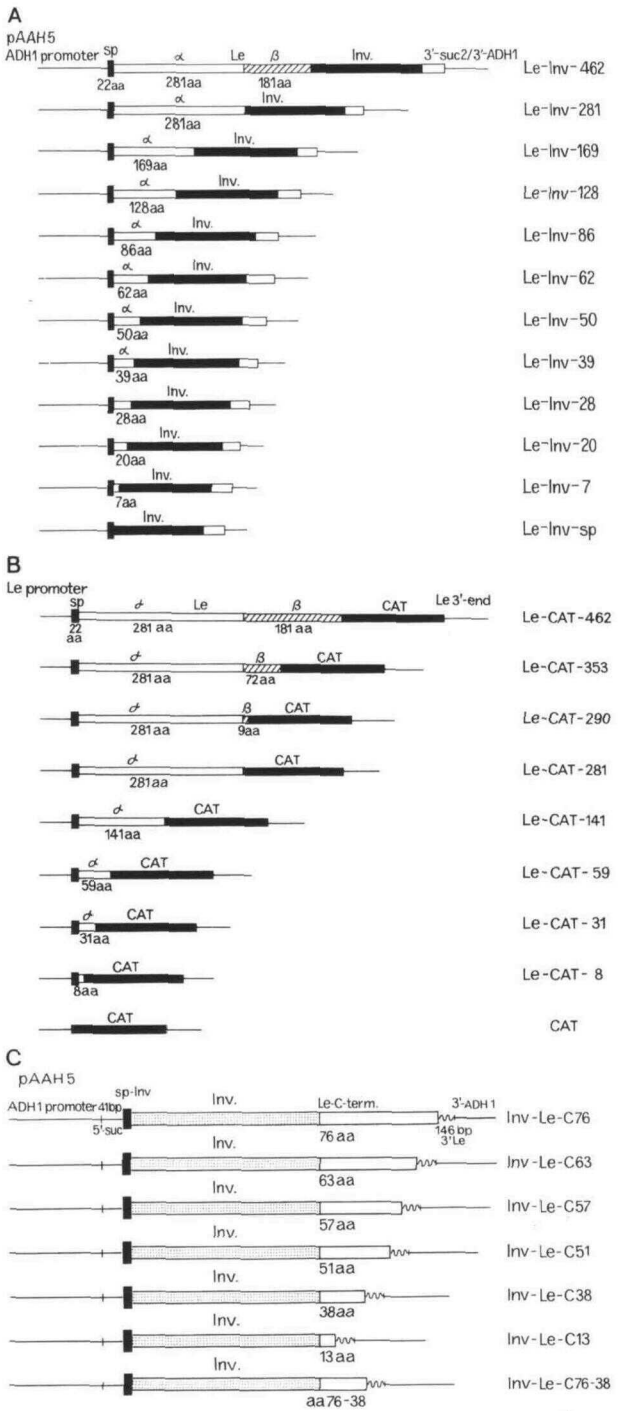

Figure 4. Schematic Representation of the Gene Fusion Constructs Used for the Targeting Experiments in Yeast and Tobacco.

(A) Fusions between amino-terminal legumin segments and yeast invertase.
(B) Fusions between amino-terminal legumin segments and CAT.
(C) Fusions between invertase and carboxy-terminal legumin segments.

Table 1. Invertase Activity Produced by Amino-Terminal Legumin-Invertase Fusions in Yeast

Fusion	Invertase Activity[a]		
	Total	External	% Secretion
Le-Inv-sp	350	350	100
Le-Inv-7	2500	2500	100
Le-Inv-20	1870	1870	100
Le-Inv-28	2000	2000	100
Le-Inv-39	1100	1040	94
Le-Inv-50	670	570	85
Le-Inv-62	1800	1705	95
Le-Inv-86	320	214	66
Le-Inv-128	330	220	66
Le-Inv-169	705	350	50
Le-Inv-281	600	45	7
Le-Inv-462	–[b]		

[a] Invertase activity is in units (nanomoles of glucose per minute at 30°C) per OD_{600} yeast cells.
[b] Fusion unstable in pAAH5.

128 amino acids. A segment with 169 amino acids kept 50% intracellular, and the whole α chain (281 amino acids) was necessary to retain more than 90% inside the yeast cells.

Native legumin is transported to the yeast vacuoles. To determine whether the legumin segments also target the intracellular portions of invertase activity to the vacuoles, we isolated vacuoles from the strain harboring the Le-Inv-86 fusion, retaining 33% of the activity inside the cell. Recovery of vacuoles was calculated from the α-mannosidase (vacuolar marker) activity in the spheroplast lysate and in the vacuole fraction. Contamination with ER or cytoplasma was low (approximately 10%), as determined by assaying the fractions for cytochrome *c* reductase and α-glucosidase, respectively. The recovery of intracellular invertase in the vacuole fraction was calculated from the activities in the whole cells, in the spheroplast supernatant, in the spheroplast lysate, and in the vacuole fraction. Figure 6 shows that the intracellular invertase activity cofractionated with the vacuolar marker α-mannosidase, indicating that the legumin fusions are targeted to the vacuoles.

A Short Carboxy-Terminal Legumin Segment Efficiently Targets Invertase to Yeast Vacuoles

The carboxy-terminal invertase-legumin fusions were analyzed in yeast in the same way as described for the amino-terminal fusions. The results are shown in Table 2. A short segment of 13 amino acids caused practically no reduction of secretion. In the case of the Inv-Le-C38 fusion, the total cellular invertase activity was very low, indicating a structural interaction between a specific legumin segment and invertase, and 28% of that activity was retained inside the cells. With an additional 13 amino acids (Inv-Le-C51), activity was normal and only 17% was retained. Six additional amino acids (Inv-Le-C57) caused an increase in the retention to 43% inside the cells and another 6 amino acids (Inv-Le-C63) increased that value to only 54%. With the addition of the last 76 carboxy-terminal amino acids of legumin (for the sequence, see Figure 5) to invertase, practically complete (93%) retention was achieved. This activity was also transported efficiently to the yeast vacuoles, as shown by vacuole isolation described above (Figure 6). These results indicated that the essential sequence in the carboxy-terminal signal might be located in the amino-terminal part of the 76-amino acid segment. However, deletion of the carboxy-terminal half (Inv-Le-C76-38) resulted in considerable loss of vacuolar transport to only 50%, indicating that the complete segment is necessary to form the signal.

Does the Level of Expression Influence the Secretion/Retention Ratio?

The total invertase activities expressed from the different gene fusions ranged from about 200 units per OD of cells (Inv-Le-C76) to 2500 units per OD of cells (Le-Inv-7). These differences could be due to the influence of the legumin segments on the specific activities of the fusion molecules. In case of CPY-invertase and PHA-invertase fusions, activities were not as variable. Values of approximately 200 to 300 and 350 to 750 units per OD of cells, respectively, were observed (Johnson et al., 1987; Tague et al., 1990). About threefold enhancement of the expression of a PHA-Inv fusion caused an increase in secretion from 10% to 22%, probably because of the saturation of the vacuolar sorting machinery (Tague et al., 1990). To analyze whether

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     TSSEFDRLNQ CRLDNINALE PDHRVESEAG LTETWNPNHP   40
     ELRCAGVSLI RRTIDPNGLH LPSYSPSPQL IYIIQGKGVI   80
     GLTLPGCPQT YQEPRSSQSR QGSRQQQPDS HQKIRRFRKG  120
     DIIAIPSGIP YWTYNNGDEP LVAISLLDTS NIANQLDSTP  160
     RVFYLGGNPE VEFPETQEEQ QERHQQKHSL PVGRRGGQHQ  200
     QEEESEEQKD GNSVLSGFSS EFLAQTFNTE EDTAKRLRSP  240
     RDKRNQIVRV EGGLRIINPE GQQEEEEQEE EEKQRSEQGRN 281

181  GLEETICSLK IRENIAQPAR ADLYNPRAGS ISTANSLTLP
141  ILRYLRLSAE YVRLYRNGIY APHWNINANS LLYVIRGEGR
101  VRIVNSQGNA VFDNKVRKGQ LVVVPQNFVV AEQAGEEEGL
 61  EYLVFKTNDR AAVSHVQQVF RATPADVLAN AFGLRQRQVT
 21  ELKLSGNRGP LVHPQSQSQS N
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Figure 5. Amino Acid Sequence of the Legumin Propolypeptide.

The sequence was derived from a genomic DNA sequence (Bäumlein et al., 1986) and is shown without the signal peptide. The upper part numbered from 1 to 281 (starting at the amino terminus) is the sequence of the α chain. The lower part numbered from 1 to 181 (starting at the carboxy terminus) is the sequence of the β chain.

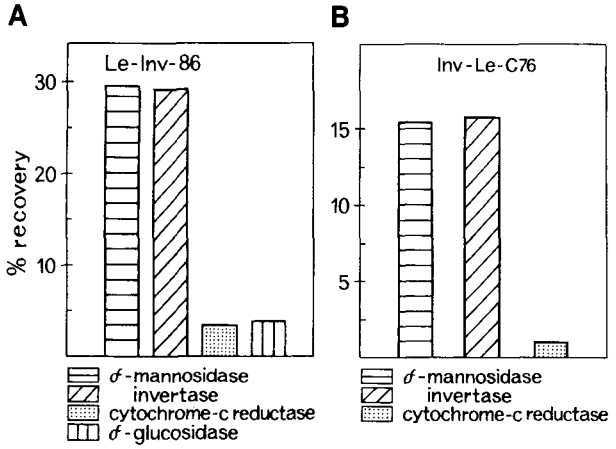

Figure 6. Localization of Legumin-Invertase Fusions in Isolated Yeast Vacuoles.

Vacuoles were isolated (see Methods) and the indicated marker enzyme activities were determined in the total lysate and in the vacuole fraction.
(A) and **(B)** Cofractionation of invertase activity of fusions Le-Inv-86 **(A)** and Inv-Le-C76 **(B)** with the vacuolar marker enzyme α-mannosidase.

the level of expression of our legumin-invertase fusions allowed correct results, we also expressed the fusion Le-Inv-86 at a much lower level by transferring the fusion including *ADH1* promoter and terminator from the multicopy plasmid pAAH5 to the yeast plasmid YCp50 (Sherman et al., 1986). After transformation with this plasmid, only one copy of the fusion gene was present per yeast cell, resulting in a very low expression. With this low activity, variable results were obtained because of background interference ranging from 30 to 70% (average 50%) secretion (data not shown). This result indicated a certain overloading of the vacuolar transport pathway. On the other hand, retention values went up to more than 90% with long legumin segments. In addition, through the use of immunocytochemistry, native legumin expressed with pAAH5 could only be detected in yeast vacuoles, and only trace amounts of legumin were detected in periplasma and medium samples (see above). Together, these results indicated that the conclusions derived from the use of the yeast expression system are essentially correct.

Glycosylation of Invertase Provides Additional Evidence for Passage through the Secretory System

Secretory invertase is highly and heterogeneously glycosylated. This glycosylation occurs in the ER and in the Golgi apparatus. Wild-type yeast cells also produce a cytoplasmic and, therefore, unglycosylated form of invertase (Carlson and Botstein, 1982; Perlman et al., 1982). These two forms can be distinguished easily by their different mobility in a native acrylamide gel (Gabriel and Wang, 1969; Carlson et al., 1981).

All amino-terminal and carboxy-terminal legumin-invertase fusions (Figures 4A and 4C) produced in yeast were analyzed for invertase glycosylation. In all cases, the invertase was highly glycosylated, as illustrated in Figure 7 for some examples. This means that all fusions enter the secretory system and pass the Golgi apparatus, yielding additional evidence for the localization of all intracellularly retained fusions in the vacuole.

Analysis of the Expression of Legumin-CAT Fusions in Plants

Although the legumin propolypeptide (this paper) and PHA (Tague et al., 1990) are sorted into the vacuoles of yeast, it remains to be demonstrated that these cells use the same sorting mechanism and recognize the same targeting signals as the storage tissue cells in developing plant seeds. Field bean, the donor of the legumin gene, still cannot be transformed and regenerated efficiently. Therefore, we have used the tobacco transformation system to verify our results in plants. Like many other seed storage protein genes, the legumin gene used in our study is correctly expressed in tobacco seeds (Bäumlein et al., 1987). It has also been shown that plant vacuolar proteins such as phaseolin and PHA from common bean and the vegetative storage protein patatin from potato are transported to the vacuoles in transgenic tobacco (Greenwood and Chrispeels, 1985; Sturm et al., 1988; Sonnewald et al., 1989).

Legumin-*CAT* gene fusions (Figure 4B) were transformed into tobacco using the Ti plasmid system. Transformation and expression of the gene fusions were verified by DNA and RNA blotting techniques. The RNA gel blot shown in Figure 8 reveals that mRNA is formed from all

Table 2. Invertase Activity Produced by Carboxy-Terminal Legumin-Invertase Fusions in Yeast

	Invertase Activity		
Fusion	Total	External	% Secretion
Inv-Le-C13	900	845	94
Inv-Le-C38	5	3.6	72
Inv-Le-C51	500	415	83
Inv-Le-C57	440	250	57
Inv-Le-C63	550	250	46
Inv-Le-C76	190	13	7
Inv-Le-C76-38	580	290	50

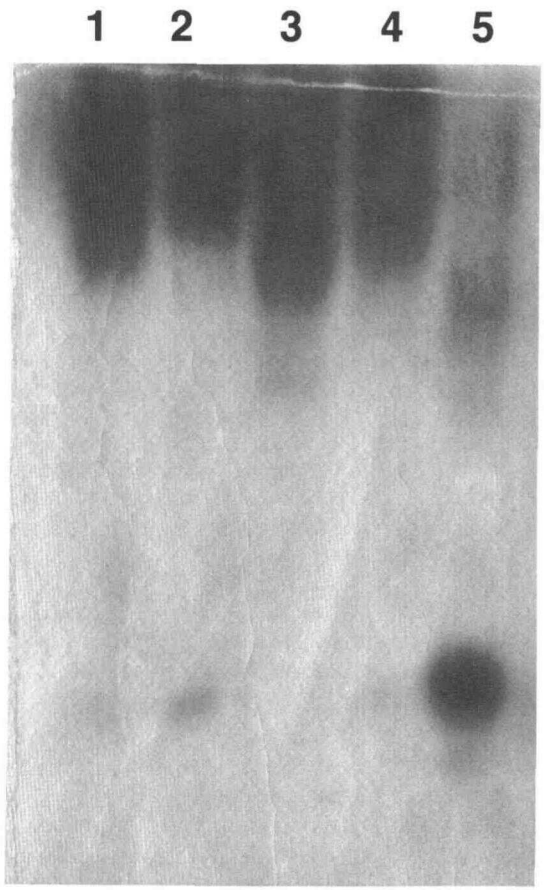

Figure 7. Invertase Activity Gel Demonstrating the Glycosylation of Legumin-Invertase Fusions in Yeast.

Lane 1, wild-type invertase (purchased sample, Boehringer Mannheim) as marker; other lanes show invertase activity of fusions Le-Inv-sp (lane 2), Le-Inv-86 (lane 3); Inv-Le-C76 (lane 4). All invertase fusion proteins are highly glycosylated, as can be seen from comparison with a cytoplasmic unglycosylated invertase activity in lane 5 resulting from a construct with incomplete signal sequence.

transformed fusion genes at about the same steady-state level in seeds when controlled by the legumin promoter and in seeds and leaves when programmed by the CaMV 35S promoter.

Protein level expression was analyzed by the CAT activity assay and by immunoblotting with anti-legumin and anti-CAT antibodies. CAT activity could only be detected in seeds with the fusions Le-CAT-59, Le-CAT-8, and CAT. Four of the fusions without CAT activity were expressed in leaves with the CaMV 35S promoter. Also in this case no CAT activity could be detected. That the long legumin-CAT fusions can in principle exhibit CAT activity was demonstrated by expression of the Le-CAT-462 fusion in yeast. As shown in Figure 9, high CAT activity could be observed.

The results from the CAT activity assays were confirmed by immunoblotting using a CAT antiserum. CAT protein could only be detected in seeds transformed with Le-CAT-59, Le-CAT-8, and CAT (data not shown). Figure 10 shows that by using anti-legumin antibodies, the α chain and a degradation product of legumin could be detected in seeds for fusion constructs Le-CAT-290, Le-CAT-353, and Le-CAT-462. No legumin could be detected with Le-CAT-281. In this fusion, the β chain was removed and substituted exactly by CAT, probably destroying the recognition and cleavage sequences for the α-β cleavage. These results indicated that as long as the proteolytic cleavage sequence is preserved in the fusions (which is obviously the case also with only 9 amino acids of the β chain in the fusion Le-CAT-290), proteolytic cleavage occurs at this site.

It is suggested, therefore, that these fusions are transported into the protein bodies because it is known that specific processing of the propolypeptides of 11S globulin subunits takes place in the protein storage vacuoles (Chrispeels et al., 1982; Hara-Nishimura and Hara-Nishimura, 1987). The β chain-CAT portion is obviously degraded because no CAT activity is present and β chain-specific antibodies do not detect any additional band (data not shown). The occurrence of a degradation product larger in size than the β chain indicates that the α chain is also partially degraded (Figure 10). The shorter the β chain portion in the fusions, the greater is the degradation. In

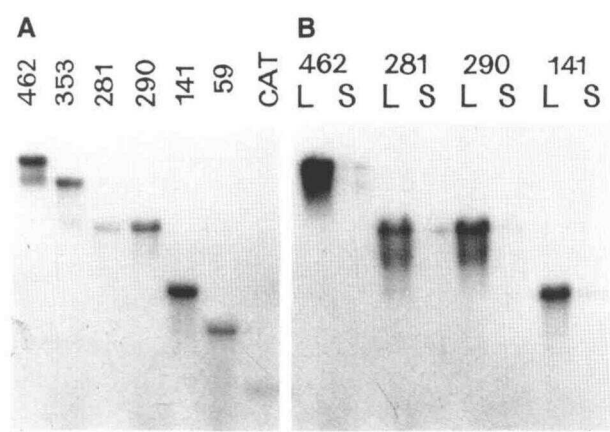

Figure 8. RNA Gel Blot Showing the Expression of Legumin-*CAT* Fusion Genes in Transgenic Tobacco.

Le-CAT fusions are indicated by the number of legumin amino acids.
(A) Expression of the legumin promoter in seeds.
(B) Expression of the CaMV 35S promoter in leaves (L) and seeds (S).

the leaves of the CaMV 35S promoter transformants, no legumin could be detected by immunoblotting, indicating complete degradation of the fusions in the leaf cells where these long fusions are probably also transported to the vacuoles (see Discussion).

Vacuolar Targeting of Legumin-CAT Fusions in Tobacco Seeds

Several fusions were analyzed for their intracellular localization. For this purpose, protein bodies were isolated from tobacco seeds using a potassium iodide step gradient in glycerol (Sturm et al., 1988). The fractions were analyzed either for CAT activity (in the case of the CAT fusions Le-CAT-8 and Le-CAT-59) or for the presence of the legumin α chain and its degradation product (in the case of the fusions Le-CAT-290 and Le-CAT-462). As shown in Figure 9, the CAT activity produced by the three short legumin-CAT fusions could not be found in the protein body fraction, whereas the α chain (in the case of Le-CAT-462) or the degradation product (in the case of Le-CAT-290) was present in the isolated protein bodies, as demonstrated in Figure 10. These results showed that in plants, as in yeast, short amino-terminal segments of legumin are not sufficient for vacuolar targeting, whereas segments as long as the α chain are able to direct the fusions to the vacuoles.

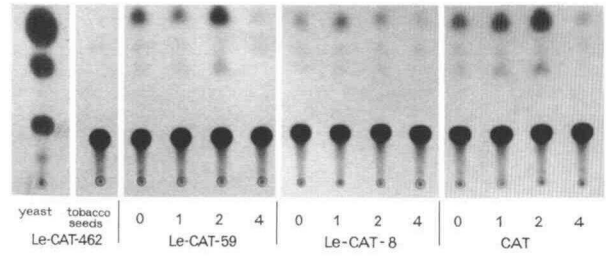

Figure 9. CAT Activity Assay of Cell Fractions from Transgenic Tobacco Seeds.

Left two lanes, comparison of expression of Le-CAT-462 in yeast and tobacco. The fusion with complete legumin yields CAT activity in yeast but not in tobacco seeds. Right three sections, detection of CAT activity in different fractions from the potassium iodide step gradient. Lanes 4 indicate the protein body fraction prepared from seeds expressing the indicated short legumin-CAT fusions; no CAT activity was found in the protein body fractions.

DISCUSSION

Vacuolar Targeting Information in the Legumin Propolypeptide

The aim of this work was to analyze the targeting information of the plant vacuolar protein legumin, the major field bean seed storage protein. We used both yeast and plants for our intracellular transport analysis. Yeast has been used for the investigation of vacuolar targeting information of yeast proteases (Johnson et al., 1987; Valls et al., 1987, 1990; Klionsky et al., 1988) and has been shown to be suitable for the analysis of the plant vacuolar protein PHA (Tague and Chrispeels, 1987; Tague et al., 1990; Chrispeels, 1991).

We expressed native legumin in yeast and found that the legumin subunit precursor accumulates inside the yeast cells. We also demonstrated that this plant vacuolar protein is efficiently transported to yeast vacuoles. To localize the vacuolar targeting information in the legumin propolypeptide, a series of gene fusions between segments of legumin and yeast invertase were constructed and analyzed in yeast. We found that in contrast to the results obtained with yeast proteases and PHA, short amino-terminal segments of legumin up to about 40 amino acids additional to the signal peptide were not able to retain any portion of the fusions inside the cells. The vacuolar portion increased with increasing length of the legumin segment. A segment of 86 additional amino acids targets 33% of the invertase activity to the vacuole and only the whole α chain (281 amino acids) targets more than 90% to the vacuole.

The results obtained with the amino-terminal fusions in yeast were principally confirmed in a plant system. Legumin-CAT fusions were expressed in tobacco leaves and seeds. If the legumin signal peptide plus an additional 8 or 59 amino acids were fused to CAT, it did not reach the vacuoles. The whole legumin (462 amino acids) or the α chain plus 9 amino acids of the β chain allowed for targeting of CAT fusions to the vacuoles, indicating that, as in yeast, short amino-terminal segments of legumin are not sufficient for vacuolar targeting, whereas the α chain is able to direct the fusions to the vacuoles.

The analysis of fusions with carboxy-terminal legumin segments indicated that this region also contains vacuolar targeting information. As in the case of the amino-terminal segments, vacuolar transport increased with increasing length of the carboxy-terminal segments. However, a much shorter segment (76 amino acids) was sufficient for efficient targeting. We conclude from these results that there are at least two regions in the legumin propolypeptide containing vacuolar targeting information and that these regions can act independently of each other.

On the Nature of the Vacuolar Targeting Signal

Interaction of a specific signal and a corresponding receptor is likely for vacuolar targeting of the yeast CPY protein (Valls et al., 1990). The first 30 amino-terminal amino acids

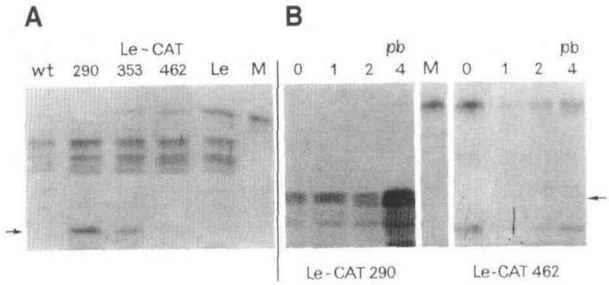

Figure 10. Detection by Immunoblotting of the Legumin Part of Le-CAT Fusions in Transgenic Tobacco Seeds.

(A) Detection of the α chain (upper band) and a degradation product, indicated by the arrow, in seed extracts.
(B) Detection of the α chain (upper band) and a degradation product (arrow) in protein body fractions (pb) isolated from seeds expressing the indicated fusions.
wt, untransformed tobacco; Le, original legumin expressed in tobacco; M, native legumin.

of the CPY propeptide are sufficient and necessary for efficient targeting of CPY-invertase fusions to the vacuole (Johnson et al., 1987). The tetrapeptide QRPL located within this small domain (positions 4 to 8 of proCPY) was found to be critical for targeting (Valls et al., 1990). However, the vacuolar sorting signal of yeast proteinase A (Klionsky et al., 1988) is not similar to the CPY sorting sequence and an element comparable to the QRPL tetrapeptide is not present. This indicates that either different signals exist or the character of the signal is formed by physicochemical and/or structural properties of the targeting element and is not dependent on a highly conserved amino acid sequence.

An amino-terminal segment of about 30 residues of the plant vacuolar protein PHA also contains information for targeting to yeast vacuoles. The sequence resembles that of CPY, and the tetrapeptide LQRD, partially homologous to the QRPL of CPY at positions 18 to 21 of mature PHA, forms the essential core of the signal (Tague et al., 1990). However, this amino-terminal segment is sufficient but not necessary for vacuolar targeting of PHA in yeast, indicating that there is more information in the PHA protein for targeting to the vacuole.

We also found multiple targeting information in the plant vacuolar protein legumin. However, with respect to the nature of the signal, our results differed from those reported by others. In both the amino-terminal and the carboxy-terminal regions, long and complete sequences are necessary for efficient targeting, indicating that the signal is not formed by the primary structure of a short polypeptide sequence. Long polypeptide sequences could form the targeting signal by establishing a "signal patch" on the surface of the protein structure after folding.

On the other hand, this result could also mean that a different sorting mechanism might be involved. In animal cells, secreted proteins (zymogens) are sorted in the Golgi (and in the ER if a certain concentration is reached) by forming protein aggregate granules that specifically exclude other luminal ER proteins (i.e., condensation sorting; see Burgess and Kelly, 1987; Tooze et al., 1989). Such an interaction between the sorted proteins with each other and with a membrane component that is not necessarily a high-affinity receptor could depend on the length of the polypeptide segment. One has also to consider that in the plant, legumin propolypeptides form trimers in the ER and that these trimers are transported from the ER to the Golgi apparatus, where they are sorted in the vacuoles. It is very unlikely that comparable structures are formed by the legumin-invertase hybrid proteins in yeast. It remains to be demonstrated whether the structures found to act as sorting signals in yeast act by way of the same mechanism in plant cells and, if so, how these structures are exposed by the trimeric conformation.

Another possible explanation for the results obtained with plant vacuolar proteins and fusion proteins in yeast is that plant proteins and hybrid proteins are not correctly folded by the protein transport and folding machinery of polypeptide chain-binding proteins (for review, see Rothman, 1989) in yeast. A so-called scavenger pathway might exist, as suggested by Johnson et al. (1987), recognizing misfolded proteins and directing them to the vacuole for degradation. The efficiency of such a mechanism obviously would be highly variable and would depend on the degree of misfolding. On the other hand, our legumin-CAT fusion results in tobacco indicated that the long segments truly might act as transport signals. Preliminary data from the analysis of the legumin-invertase fusions in tobacco mesophyll cells suggest that the legumin α chain also efficiently directs invertase to the vacuoles.

There is further evidence that very complex structures might be necessary to form a plant vacuolar targeting signal. An amino-terminal segment of 113 amino acids of a vacuolar proteinase inhibitor from potato that is only 123 amino acids long still allowed complete secretion of invertase from *Arabidopsis* cells (von Schaewen et al., 1990). The short PHA segments directing invertase to yeast vacuoles also are not sufficient for vacuolar targeting in plant cells (Chrispeels, 1991). In barley lectin, a carboxy-terminal propeptide domain (only 15 amino acids long) was found to be necessary for targeting to plant vacuoles (Bednarek et al., 1990). It has yet to be shown whether this segment is sufficient for targeting or whether it is involved in the formation of a special protein conformation. The domain has the potential to form an amphipathic helix that might interact with other lectin domains (Bednarek et al., 1990).

Despite the observed differences between the legumin sorting signals and those of PHA, for example, we compared the protein sequences for the tripeptide LQR (part

of the tetrapeptide LQRD, see above) because Tague et al. (1990) found this tripeptide also in the carboxy-terminal region of legumin A from pea, which is homologous to the carboxy-terminal part of field bean legumin found to act as a vacuolar sorting signal. However, in field bean legumin, GLRQR occurs at positions 29 to 25 (numbered from the carboxy terminus as in Figure 5) instead of the NLQRN in pea legumin. Finally, these sequences are located in the carboxy-terminal half of the sorting signal, the deletion of which did not lead to complete loss of vacuolar targeting but only to a reduction from more than 90% with the fusion Inv-Le-C76 to 50% with Inv-Le-C76-38.

In addition, we screened 67 sequences from our data bank of plant vacuolar proteins, mostly targeted into storage vacuoles of seeds, for the occurrence of the sequence LQR. The results are shown in Table 3. Even allowing conservative amino acid substitutions (isoleucine and valine for leucine, asparagine for glutamine, lysine for arginine), only about half of the sequences contained this putative signal. For example, in 11S globulins, only the A-type subunits (e.g., legumin A of pea) contained the tripeptide, even multiple at different sites, whereas it did not occur in B-type subunits such as the one used in our studies.

Taken together, no generalizations can be made about vacuolar sorting signals and mechanisms. On the one hand, relatively short amino acid stretches with similar essential core sequences can act as sorting signals, very likely by way of a specific receptor, and the deletion of short propeptide domains leads to secretion (Chrispeels, 1991). On the other hand, we found both in yeast and in plant cells that vacuolar targeting of legumin is mediated by very different, much more complex sequences that might form the signal by higher structures and could act by way of a less specific mechanism.

Table 3. Occurrence of the Tripeptide LQR in Plant Vacuolar Proteins

Types of Plant Vacuolar Proteins	No. of Sequences With LQR	Without LQR	Total
11S globulins	13	11	24
7S globulins			
Vicilin-like	8	0	8
Convicilin-like	4	0	4
Lectins	4	3	7
Albumins	3	2	5
2S proteins	1	3	4
Prolamins	0	11	11
Vegetative storage proteins	0	4	4
Total	33	34	67

METHODS

Reagents

DNA-modifying enzymes were obtained from Boehringer Mannheim and from Bethesda Research Laboratories and were used according to the manufacturers' instructions. DNA sequencing reagents were from Pharmacia and Boehringer Mannheim. Radioactive deoxynucleotides and nucleotides were from Amersham. For protein gel blot staining, Promega's ProtoBlot Western Blot AP System and Amersham's streptavidin-biotin system were used. Novozym 234 and Lysing Enzyme (cell wall lysing enzymes from *Trichoderma harzianum*) were from Calbiochem and Sigma, respectively. *o*-Dianisidin, *N*-ethylmaleimide, and Ficoll 400 were obtained from Sigma. NADPH, *p*-nitrophenyl-α-D-glucopyranoside, *p*-nitrophenyl-α-D-mannopyranoside, and low melting agarose were from Serva; horseradish peroxidase, glucose oxidase, and invertase from Boehringer Mannheim; and cytochrome *c* from Biomed (Cracow, Poland). Antibiotics for selection of transformed bacteria and transformed plants were obtained mainly from Serva, and ^{14}C-chloramphenicol was from Amersham. Antisera against CAT were from Hoffmann-LaRoche Inc. (Burns and Crowl, 1987) and from 5 Prime-3 Prime Inc.; the legumin antibody used for immunocytochemistry was a gift of R. Manteuffel (Gatersleben). Affi-Gel was from Bio-Rad and Protein A was from Pharmacia.

Strains and Media

Escherichia coli strains were used for plasmid maintenance. *E. coli* JM101 was used for production of single-stranded DNA with the helper phage M13K07 (Vieira and Messing, 1988). *E. coli* GJ23 was used as helper strain for conjugation (van Haute et al., 1983). *Agrobacterium tumefaciens* C5801 (rifampicin) containing the disarmed Ti plasmid pGV3850 (Zambryski et al., 1983) was used for transformation of tobacco (*Nicotiana tabacum* Petit Havana cv SR1, Horsch et al., 1985). Nutrient broth (Immunpräparate Berlin) was used for growth of *E. coli* strains; YEB medium (Vervliet et al., 1975) was used for growth of *Agrobacterium* strains.

Yeast (*Saccharomyces cerevisiae*) strain SHY2 (*ura3*, *trp1*, *leu2*, *his3*, *can*) (Botstein et al., 1979) was used for expression of native legumin; strain SEY6210 (*ura3*, *trp1*, *leu2*, *his3*, *lys2*, *can*, *suc2-Δ9*) (obtained from S. Emr, Pasadena, CA) was used for expression of legumin-invertase fusions. For growth of legumin-bearing SHY2 strains, minimal medium with 2% glucose was used according to Tanaka et al. (1967). For SEY6210 strains bearing legumin-invertase fusions, the medium contained 2% fructose as carbon source. Cells for spheroplast formation and vacuole isolation were grown in minimal medium according to Wickerham (1946).

Plasmid Constructions

All basic constructions were performed in the phagemid pBS– (Stratagene). The intron-free legumin gene was constructed by substituting a BamHI-KpnI restriction fragment of a legumin cDNA clone, B273, isolated in our laboratory (G. Saalbach, unpublished

results) for the corresponding intron-containing fragment of a legumin genomic clone, *LeB4* (Bäumlein et al., 1986). An appropriate restriction fragment from pSV2CAT (Gorman et al., 1982) containing the entire coding sequence of the *CAT* gene was inserted 3′ of the legumin gene and Le-*CAT* fusions generated by oligonucleotide-directed deletion mutagenesis. Approximately 470 bp of legumin 3′-untranslated region was fused behind the stop codon of *CAT* as a polyadenylation signal.

For transformation into tobacco, fusions including the legumin promoter were subcloned into the SmaI site of the plant intermediary vector pMLJ1 (gift of L. Herrera-Estrella). A SalI site was generated 6 bp 5′ to the ATG translation start of the legumin gene, and corresponding SalI-HindIII fragments of several fusions were inserted into the SmaI site of pRT103 (Töpfer et al., 1987) for expression with the CaMV 35S promoter. HindIII fragments of these constructs were also subcloned into pMLJ1. An SphI fragment comprising the coding region plus 148 bp of 5′-flanking and 147 bp of 3′-flanking regions was isolated from the intron-free legumin gene and inserted into the SphI site of pBS−, resulting in pLeSph.

Plasmid pSUC23 containing a *SUC2* gene (Taussig and Carlson, 1983) was obtained from T. Rapoport (Berlin). A HindIII fragment of this gene spanning bases 12 to approximately 2700 was inserted into the HindIII site of the pLeSph such that both genes were in the same orientation. The legumin-invertase gene fusions were generated by deletion mutagenesis. For the fusion of carboxy-terminal legumin segments to invertase, an EcoRI-PstI fragment from pSUC23 spanning bases −26 (plus polylinker) to approximately 2270 of the *SUC2* gene was subcloned into pBS− by way of EcoRI-PstI. The resulting plasmid pSU was opened with PstI, blunt ended by T4-DNA polymerase, and then cut with SphI (polylinker). A KpnI(blunt)-SphI fragment spanning bases 1473 to 1850 of the legumin gene with 230 bp of 3′-coding region and 147 bp of 3′-noncoding region was inserted into the opened pSU plasmid such that the SUC2 gene and the legumin gene fragment were in the same orientation.

Oligonucleotide-directed in vitro mutagenesis for generation of restriction sites and deletions was performed essentially according to Zoller and Smith (1983). Oligonucleotides were synthesized on an Applied Biosystems 391 DNA synthesizer (F. Machemehl, Gatersleben). For deletions, oligonucleotides encoding the junction sequences were 30 to 40 bases long. The fusion sequences were verified using dideoxy DNA sequencing (Sanger et al., 1977).

For expression of legumin and legumin-invertase fusions in yeast, SalI-HindIII (polylinker) restriction fragments starting 6 bp in front of the ATG (see above) were isolated from the pBS− plasmids and inserted by way of blunt-end ligation into the HindIII site of the yeast shuttle plasmid pAAH5 (Ammerer, 1983). For the carboxy-terminal invertase-legumin fusions, EcoRI-SphI fragments were used in the same way. For low-level expression in yeast, a BamHI fragment was isolated from the pAAH5 clone containing Le-Inv-86. This fragment comprising the *ADH1* promoter, the legumin invertase gene fusion, and the *ADH1* terminator was inserted into the BamHI site of YCp50 (Sherman et al., 1986; obtained from S. Emr, Pasadena, CA).

Transformation of Yeast and Tobacco

Yeast was transformed according to Ito et al. (1983), and transformed cells were selected on minimal medium with the required supplements.

Tobacco leaves were used for *Agrobacterium*-mediated leaf disc infection as described by Horsch et al. (1985). Transformants were selected on 100 μg/mL kanamycin. Transformation of the genes was verified by DNA gel blotting (Southern, 1975), and expression of the genes by RNA gel blotting using the method of Thomas (1983).

Immunological Procedures and Electron Microscopy

Antibodies against a B-type subunit of legumin (gift of C. Horstmann, Gatersleben) and against α and β chains isolated from a denaturing reducing polyacrylamide gel were raised in mice. The antigens were bound to Affi-Gel and used for affinity purification of the antibodies.

Yeast cells were homogenized with dry ice and glass beads in a mortar, and the frozen powder was extracted by boiling in SDS sample buffer. Tobacco seeds were ground in a mortar under liquid nitrogen, and the powder was extracted in 0.1 M phosphate buffer, pH 7.5, with 1 M KCl. Spheroplasts and isolated vacuoles from yeast as well as cell fractions from tobacco seeds were lysed by addition of concentrated SDS sample buffer and boiling. Samples were separated on polyacrylamide gels (Laemmli, 1970) and blotted to nitrocellulose according to Towbin et al. (1979). Legumin bands were visualized by treating the blots with legumin antibodies, followed by biotinylated anti-mouse immunoglobulin G and streptavidin-alkaline phosphatase or by alkaline phosphatase immunoglobulin G conjugates, and staining with nitro blue tetrazolium and 5-bromo-4-chloroindolylphosphate.

For electron microscopy, yeast cells were prefixed with 2% glutaraldehyde in cacodylate buffer, pH 7.2, with 1 M sorbose. After centrifugation, the cells were wrapped in 3% low melting agarose (gel point 26 to 29°C). Pieces of 1 mm^3 were quickly frozen in liquid propane (−185°C) and stored under liquid nitrogen. Freeze-substitution was carried out according to Müller et al. (1980). At −35°C, the samples were embedded in Lowikryl K4M resin, which yields a good preservation of cellular structures and low nonspecific antibody binding (Roth et al., 1981; Craig and Goodchild, 1982). Embedding was carried out in gelatin capsules by UV light polymerization under nitrogen for 24 hr. After 2 days of curing, thin sections were cut using glass knives on an LKB-Ultrotome. Sections were collected on Formvar film-coated nickel grids. Nonspecific binding was blocked by treatment with 0.1% BSA in 0.15 M phosphate buffer, pH 7.2, containing 0.5% Tween 20, 0.1% PEG, and 5 mM ammonium chloride for 10 min. The grids were incubated on drops of diluted legumin antibody solution for 15 min and then extensively washed in blocking solution with reduced BSA content (100 μg/mL). Thereafter, the grids were treated with protein A-gold conjugate prepared according to Roth (1983) for 15 min and washed again. Finally, grids were washed in distilled water and contrasted with uranyl acetate (1% in ethanol). After washing with pure ethanol and drying, specimens were evaluated in a TESLA BS500 transmission electron microscope at 60 kV. Controls were run without antibody treatment and with preimmune serum.

Assays

Quantitative invertase assays were performed using the method of Goldstein and Lampen (1975), as described by Johnson et al.

(1987). External activity was measured using intact cells, total activity after lysis of the cells with 0.5% Triton X-100. Invertase activity gels were performed using triphenyltetrazolium chloride according to Gabriel and Wang (1969). CAT activity was assayed according to Gorman et al. (1982).

Spheroplasts of yeast were prepared as described by Maraz and Subik (1981) using lysing enzymes at 3 to 5 mg/mL. Spheroplasts were lysed and vacuoles isolated on a Ficoll step gradient according to Stevens et al. (1982). The vacuole fraction and the original spheroplast lysate were assayed for invertase and marker enzyme activities. α-Mannosidase was assayed according to Opheim (1978), NADPH cytochrome *c* reductase was assayed as described by Kubota et al. (1977), and α-glucosidase was assayed according to Halvorson and Elias (1958).

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