The Vacuolar Targeting Signal of the 2S Albumin from Brazil Nut Resides at the C Terminus and Involves the C-Terminal Propeptide as an Essential Element

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Genetic constructs in which different N- and C-terminal segments of Brazil nut (Bertholletia excelsa H.B.K.) 2S albumin were fused to secretory yeast invertase were transformed into tobacco (Nicotiana tabacum) plants to investigate the vacuolar targeting signal of the 2S albumin. None of the N-terminal segments, including the complete precursor containing all propeptides, was able to direct the invertase to the vacuoles. However, a short C-terminal segment comprising the last 20 amino acids of the precursor was sufficient for efficient targeting of yeast invertase to the vacuoles of the transformed tobacco plants. Further analyses showed that peptides of 16 and 13 amino acids of the C-terminal segment were still sufficient, although they had slightly lower efficiency. When segments of 9 amino acids or shorter were analyzed, a decrease to approximately 30% was observed. These segments included the C-terminal propeptide of four amino acids (Ile-Ala-Gly-Phe). When the 25 albumin was expressed in tobacco, it was also localized to the vacuoles of mesophyll cells. If the C-terminal propeptide was deleted from the 25 albumin precursor, all of this truncated 25 albumin was secreted from the tobacco cells. These results indicate that the C-terminal propeptide is necessary but not sufficient for vacuolar targeting. In addition, an adjacent segment of at least 12 amino acids of the mature protein is needed to form the complete signal for efficient targeting.

Plant seed-storage proteins accumulate in storage vacuoles. Precursor forms of these proteins enter the lumen of the ER and in many instances appear to follow the bulk flow to the Golgi apparatus, where they are sorted into vesicles for further transport to the vacuole.

Studies of a number of different plant vacuolar proteins have shown that positive sorting information residing in the polypeptide structure is required for this process (for reviews, see Bednarek and Raikhel, 1992; Chrispeels and Raikhel, 1992). In some cases this information is localized in ATPPs or CTPPs, respectively, whereas in others, vacuolar targeting is located in terminal or internal regions of the mature proteins. N-terminal signals were identified in sporamin from sweet potato (Matsuoka and Nakamura, 1991) and in aleurain from barley (Holwerda et al., 1992), whereas C-terminal signals were found in lectin from barley (Bednarek and Raikhel, 1991) and in chitinase from tobacco (Neuhaus et al., 1991). In legumin from the field bean, two segments of the mature protein were shown to be effective in targeting to the plant vacuoles: an N-terminal segment of 281 amino acids and a C-terminal segment of 76 amino acids (Saalbach et al., 1991; G. Saalbach and U. Schumann, unpublished data). In phytohemagglutinin of bean an internal segment (amino acids 83–113) was both sufficient and necessary for directing yeast invertase to vacuoles in transgenic plants (von Schaewen and Chrispeels, 1993).

Among the targeting determinants so far identified there is little or no sequence similarity. The C-terminal signals are rich in amino acids with hydrophobic side chains. A detailed analysis of barley lectin CTPP revealed that the hydrophobic tripeptide VFA, as well as several other artificial hydrophobic sequences, was sufficient (Dombrowski et al., 1993). Similar results were obtained in a mutational analysis of the CTPP of tobacco chitinase. Vacuolar targeting by this hydrophobic CTPP was severely impaired when only a single amino acid was deleted, whereas exchanges of amino acids and substitutions of the targeting signal with random sequences had effects that covered the whole range from retention to secretion (Neuhaus et al., 1994). In contrast to the C-terminal targeting signals, the N-terminal targeting determinants of sporamin and aleurain share a common sequence motif (NP1FL/P), which has been shown to be essential for efficient sorting of sporamin (Nakamura and Matsuoka, 1993).

Seeds of Brazil nut (Bertholletia excelsa H.B.K.) contain a small, sulfur-rich storage protein that is a member of the group of 25 albumins (Sun et al., 1987). It is deposited in protein bodies in the hypocotyl of the Brazil nut embryo (Harris et al., 1993). The 25 albumins undergo extensive posttranslational processing (Ampe et al., 1986; Krebbers et al., 1988). Like other proteins (Holwerda et al., 1992; Nakamura and Matsuoka, 1993), it might be anticipated that the vacuolar targeting information would reside in one of the propeptide segments. To investigate this possibility we prepared constructs in which N-terminal and C-terminal segments of the Brazil nut 2S albumin precursor were fused to yeast secretory invertase. We subsequently used the constructs to transform tobacco (Nicotiana tabacum) and

Abbreviations: ATPP, amino-terminal polypeptides; CaMV, cauliflower mosaic virus; CTPP, carboxyl-terminal polypeptides.
analyzed the vacuolar targeting of the invertase. We found that a C-terminal segment of 20 amino acids is sufficient for transport of the yeast invertase to the vacuoles of mesophyll cells in transformed tobacco. This fragment includes a C-terminal propeptide of four amino acids (IAGF). Deletion of this propeptide resulted in complete secretion of the 2S albumin, thus showing that it is an essential part of the targeting sequence.

**MATERIALS AND METHODS**

**Gene Constructions and Plant Transformation**

Gene cloning, modification, and analysis were done as described previously (Sambrook et al., 1989). Basic gene constructions were performed in the phagemid pBS- (Stratagene). The Brazil nut (Bertholletia excelsa H.B.K.) 2S albumin clone was assembled in vitro from synthetic oligonucleotides as described previously (Saalbach et al., 1994) and according to the cDNA sequence published by Altenbach et al. (1987). Plasmid pSUC23 containing the yeast secretory invertase gene SUC2 (Tausig and Carlson, 1983) was obtained from T. Rapoport (Berlin, Germany). Gene fusions were constructed by using oligonucleotide-mediated deletion mutagenesis as described previously (Saalbach et al., 1991). Fragments encoding the gene fusions were placed under the control of the CaMV 35S promoter by subcloning into pRT103 (Topfer et al., 1987). In N-terminal fusions, the translation start of the 2S albumin was fused to the translation start site of pRT103 via the Ncol site. To ensure import into the ER of C-terminal fusions, the signal peptide of legumin was N-terminally fused to the translation start site of pRT103 (Töpfer et al., 1987). Gene fusions were cloned into the binary plant transformation vector pGA471 (An, 1987). The resulting plasmids were transformed by electroporation (Gene Pulser, Bio-Rad) into Agrobacterium tumefaciens strain LBA4404 harboring the tumor-inducing plasmid pGV2260 (Deblaere et al., 1985). Leaf discs of tobacco (Nicotiana tabacum Samsun NN) were used for the transformation, as described previously by Horsch et al. (1985). Transformed protoplasts were isolated from tobacco leaves by incubation of protoplasts in 15 mM phosphate buffer, pH 5.6-5.8 containing 1.7 mg mL\(^{-1}\) buffer and subsequent centrifugation at 8 mg/mL buffer. After the sample was centrifuged at 5000g for 20 min vacuoles were collected from the 7 to 4% and 4 to 0% interfaces. The yield of isolated vacuoles was subsequently carried out as described above.

**Vacuolar Targeting of Invertase and 2S Albumin**

The efficiency of vacuolar targeting of invertase was determined by comparing the amount of invertase activity contained in a total leaf homogenate (amount of invertase activity secreted plus the amount of invertase activity retained in leaf cells) with the amount of invertase activity contained in leaf protoplasts (invertase activity retained in cells) and with the amount of invertase activity contained in vacuoles. The amount of invertase activity contained in protoplasts was calculated from the amount of activity detected in isolated protoplasts and the percentage of recovery of protoplasts from leaves. The amount of invertase activity contained in vacuoles was calculated from the amount of invertase activity detected in isolated vacuoles and the percentage of recovered vacuoles from protoplasts.

Total leaf extracts were prepared by homogenizing leaves from transformed plants in 50 mM sodium acetate buffer, pH 5.2, at 5 mg fresh weight mL\(^{-1}\) buffer and subsequent centrifugation at 12,000g for 10 min.

Protoplasts were isolated from tobacco leaves by incubation of protoplasts in 0.5 mM Gly (pH 5.6-5.8) containing 1.7 mg mL\(^{-1}\) Cellulase Onozuka R-10 (Serva, Heidelberg, Germany) and 0.27 mg mL\(^{-1}\) Macerozyme R-10 (Serva), as well as 0.82 mg mL\(^{-1}\) Driselase (Kyowa Hakko Kogyo, Tokyo, Japan) for 14 to 16 h at 23°C. Protoplasts were collected after centrifugation at 100g for 5 min and washed three times in 0.5 mM Gly. The yield of protoplasts was estimated from the ratio of acidic α-mannosidase activities recovered from protoplasts to those detected in the total leaf extract.

Vacuoles were isolated from protoplasts using the method of Glund et al. (1984). Protoplasts were lysed in 15 mM phosphate buffer, 5 mM EDTA, pH 8.0, containing 0.4 mM Gly as osmoticum. The lysate was adjusted to 8% (w/v) Ficoll and overlayered in an ultracentrifuge tube with layers of 7, 4, and 0% (w/v) Ficoll in the lysis buffer. After the sample was centrifuged at 5000g for 20 min vacuoles were collected from the 7 to 4% and 4 to 0% interfaces. The yield of isolated vacuoles was estimated from the ratio of α-mannosidase activities contained in the protoplasts to those detected in isolated vacuoles.

Vacuolar targeting of 2S albumin was determined by comparing the amount of 2S albumin detected in protoplasts with the amount of 2S albumin detected in the culture medium. For this purpose, protoplasts were radiolabeled for various times. 2S albumin was subsequently immunoprecipitated from both protoplasts and culture medium (see below). Finally, the immunoselected 2S albumin was analyzed by SDS-PAGE and fluorography.
Protoplasts isolated from transformed plants were cultured in the presence of 50 mCi mL⁻¹ EXPRES³⁵S³⁵S Protein Labeling Mix (NEN). This mixture contains L-[³⁵S]Met (73% on molar basis) and L-[³⁵S]Cys (22%) and therefore represents a suitable label for the sulfur-rich 2S albumin. After variable incubation times, protoplasts were separated from the culture medium by centrifugation at 100 g for 5 min. The protoplast pellets were lysed in lysis buffer (50 mM phosphate buffer, pH 7.5, 0.2 M NaCl, 1% Triton X-100, 0.5% SDS, and 1.6 mg mL⁻¹ Complete Anti-Protease Mix (Boehringer Mannheim) at a concentration of 1 mL buffer g⁻¹ protoplasts. After the proteins were denatured for 5 min at 95°C, about 2 to 3 volumes of lysis buffer containing 1% BSA and no SDS were added. After further incubation for 10 min at room temperature the mixture was transferred to ice. For immunoselection of the 2S albumin, 20 µL of anti-2S albumin antibodies was added. After incubation of the mixture on ice for 2 h, 200 µL of a 10% (w/v) suspension of Protein A-Sepharose (Pharmacia) was added and the mixture was incubated for another 2 h while being slowly agitated. The Protein A-Sepharose was pelleted in a microfuge and washed six times with 10 mM phosphate buffer, pH 7.2, 150 mM NaCl, and 0.1% Tween 20 (Serva). Proteins were subsequently eluted by adding an equal volume of 2X SDS sample buffer to the final Protein A-Sepharose pellet and heating to 95°C for 5 min. The Protein A-Sepharose was removed from the solubilized proteins by centrifugation in a microfuge.

Proteins in the culture medium were concentrated by adding solid ammonium sulfate to 70% saturation. After incubation on ice for 2 h, precipitated proteins were collected by centrifugation (12,000g for 10 min). The precipitated proteins were resuspended in 50 mM phosphate buffer, pH 7.5, 0.2 M NaCl, 1% Triton, 1% BSA, and 1.6 mg mL⁻¹ Complete Anti-Protease Mix, and the volume was adjusted to the one of the protoplast extracts (see above). Immunoselection of 2S albumin was carried out as described above.

**Monitoring Expression of 2S Albumin in Transgenic Tobacco**

Expression of 2S albumin in transgenic tobacco was monitored by immunoblotting. Total proteins were extracted with SDS sample buffer from leaves and protoplasts of transgenic plants. Vacular proteins were precipitated by adding solid ammonium sulfate to 30% TCA to the vacuole fraction obtained from the Ficol gradient. These samples were kept on ice for 2 h and pelleted at 12,000g for 15 min. The pellets were dissolved in SDS sample buffer. Proteins were separated on 17% SDS-polyacrylamide gels (Laemmli et al., 1970) under reducing conditions and transferred onto 0.22-µm nitrocellulose membranes (Schleicher & Schuell) by electroblotting according to the method of Towbin et al. (1979). The proteins were fixed by treatment with 1% glutaraldehyde according to the method of Karray and Sirbasku (1989). The blots were blocked with 5% BSA in 10 mM phosphate buffer, pH 7.2, 150 mM NaCl, and 0.1% Tween 20 for at least 1 h and subsequently incubated with the 2S albumin antibodies (dilution 1:500) for 2 h. Blots were washed four times in 10 mM phosphate buffer, pH 7.2, 150 mM NaCl, and 0.1% Tween 20 and subsequently incubated for 30 min with secondary antibodies coupled to alkaline phosphatase (Sigma) (dilution 1:10,000). Proteins were visualized with the chromogenic substrates 5-bromo-4-chloro-3-indolyl-phosphate and nitroblue tetrazolium chloride.

**Preparation of Antibodies**

Brazil nut 2S albumin was purified by C. Horstmann (Gatersleben, Germany) as described previously (Sun et al., 1987) and used for the preparation of antibodies in rabbits. Rabbits were immunized according to a standard protocol by three injections with 2S albumin (first injection, 1 mg; second and third injections, 200 µg each) at intervals of 4 weeks. The antibodies were affinity-purified from crude serum on a column (Affi-Gel 15, Bio-Rad) to which 2S albumin had been coupled. Bound antibodies were eluted with 50 mM Gly, pH 2.2.

**SDS-PAGE and Fluorography**

SDS-PAGE was done according to the method of Laemmli et al. (1970) on 17% gels. Prior to electrophoresis, proteins were extracted in SDS sample buffer (50 mM Tris, 2% SDS, 50 mM DTT, 10% glycerol, 0.1% bromphenol blue, pH 6.8). For fluorography gels were fixed in methanol:water:acetic acid (45:45:10, v/v/v) for 30 min and subsequently soaked with Amplify (Amersham) for 20 min. After drying, gels were exposed to x-ray film for 1 to 4 weeks.

**RESULTS**

**The Vacular Sorting Information Resides at the C Terminus**

The 17-kD precursor of the 2S albumin from Brazil nut (Fig. 1A) undergoes extensive posttranslational processing (Ampe et al., 1986; Altenbach et al., 1987; De Castro et al., 1987). A signal peptide (2.1 kD) is co-translationally cleaved off from the 2S albumin upon entry into the ER. Prior to or concomitantly with the arrival of the 2S albumin at the vacuole, three more peptides are removed: an N-terminal propeptide of 14 amino acids, a C-terminal propeptide of 4 amino acids, and a linker peptide of 5 amino acids, which, in the precursor, joins the small and large subunits of 2S albumin. Finally, in the mature protein, the large subunit (8.6 kD) is connected with the small subunit (3.6 kD) by disulfide bridges.

A series of gene constructions were made (Fig. 1B) in an attempt to localize, identify, and characterize the targeting information of the 2S albumin from Brazil nut. One series was aimed at investigating the possible roles of N-terminal sequences. Fusion protein N1 contained the signal peptide, the N-terminal propeptide, and the small subunit of 2S albumin linked to invertase. In addition N2 contained the linker peptide and the first 22 amino acids of the large subunit of 2S albumin, whereas N3 contained the entire precursor. A second series of constructs was made to examine the role of C-terminal sequences in vacuolar targeting. These constructs en-
ER, C1 and C2 contained the signal peptide of legumin in the sequence hy colons. In addition to the N-terminal of 2s albumin, and fusion protein C2 contained the last protein C1 contained the last 20 C-terminal amino acids albumin linked to the C terminus of invertase. Fusion protein C2, which included the code for the \(2.1\) kD signal peptide linked to invertase. A control construct encoded a protein (SEC), which consisted solely of the legumin signal peptide linked to invertase. SP, 2s albumin signal peptide; Leg.SP, signal peptide and C-terminal (C1 and C2) fragments of 2s albumin were fused to yeast invertase. SP, 2s albumin signal peptide; Leg.SP, signal peptide of legumin from \textit{Vicia faba}; SS, small subunit; LP, linker peptide; LS, large subunit; aa, amino acids.

Figure 1. Structure and sequence of the 2S albumin precursor of \textit{B. excelsa} and construction of gene fusions with yeast invertase as reporter. A. Structure and sequence of the 25 albumin precursor of \textit{B. excelsa} (see text for refs.). The amino acid sequence (one-letter code) is printed below the bars representing the different domains of the complete 2S albumin precursor (17 kD). Processing sites are indicated in the sequence by colons. In addition to the N-terminal ER signal peptide (2.1 kD), another three peptides (ATTP, linker peptide, and CTPP) are removed during proteolytic processing. The two remaining peptides (black bars) represent the small (3.6 kD) and large (8.6 kD) subunits, which are connected via disulfide bridges in the mature protein. B. Schematic representation of gene fusions used to locate the 2S albumin-targeting information. N-terminal (N1–N3) and C-terminal (C1 and C2) fragments of 2S albumin were fused to yeast invertase. SP, 2S albumin signal peptide; Leg.SP, signal peptide of legumin from \textit{Vicia faba}; SS, small subunit; LP, linker peptide; LS, large subunit; aa, amino acids.

Figure 2. Diagram demonstrating the in vivo vacuolar targeting efficiency of N-terminal 2S albumin-invertase fusions depicted in Figure 1B (fusions N1–N3). Invertase activities (\(\mu\)mol Glc min\(^{-1}\) g\(_{fw}\)\(^{-1}\); \(g_{fw}\), fresh weight) of total leaf extracts (white columns), isolated protoplasts (hatched columns), and isolated vacuoles (black columns) are compared (see “Materials and Methods” for details). Numbers in parentheses indicate individual transformants expressing the corresponding fusion protein. WT, Wild-type (untransformed) tobacco plant; SEC, secretory yeast invertase with legumin signal peptide; \(g_{fw}\), grams fresh weight of the leaf material.

coded fusion proteins with C-terminal segments of 2S albumin linked to the C terminus of invertase. Fusion protein C1 contained the last 20 C-terminal amino acids of 2S albumin, and fusion protein C2 contained the last 36 amino acids of 2S albumin. To ensure import into the ER, C1 and C2 contained the signal peptide of legumin linked to the N terminus of the invertase. A control construct encoded a protein (SEC), which consisted solely of the legumin signal peptide linked to invertase. Since this invertase fusion protein lacks any vacuolar targeting information, it should be completely secreted from the cells and therefore represents a negative control. All of the constructs were placed under the control of the constitutive CaMV 35S promoter and introduced into tobacco plants. Transformants were selected by assaying for enhanced invertase activity in leaf extracts. Since the additional invertase activity impaired growth, the yield of individual transformants was low. In some cases, such as in N1 and C1, only a few of the transformants provided sufficient material to assay for invertase activity.

The secretion or vacuolar localization of the invertase was estimated based on the relative recoveries of invertase and \(\alpha\)-mannosidase in leaf homogenates, protoplasts, and vacuole preparations (see “Materials and Methods”). As can be estimated from the data in Figure 2, the invertase produced from the control construct (SEC) was predominantly secreted with only approximately one-seventh of the total invertase activity being contained in the vacuole preparations. All of the N-terminal constructs led to distributions of invertase similar to the control construct (Fig. 2). About 25\% of the total invertase activity was recovered in the vacuoles for the construct encoding the N2 fusion protein. The very low amounts of invertase in vacuoles for the construct encoding the N3 fusion protein (containing the whole 2S albumin precursor) indicated efficient secretion of this fusion protein.

Transformants containing either of the two C-terminal constructs, however, retained an average of 100\% of the invertase activity inside the cell. This invertase activity could be completely recovered from vacuoles (Fig. 3). We tested five individual transformants containing construct C2, which included the code for the 36 C-terminal amino acids of 2S albumin. Two of these transformants (4 and 6) expressed very high levels of invertase activity. In all in-
stices the invertase was efficiently (100%) targeted to the vacuole. Similar results were obtained with transformants containing the C1 construct. This construct contained the code for only the last 20 C-terminal amino acids of the 2s albumin precursor. We tested two individual transformants, C1(2) and C1(6). The results obtained with transformant C1(2) were confirmed in six independent measurements for the protoplasts and three measurements for the vacuoles. Transformant C1(6) grew poorly; therefore, this transformant did not provide enough material to isolate vacuoles. Yet the amount of invertase activity contained in the protoplasts recovered from this transformant was similar to its total leaf activity, indicating that this transformant retained most of the invertase activity inside the cell. We performed a second transformation with construct C1. Protoplasts isolated from respective transformants contained the major portion (90% on average) of the invertase activity contained in the protoplasts recovered from this transformant was similar to its total leaf activity, indicating that this transformant retained most of the invertase activity inside the cell. We performed a second transformation with construct C1. Protoplasts isolated from respective transformants contained the major portion (90% on average) of the invertase activity (data not shown). The data presented here show that the targeting information of Brazil nut 2s albumin is attached to the C terminus of invertase (Table I). Although transformation was difficult, several independent transformants were identified for each construct. Among different transformants with the same construct, the vacuolar targeting efficiencies varied. Data obtained with a given construct have therefore been listed individually for each transformant, together with a mean value representing the average targeting efficiency among transformants with the same construct. It appeared that the segment of 16 amino acids was still efficient in directing vacuolar localization of the invertase. One hundred percent vacuolar localization was observed in two of the transformants containing this construct. In comparison, deletion of three more amino acids (NLS) led to slightly reduced targeting efficiencies. In the respective transformants a lower portion of the total invertase activity (74% on average) was retained in the cell, and in no instances was 100% targeting observed. A notable reduction in targeting efficiencies was observed in transformants with the C9 construct. An average of only approximately one-third of the invertase was targeted to the vacuoles in these transformants. Deletion of the next five amino acids (PMGGGS) did not significantly decrease the average targeting efficiency any further, although a comparison of the individual data indicated a slightly higher efficiency with construct C9 (up to 50%) than with C6 and C4. The low level of invertase in the protoplasts from transformants with constructs C6 and C4 is comparable to the level observed in protoplasts from plants transformed with the SEC construct encoding yeast invertase with the ER signal peptide from legumin but lacking any vacuolar targeting signal (Figs. 2 and 3). In some cases we isolated vacuoles from transformants expressing C9, C6, and C4. We found an average of 20% of the total leaf invertase in the vacuole preparations (data not shown). This is slightly above the value observed with the SEC construct (approximately 15%, Figs. 2 and 3).

### Table 1. Deletion analysis of the C-terminal segment of 2s albumin

<table>
<thead>
<tr>
<th>Name</th>
<th>Reporter</th>
<th>C-Terminal 2s Albumin Amino Acids</th>
<th>Percentage of Invertase in Protoplasts</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Average</td>
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<tr>
<td>C16</td>
<td>Invertase</td>
<td>NLSPNRCMPMGGSIAGF</td>
<td>81</td>
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<tr>
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<td>Invertase</td>
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<tr>
<td>C4</td>
<td>Invertase</td>
<td>IAGF</td>
<td>30</td>
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Analysis of the C-Terminal Targeting Signal

Another series of constructs was designed to identify the minimum vacuolar targeting information of Brazil nut 2s albumin. These constructs encoded progressively shorter C-terminal segments of 16, 13, 9, 6, and 4 amino acids linked to the C terminus of invertase (Table I). Although transformation was difficult, several independent transformants were identified for each construct. Among different transformants with the same construct, the vacuolar targeting efficiencies varied. Data obtained with a given construct have therefore been listed individually for each transformant, together with a mean value representing the average targeting efficiency among transformants with the same construct. It appeared that the segment of 16 amino acids was still efficient in directing vacuolar localization of the invertase. One hundred percent vacuolar localization was observed in two of the transformants containing this construct. In comparison, deletion of three more amino acids (NLS) led to slightly reduced targeting efficiencies. In the respective transformants a lower portion of the total invertase activity (74% on average) was retained in the cell, and in no instances was 100% targeting observed. A notable reduction in targeting efficiencies was observed in transformants with the C9 construct. An average of only approximately one-third of the invertase was targeted to the vacuoles in these transformants. Deletion of the next five amino acids (PMGGGS) did not significantly decrease the average targeting efficiency any further, although a comparison of the individual data indicated a slightly higher efficiency with construct C9 (up to 50%) than with C6 and C4. The low level of invertase in the protoplasts from transformants with constructs C6 and C4 is comparable to the level observed in protoplasts from plants transformed with the SEC construct encoding yeast invertase with the ER signal peptide from legumin but lacking any vacuolar targeting signal (Figs. 2 and 3). In some cases we isolated vacuoles from transformants expressing C9, C6, and C4. We found an average of 20% of the total leaf invertase in the vacuole preparations (data not shown). This is slightly above the value observed with the SEC construct (approximately 15%, Figs. 2 and 3).
The C-Terminal Propeptide IAGF Is an Essential Element of the Targeting Signal

To investigate the role of the C-terminal propeptide IAGF in targeting of the 2S albumin precursor, we assembled another set of constructs from a clone encoding the wild-type 2S albumin precursor (Fig. 4A). In addition to the 2S albumin wild-type clone (2Swt), this set included three gene constructs that encoded modified 2S albumin precursors: 2SΔ20 lacking the last 20 C-terminal amino acids, 2SΔ4 lacking the last four amino acids, and 2S+4 containing four additional amino acids (SQSN). The constructs were transformed in tobacco plants and the expression of the wild-type and mutant genes in the tobacco leaves was monitored by immunoblotting.

As Figure 4B shows, 2Swt, 2SΔ4, and 2S+4 were all expressed in tobacco leaves. After electrophoresis under reducing conditions the predominant bands of these three proteins co-migrated at about 15 kD. This main band should represent the precursor of the corresponding 2S albumin lacking the ER signal peptide (Fig. 1A). Two closely spaced bands of equal intensity appeared in 2S+4. There were minor bands around the main bands and a broad band (smear) below 7 kD could be observed in 2Swt and 2S+4. The main band (precursor) of 2SΔ4 migrated slightly faster than the 2Swt band. The small and large subunits of mature 2S albumin (Fig. 4B, lane 2S) were not present in the samples from the leaves of the transformed plants.

In contrast, 2SΔ20 lacking the 20 C-terminal amino acids could barely be detected, although its expression had been demonstrated by RNA gel blotting (data not shown). In the labeling experiments (see below) only a very weak band could be detected on fluorographs (data not shown); therefore, the targeting of this modified 2S albumin could not be analyzed.

Vacuoles were isolated from mesophyll protoplasts of transformants expressing 2Swt protein. Based on α-mannosidase activity, equal amounts of protoplasts and vacuoles were applied to an SDS gel, and the occurrence of the 2S albumin was analyzed by immunoblotting. As Figure 5 shows, 25 albumin was present in the vacuoles at the same level as in the protoplasts, showing that Brazil nut 2S albumin is targeted to the vacuoles in tobacco mesophyll cells.

To study the intracellular transport of wild-type and mutant 2S albumins, protoplasts from transformed tobacco cells were cultured in the presence of L-[35S]Met/Cys preparations. 2S albumin was immunoselected from the radiolabeled protoplasts and from the culture medium and analyzed by SDS-PAGE and fluorography. After 18 h of continuous radiolabeling of protoplasts immunoselected 2Swt on SDS gels displayed a discrete band at approximately 15 kD that corresponded to the 25 albumin precursor (lacking the ER signal peptide), as well as a broad smear of lower molecular mass (Fig. 6A, 2Swt). Compared with the total amount of wild-type 2S albumin precursor and proteolytic fragments (smear) isolated from protoplasts, only a small amount of precursor and breakdown products was detected in the culture medium (Fig. 6A).

Thus, the majority of the 2S albumin was retained in the protoplasts and appeared to be correctly targeted to the vacuoles. A similar distribution of albumin precursor and breakdown products was observed from transformants that...
In a second experiment protoplasts from the respective transformants were pulse-labeled for 2 h and chased for another 2, 4, 6, or 20 h. We found that the wild-type 2S albumin (Fig. 6B, 2Swt) was always predominantly contained within the protoplasts. After 2 h of pulse labeling, the predominant form of 2S albumin detected in the protoplasts was the 25 albumin precursor of 15 kD (Fig. 6B). After 6 h of chase almost all of this precursor form was degraded, indicating the arrival of the protein in the vacuoles. After 20 h of chase the total amount of 2S albumin products in the protoplasts was clearly lower than after 6 h of chase, indicating a continuous proteolytic degradation of the 2S albumin inside the protoplasts (vacuoles). The amount of 2S albumin in the culture medium was barely detectable. In contrast, protoplasts from transformants expressing 2SΔ4 secreted the precursor into the culture medium (Fig. 6C). After 2 h of chase already more than 50% was secreted, and after 6 h most of the corresponding precursor was found in the culture medium. After 20 h of chase this precursor was barely detectable in the protoplasts. No degradation products were observed either in the protoplasts or in the medium, even after 20 h of chase. Taken together, these data indicate that most of the normal 2S albumin precursor (2Swt) was correctly transported to the vacuole within 6 h, whereas the 2S albumin lacking the C-terminal propeptide (2SΔ4) was efficiently secreted.

**DISCUSSION**

**Determination of the Vacuolar Targeting Signal in the Brazil Nut 2S Albumin**

The 2S albumin investigated in this study is a seed-storage protein deposited in protein bodies of the hypocotyl of the Brazil nut embry (Harris et al., 1993). Because of its extremely high content of sulfur-containing amino acids this protein has attracted much attention. It has been expressed in heterologous plants such as tobacco (Altenbach et al., 1989; Saalbach et al., 1994), canola (Altenbach et al., 1992), and *Vicia narbonensis* (Saalbach et al., 1994, 1995). The intracellular localization of the 2S albumin in these plants was also addressed. The 2S albumin was detected in vacuoles of seeds from *V. narbonensis* by immunocytochemistry (Saalbach et al., 1995) and was also contained in vacuoles isolated from mesophyll cells of transformed tobacco (Saalbach et al., 1994).

In the present study we have analyzed the vacuolar targeting of the Brazil nut 2S albumin in more detail. To this end, two approaches were used. First, to localize and characterize the targeting information, genetic constructs were generated, encoding fusions of secretory yeast invertase with different N- and C-terminal domains of the 2S albumin. Second, to analyze the involvement of C-terminal domains in 2S albumin targeting, constructs were generated, encoding mutated forms of Brazil nut 2S albumin. All constructs were stably transformed into the tobacco plants. The intracellular transport was analyzed in mesophyll cells of the transformed plants. This cell system has proven useful in several studies of targeting of vacuolar proteins (see the introduction). Yeast expressed the mutant 2S albumin with four additional amino acids at the C terminus (Fig. 6A, 2S + 4). In this case, too, very little 2S albumin was detected in the culture medium. In contrast, transformants expressing the 2S albumin lacking the C-terminal propeptide predominantly secreted the modified protein. As can be seen in Figure 6A (2SΔ4) high amounts of this modified 2S albumin precursor (but only a small amount of breakdown products) accumulated in the culture medium, whereas the amount of 2S albumin products contained in the protoplasts was significantly lower. In the protoplasts a higher proportion of breakdown products was observed.
invertase has been shown to be a suitable reporter for targeting studies in yeast (Johnson et al., 1987; Tague et al.,
1990; Saalbach et al., 1991), as well as in plants (von Schae-
wen et al., 1990; von Schaewen and Chrispeels, 1993). It is
very stable (see below) and efficiently passes the secretory
system. However, overexpression of this invertase in
plants is known to interfere with plant growth (von Schae-
wen et al., 1990; Dickinson et al., 1991), which might ex-
plain why the yield of transformants was mostly low in our
experiments.

To study the cellular localization of the invertase fusions
protoplasts and vacuoles were isolated from leaves of
transformed plants and the recovered invertase activity
was considered intracellular and vacuolar, respectively. It
has to be considered when using this approach that the
steady-state level of invertase in the cells might be affected
mainly by proteolytic breakdown or alterations of trans-
scriptional/translational activities occurring during the
protoplast/vacuole isolation procedure. However, yeast
invertase is known to be very stable. When it was used in
targeting studies of yeast carboxypeptidase Y it was shown
that the carboxypeptidase Y portion of the fusion proteins
was rapidly degraded (50% within 15 min), whereas the
invertase portion remained stable during several hours
(Johnson et al., 1987). We tested the stability of the inver-
tase in crude leaf extracts by measuring the activity after
various storage times at room temperature; no reduction in
invertase activity was observed, even after 48 h (data not
shown).

On the other hand, it is known that certain promoters can
be induced by the stress conditions occurring during pro-
toplast isolation. For example, the promoter of an unknown
seed protein (USP) gene from field bean (Baumlein et al.,
1991) is significantly activated during the isolation of pro-
toplasts from transgenic tobacco plants (H. Baumlein, per-
sonal communication). To our knowledge this is not the
case with the constitutive CaMV 35S promoter used in our
studies. We tested this by gel blotting RNA from trans-
formed plants expressing 2Swt (Fig. 4). We observed a
somewhat lower relative level of 2Swt mRNA in the total
RNA prepared from protoplasts compared with that from
leaves (data not shown). Taken together, the great stability
of the invertase and the use of the CaMV 35S promoter
should ensure that there is no significant alteration of the
invertase level during the protoplast isolation.

The Vacuolar Targeting Signal of the 2S Albumin
Resides at the C Terminus

By comparing the results obtained with the N-terminal
2S albumin-invertase fusions (Fig. 2) with those obtained
with the C-terminal fusions (Fig. 3), one can conclude that
the vacuolar targeting information of the 2S albumin is
localized within the 20 C-terminal amino acids of
pro-2S albumin. Protoplast preparations from plants ex-
pressing the control construct SEC (invertase with the
legumin signal peptide) contained about 30% of the in-
vertase. This portion might represent intracellular inver-
tase localized in the ER (which is en route to the apo-
plast). The proportion of intracellular invertase was
much lower in plants expressing the N-terminal con-
struct N3 (Fig. 3) compared with those expressing SEC. Te
differences might be the result of different rates of
transport through the secretory system. Vacuoles iso-
lated from plants expressing SEC contained only approx-
imately 15% of the invertase contained within the leaves.
When the C-terminal domains from the 2S albumin (36
or 20 amino acids) were linked to the C terminus of
invertase (i.e. to SEC), 100% of the invertase was recov-
ered in the vacuoles. This result clearly shows that these
C-terminal domains contain vacuolar targeting informa-
tion.

When the complete 2S albumin precursor was linked to
the N terminus of invertase (according to construct N3), the
resulting fusion protein was efficiently secreted from to-
bacco cells (Fig. 2). This shows that the C-terminal 2S
albumin-targeting information, even though present in this
fusion protein, is nonfunctional when located in this part of
the protein. This could be true for further internal or N-
terminal domains of the 2S albumin. In legumin from field
bean, vacuolar targeting information was found in both
N-terminal and C-terminal domains (Saalbach et al., 1991).
Therefore, we generated constructs encoding fusion pro-
teins with shorter N-terminal segments of the 2S albumin.
Because of the difficulties with plant transformation (see
above), two of five generated constructs (data not shown)
could not be analyzed at all. Protoplasts and vacuoles
isolated from plants expressing N2 (Fig. 1B) contained
approximately one-third of the invertase of the total leaf
(Fig. 2). This might indicate that vacuolar targeting infor-
mation is also contained in N-terminal domains of the 2S
albumin. On the other hand, the data obtained with mu-
tated forms of the 2S albumin do not indicate this to be the
case.

Deletion analysis of the C-terminal targeting sequence
revealed that amino acids 16 to 10 (counted from the C
terminus of the precursor), representing the sequence
NLSPMRC, are important for efficient vacuolar target-
ing. The deletion of this sequence reduced the intracel-
lular invertase to a level comparable to that observed
with the control construct SEC. This shows that the
C-terminal propeptide is not sufficient for vacuolar tar-
geting. On the basis of the presented data it is not
possible to decide whether the C-terminal propeptide is
capable of directing a low level of invertase to the vacu-
ules. However, a low level of targeting would be sup-
ported by the fact that a putative vacuolar targeting
receptor binds to a peptide corresponding to the last
seven C-terminal amino acids of the 2S albumin precur-
sor (see below).

The C-terminal Propeptide of the 2S Albumin Is
Necessary for the Vacuolar Targeting

To investigate whether the C-terminal domain of pro-2S
albumin is necessary for the vacuolar targeting of the 2S
albumin, wild-type 2S albumin and forms mutated at the C
terminus were expressed in tobacco. Wild-type 2S albumin
(2Swt) was completely recovered in vacuoles isolated from
transformed plants (Fig. 5), indicating efficient localization
of the 2S albumin to the vacuoles of tobacco mesophyll cells. In accordance, pulse-chase experiments showed that 2Swt was efficiently retained inside cultivated protoplasts of the 2s albumin to the vacuoles of tobacco mesophyll cells. In accordance, pulse-chase experiments showed that pro-2S albumin (without the ER signal peptide) and a smear below 7 kD were observed both on western blots of total leaf extracts (Fig. 4B) and on fluorographs from pulse-chase experiments (Fig. 6, A and B). The smear may represent products resulting from proteolytic breakdown occurring in the vacuole. Mature 2S albumin consisting of the small (3.6 kD) and the large (8.6 kD) subunits was not found in these samples (Fig. 4), indicating that the processing of 2S albumin does not occur in tobacco leaf cells. This is in contrast to the specific expression of the Brazil nut 2S albumin in seeds of tobacco. In this case the 8.6-kD subunit was detected, suggesting correct processing in the seeds (Altenbach et al., 1989).

When the last four amino acids representing the CTPP were deleted the resulting protein (2SΔ4) was stably expressed. On gels the truncated precursor could be detected as a band migrating slightly faster (in accordance to its smaller size) than the wild-type 2S albumin precursor (Fig. 4B). In pulse-chase-labeling experiments 2SΔ4 was predominantly secreted into the culture medium of isolated protoplasts. The amount of degradation products was clearly lower than in the case of wild-type 2S albumin (being located to the vacuole). These results demonstrate that the CTPP, although not sufficient for efficient targeting, is an essential element of the 2S albumin-targeting signal.

The targeting of 2SΔ20, a mutated 2S albumin lacking the last 20 C-terminal amino acids, could not be analyzed and, in fact, could barely be detected on fluorographs after pulse labeling of protoplasts. We speculate that the lack of the 20 C-terminal amino acids, which include two Cys residues, causes improper folding, leading to degradation. This is comparable to data obtained with a truncated form of phaseolin, a 7S globulin from common bean. Unlike wild-type phaseolin, the truncated protein lacking the last 59 C-terminal amino acids was not able to form trimers. It was found to be associated with the binding protein BiP and to be rapidly degraded in transiently transformed protoplasts (Pedrazzini et al., 1994).

When a small peptide of four hydrophilic amino acids (SQSN) was added to the C terminus of the 2S albumin precursor, the resulting fusion protein (2S + 4) was retained inside the protoplasts, with similar efficiencies as the wild-type 2S albumin. This is in contrast to the hydrophobic barley lectin-targeting signal, in which the addition of GGG to the C terminus of an otherwise functional signal (VFA) abolished vacuolar targeting completely (Dombrowski et al., 1993).

Comparison of the 2S Albumin-Targeting Signal with Other Vacular Targeting Signals

The targeting sequences of all other vacuolar proteins tested were located either in C- or N-terminal propeptides or in the mature portion of the proteins. The targeting sequence of Brazil nut 2S albumin is the first identified that is made up of both a propeptide and a portion of the mature protein: the C-terminal propeptide and the 16 C-terminal amino acids of the mature protein.

In general, there is no homology among plant vacuolar targeting sequences. The 2S albumin signal does not exhibit any obvious homology with other known signals. The tetrapeptide IAGF is highly hydrophobic and in this respect the 2S albumin signal resembles the C-terminal signal of barley lectin. The N-terminal portion of the 2S albumin-targeting signal contains the sequence NLSPMRCP. This sequence is similar to the motif NPIRL/P of N-terminal targeting sequences of the plant vacuolar proteins sporamin (Matsuoka and Nakamura, 1991) and aleurain (Holwerda et al., 1992). Our deletion analysis revealed that both the sequence NLS and the sequence PMRC (particularly the latter) are important for efficient targeting. The omission of these sequences resulted in reduced targeting efficiencies. The peptide NLS in the targeting sequence represents the only potential glycosylation site of the 2S albumin. However, as determined by Ampe et al. (1986) the Brazil nut 2S albumin is not glycosylated. Therefore, glycosylation does not play any role in the vacuolar targeting of this 2S albumin. The barley lectin-targeting signal is glycosylated. This N-linked high-Man glycan slows the rate of processing of the proprotein but is also not required for the transport of barley lectin to vacuoles (Wilkins et al., 1990; Bednarek et al., 1991).

We also found that the location of the 2S albumin-targeting sequence in the protein is important for proper function (see above). In contrast, the N-terminal targeting sequence of sporamin was also functional in vacuolar targeting when linked to the C terminus of the mature portion of barley lectin (Dombrowski et al., 1993).

The Brazil nut 2S albumin shares homology with the Arabidopsis 2S albumin, in which none of the propeptides was necessary for vacuolar targeting (D’Hondt et al., 1993). In 2S albumin from Brazil nut, however, the C-terminal propeptide participated in directing vacuolar targeting. The C-terminal propeptide of Arabidopsis 2S albumin consists of only one amino acid (Tyr); the C-terminal region is also different in other ways from that of the Brazil nut 2S albumin. In accordance, a potential receptor for vacuolar targeting signals binding to the 2S albumin signal (Kirsch et al., 1996; see below) did not bind to the Arabidopsis 2S albumin C terminus. This suggests that vacuolar targeting information may reside in different structures, even among closely related proteins.

The N- and C-terminal 2S albumin gene constructs (shown in Figs. 2 and 3) were also tested in yeast (G. Saalbach and G. Kunze, unpublished results), and efficient retention inside the yeast cells was never observed. Only very long N- and C-terminal 2S albumin segments retained 40 to 50% of the invertase activity within the yeast cells. Several plant vacuolar proteins have been tested in yeast and, as with the 2S albumin, the results indicated that in yeast different signals are used for targeting to vacuoles (Gal and Raikhel, 1994).
Binding of the 2s Albumin-Targeting Signal to a Putative Vacuolar Targeting Receptor

A potential receptor (BP-80) for vacuolar targeting signals has been isolated from clathrin-coated vesicles of pea using a synthetic peptide with the sequence of the proaleurain signal (Kirsch et al., 1994). Binding experiments of BP-80 with peptides derived from the 2s albumin signal have been conducted (Kirsch et al., 1996). It was found that BP-80 also binds to the 2s albumin signal. Contrary to the in vivo data (this paper), the last nine C-terminal amino acids were sufficient to cause this binding. Mutation of the sequence PMR (see above) to FGR in the 2s signal had no effect on binding, nor did the omission of PMRC from the targeting sequence. This shows that the PMR motif is not required for binding. In our in vivo experiments, however, we found that this motif is important for efficient vacuolar targeting of the reporter protein (yeast invertase). In other respects, the data from these binding experiments correlated with the in vivo data reported here. In particular, the deletion of the tetrapeptide IAGF led to complete loss of binding, whereas the peptide GGSIAGF weakly bound BP-80.

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