The Plant Cell, Vol. 4, 307–318, March 1992 © 1992 American Society of Plant Physiologists

Proaleurain Vacuolar Targeting Is Mediated by Short Contiguous Peptide Interactions

Barry C. Holwerda,[a,1] Hal S. Padgett,[b] and John C. Rogers[a,b,2]

[a] Division of Hematology/Oncology, Washington University School of Medicine, St. Louis, Missouri 63110
[b] Division of Biology and Biomedical Sciences, Department of Biology, Washington University, St. Louis, Missouri 63130

Targeting of soluble proteins to the plant vacuole is mediated by determinants that reside in the polypeptide. We identified the vacuolar targeting determinant of aleurain, a plant vacuolar thiol protease, by incorporating different sequences from proaleurain into the secreted thiol protease, proendoproteinase B (proEP-B), and vice versa. The targeting fates of the chimeric proteins were analyzed by transient expression in electroporated tobacco protoplasts. The targeting determinant SSSSFADSNPIR is positioned at the N terminus of the aleurain propeptide, and its substitution into the propeptide of EP-B caused vacuolar targeting of the resulting chimeric protein. This determinant can be divided into two smaller determinants, SSSSFADS and SNPIR, each of which is sufficient to target proEP-B chimeras to the vacuole, but with lower efficiency. These smaller determinants interact in a positive manner because the combined determinant SSSSFADSNPIR targeted proEP-B with an efficiency greater than each of the smaller determinants alone. Accordingly, the efficiency of aleurain targeting was decreased when either of the smaller determinants was disrupted by replacement with similarly positioned proEP-B sequences. Further experiments on proaleurain identified an additional determinant, VTDRAAST, adjacent to the SSSSFADSNPIR determinant that is also necessary for efficient vacuolar targeting. Our results provide evidence that efficient vacuolar targeting of this thiol protease in plant cells is mediated by the combined action of smaller contiguous determinants; two of these alone are sufficient for vacuolar targeting.

INTRODUCTION

The vacuole is one of the most conspicuous compartments within a plant cell. This organelle, which is a part of the secretory system, performs numerous functions vital to cellular homeostasis that include the accumulation of amino acids, inorganic ions, and metabolic intermediates (reviewed by Matile, 1976, 1978). Vacuoles also contain a number of hydrolytic enzymes that degrade macromolecules and organelles in a function analogous to animal cell lysosomes. Economically, the most important aspect of plant vacuoles is their role in protein storage; specialized vacuoles, called protein bodies, are the site of storage protein deposition in plant reserve tissues such as the seed endosperm.

Recently, there has been much interest in elucidating the mechanisms by which vacuolar proteins are selectively targeted in plant cells. Vacuolar targeting requires positive sorting information (Dorel et al., 1989); proteins cotranslationally directed into the endoplasmic reticulum (ER) are secreted by plant cells if they have no apparent targeting information (Iturriaga et al., 1989; Denecke et al., 1990). In contrast to the mannose-6-phosphate-dependent targeting of proteins to lysosomes (reviewed by von Figura and Hasilik, 1986; Kornfeld, 1987; Kornfeld and Mellman, 1989), plant vacuolar proteins do not require N-linked oligosaccharides for targeting (Voelker et al., 1989; Sonnewald et al., 1990; Wilkins et al., 1990), suggesting that a feature of the polypeptide comprises the vacuolar targeting determinant. A number of studies have shown that the propeptides of vacuolar proteins are necessary for vacuolar targeting. Transgenic tobacco cells correctly sort barley lectin (Wilkins et al., 1990) and sweet potato sporamin (Matsuoka et al., 1990) into vacuoles. However, deletion of the 15-residue C-terminal extension of barley lectin or the 16-residue N-terminal propeptide of sporamin resulted in the secretion of the respective proteins from transgenic tobacco cells (Bednarek et al., 1990; Matsuoka and Nakamura, 1991, respectively). When the C-terminal extension of barley lectin or tobacco chitinase A was engineered onto a secreted protein, cucumber chitinase, the resulting chimeric molecules were sorted to the vacuole, therefore demonstrating that these propeptides are sufficient for vacuolar targeting (Bednarek and Raikhel, 1991; Neuhaus et al., 1991, respectively). In addition, it has been shown that a long segment of the seed storage protein legumin (i.e., 290 N-terminal residues) was sufficient for targeting a chloramphenicol acetyltransferase fusion protein to protein bodies in tobacco seeds (Saalbach et al., 1991). These

[1] Current address: Searle Research, Infectious Diseases, 700 Chesterfield Parkway North, St. Louis, MO 63198.
[2] To whom correspondence should be addressed at Department of Biology, Box 1137, Washington University, St. Louis, MO 63130.

vacuolar proteins have no apparent amino acid homology, and, in view of this, Chrispeels (1991) has suggested that three-dimensional structure may play an important role in vacuolar targeting.

In this article, we identify amino acid sequences in the propeptide of a plant vacuolar protein, aleurain, that are sufficient to cause a secreted protein, endoproteinase B (EP-B), to be targeted to plant vacuoles. Aleurain is a thiol protease that is closely related to the lysosomal enzyme cathepsin H (Rogers et al., 1985) and is targeted to vacuoles in barley aleurone cells (Holwerda et al., 1990), whereas EP-B is a thiol protease that is secreted by the same barley cells (Koehler and Ho, 1990a, 1990b). Aleurain and EP-B are cotranslationally directed to the lumen of the ER as 42- and 42.5-kD proenzymes that undergo proteolytic processing to form mature enzymes of 32 kD and 30 kD, respectively (Holwerda et al., 1990; Koehler and Ho, 1990a, respectively). By using the cDNAs of these differentially sorted proteins to make chimeric molecules, we identified amino acid sequences from proaleurain that, when substituted in proendoproteinase B (proEP-B), resulted in the targeting of EP-B to the vacuole. Conversely, replacement or disruption of the targeting determinant in proaleurain resulted in the secretion of aleurain. We determined the sorting fate of the chimeric proteins by transient expression in tobacco protoplasts using electroporation.

The targeting determinant SSSSFADSNPIR is located at the N terminus of the aleurain propeptide. It can be divided into two smaller determinants, SSSSFADS and SNPIR, that each are sufficient targeting determinants. However, the combined determinant SSSSFADSNPIR targeted more efficiently than the sum of the separate determinants, indicating a positive interaction between the parts. In addition, another sequence adjacent to these determinants, VTDRAAST, is also necessary for efficient targeting. These results indicate that plant vacuolar proteins may rely on more than one determinant for efficient vacuolar sorting and that these determinants interact in a positive manner.

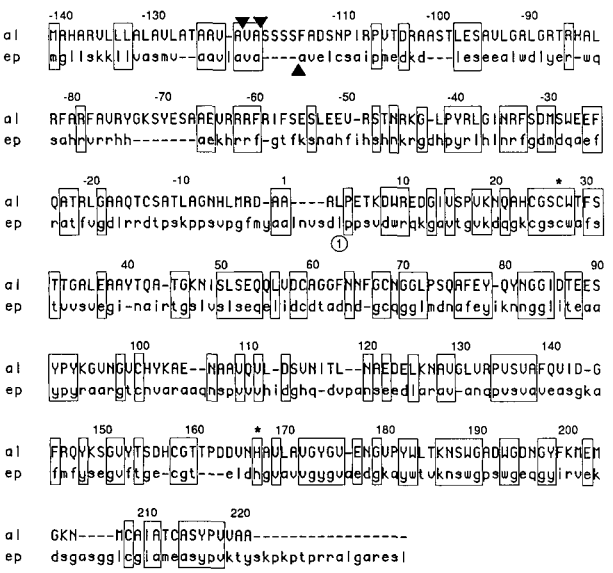

Figure 1. The Aligned Amino Acid Sequences of Proaleurain and ProEP-B.

The deduced amino acid sequences of proaleurain (al) (Rogers et al., 1985) and proEP-B (ep) (pHVEP4) (Koehler and Ho, 1990a) were aligned using the ALIGN program (Dayhoff et al., 1983). Identical amino acids are outlined, and gaps for optimal alignment are indicated by dashes. Numbering of amino acids is based on the N-terminal sequence of purified mature aleurain (B. Holwerda and J. Rogers, unpublished data). The N terminus of the mature EP-B is indicated by the number 1 enclosed by a circle (Koehler and Ho, 1990b). Signal sequence cleavage sites, denoted by solid triangles, were predicted using the SIGSEQ1 program (Folz and Gordon, 1987), which is based on the methods of von Heijne (1983, 1986). The active site C and H residues are denoted by asterisks.

RESULTS

The amino acid sequences of proaleurain and proEP-B derived from the respective cDNAs are aligned in Figure 1. Because aleurain and EP-B are both thiol proteases (46% amino acid identity in protease domains), it should be possible to make replacements of amino acid sequences between proaleurain and proEP-B with little disruption of three-dimensional structure in the recombinant protein. The targeting fate of these proteins was examined by transiently expressing them, by way of electroporation, in tobacco protoplasts.

Tobacco Protoplasts Target Aleurain to the Vacuole and Secrete ProEP-B

It was necessary to show that the barley proteins aleurain and EP-B were correctly and efficiently sorted by a heterologous system, tobacco protoplasts. The results of the pulse chase and subcellular fractionation experiments shown in Figure 2 indicated that wild-type barley aleurain is correctly and efficiently targeted to vacuoles in tobacco protoplasts. Immediately after labeling, the 42-kD proaleurain was the predominant protein recovered from immunoprecipitates of cell extracts, and after a 3-hr chase period, it was completely converted to mature 32-kD aleurain (Figure 2A). Quantitation by scintillation counting indicated that 100% of the proaleurain was converted to the mature protein by 3 hr and that neither protein was secreted into the media. The fact that 32-kD aleurain was not recovered from the media indicated that the protoplasts remained intact over the course of the pulse chase experiment. We previously showed that proaleurain was proteolytically processed in an acidified post-Golgi compartment and targeted to vacuoles in barley aleurone cells (Holwerda et al., 1990), and the results here indicated that tobacco protoplasts sort

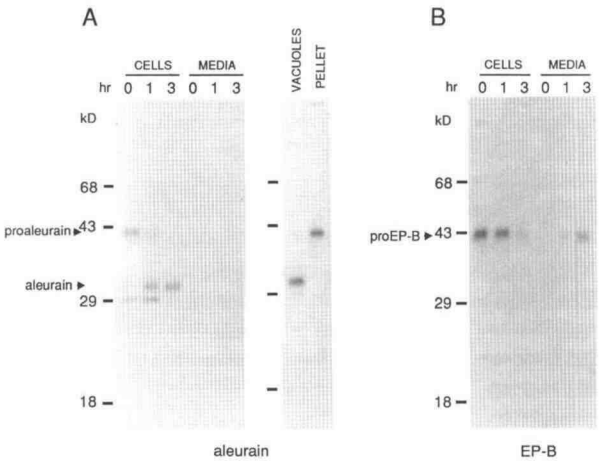

Figure 2. Aleurain and EP-B Are Correctly Sorted by Tobacco Protoplasts.

(A) Aleurain-expressing protoplasts. At left, tobacco protoplasts expressing aleurain were pulse-labeled and chased for the indicated times. Immunoprecipitates were made using aleurain antibodies and analyzed by SDS-PAGE and fluorography. At right, tobacco protoplasts expressing aleurain were subjected to subcellular fractionation immediately after labeling for 1 hr. Immunoprecipitates made with aleurain antibodies from vacuole and pellet fractions were analyzed by SDS-PAGE and fluorography.

(B) EP-B–expressing protoplasts. Tobacco protoplasts expressing EP-B were pulse-labeled and chased for the indicated times. Immunoprecipitates were made using anti–EP-B antibodies and analyzed by SDS-PAGE and fluorography.

proaleurain in the same manner as barley cells. To prove vacuolar targeting of aleurain in tobacco protoplasts, we prepared vacuoles from cells expressing aleurain and compared the enrichment of aleurain to the vacuolar marker α-mannosidase (Boller and Kende, 1979). Figure 2A (right) shows that mature 32-kD aleurain was the predominant form of the protein recovered from the vacuole fraction. Quantitation of aleurain and α-mannosidase activity in the vacuoles showed that both proteins were enriched by 2.8-fold over the unfractionated whole cell lysate. Proaleurain was the predominant protein immunoprecipitated from the pellet, indicating that this fraction contained ER and Golgi (Figure 2A, pellet). We previously reported that monensin and brefeldin A inhibited the proteolytic processing of proaleurain, indicating the post-Golgi location of this process in barley aleurone cells (Holwerda et al., 1990). The effects of brefeldin A in plant cells are not well documented; however, we have found that it causes no adverse effects: barley aleurone cells treated with the drug continued normal processing and targeting of proaleurain after it was removed (B.C. Holwerda and J.C. Rogers, unpublished results). Monensin did not block secretion of proteins by tobacco protoplasts (data not shown), but brefeldin A, which blocks forward ER to Golgi trafficking (Lippincott-Schwartz et al., 1989), prevented the conversion of proaleurain to mature aleurain in tobacco protoplasts (data not shown). A 29-kD band visible in some of the immunoprecipitates (Figure 2A) was a nonspecific contaminant that was also seen in immunoprecipitates from nontransfected tobacco protoplasts using either aleurain or EP-B antibodies (data not shown).

In contrast to the vacuolar targeting of aleurain, tobacco protoplasts secreted proEP-B. The pulse-chase experiment illustrated in Figure 2B shows that 42-kD proEP-B found in cells at 0 hr was secreted during the chase period. After a chase period of 3 hr, 50% of the proEP-B was recovered from the media, whereas 24% remained with the cells. The remaining 26% could not be recovered from any fraction and was most likely degraded by nonspecific proteases in the media. There are a number of hydrolytic activities that are difficult to remove from protoplast preparations; residual proteolytic activities probably remained from enzyme mixtures used to make tobacco protoplasts (Boller and Kende, 1979). In any case, none of the proEP-B was recovered as a smaller mature protein in either the cells or media. Barley aleurone cells normally convert proEP-B to a 30-kD mature form during and after secretion (Koehler and Ho, 1990a), and, therefore, the lack of proteolytic processing in tobacco protoplasts indicates that these cells do not have the necessary processing enzymes in their default (secretion) pathway. However, when appropriate chimeric proEP-B derivatives were targeted to the vacuole, they were proteolytically processed/degraded to smaller forms (see below). The recovery of these smaller forms served as an indicator of vacuolar targeting.

The Proaleurain Targeting Determinant Can Be Divided into Two Smaller Determinants that Each Target ProEP-B to the Vacuole

Having established that aleurain and EP-B were correctly sorted by tobacco protoplasts, we identified a sequence in proaleurain that, when substituted for similarly positioned residues in proEP-B, caused vacuolar targeting of the resulting chimeric proEP-B molecule. We focused on the propeptide of aleurain for a number of reasons. The propeptides of EP-B and aleurain show significant differences; they have lower amino acid identity (22%) compared to the mature enzymes (46%), and the aleurain propeptide is longer by 8 to 10 residues (Figure 1). Changes were made to the N-terminal portion of the propeptides because this region may be the most accessible to the putative vacuolar sorting machinery.

The results in Figures 3 and 4 show that aleurain has a determinant in its propeptide that is sufficient to cause sorting of proEP-B to vacuoles and that this determinant can be divided into smaller parts that are also sufficient for targeting. Construct 1 incorporates SSSSFADS from the aleurain propeptide (Figure 1; residues −119 to −112) into proEP-B (Figure 3, top). When construct 1 was expressed in protoplasts, some of the 43-kD product was secreted (Figure 3A, media), but in contrast to wild-type proEP-B, some of the 43-kD product was converted to 39- and 33-kD products that remained in cells

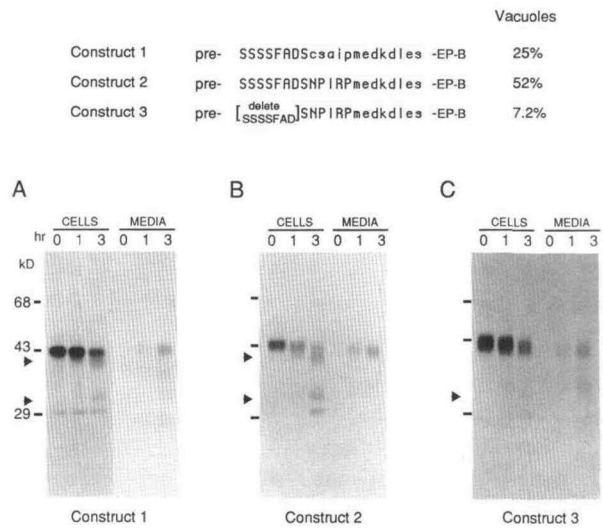

Figure 3. Determinants from the Aleurain Propeptide that Cause Targeting of ProEP-B to a Proteolytic Compartment—Vacuoles.

At the top, the deduced amino acid sequences of constructs 1, 2, and 3 are shown with aleurain residues in uppercase letters and EP-B residues in lowercase letters. The signal peptide (pre) for these constructs is from aleurain and continues toward the N terminus as shown in Figure 1. With the exclusion of the indicated amino acids from aleurain, the remainder of each construct is derived from EP-B. The column on the right indicates the vacuolar targeting efficiency (see text).
(A) Protoplast treatment. Tobacco protoplasts expressing construct 1 were pulse-labeled and chased for the indicated times. Immunoprecipitates were made using anti–EP-B antibodies and analyzed by SDS-PAGE and fluorography.
(B) Tobacco protoplasts expressing construct 2 treated as in **(A)**.
(C) Tobacco protoplasts expressing construct 3 treated as in **(A)**.
Arrowheads at 33 and 39 kD denote proteins that were recovered from a vacuole enriched fraction (see Figure 4B).

after a 3-hr chase period (Figure 3A, indicated by arrows). Construct 2 incorporates a larger portion of the aleurain propeptide SSSSFADSNPIR (Figure 1; residues −119 to −108) into proEP-B (Figure 3, top). Construct 2 produced qualitatively similar results to those for construct 1 (Figure 3B); some of the 43-kD proprotein was secreted, and some was converted into two products at 39 and 33 kD that were retained by cells. Construct 3, which substitutes SNPIR into proEP-B (Figure 3, top), also secreted some 43-kD proEP-B and accumulated a small, but reproducible, amount of cell-associated 33-kD product at 3 hr (Figure 3C); it did not, however, accumulate a discernible 39-kD product. With each of the three constructs, there was some 43-kD proEP-B remaining with the cells at 3 hr (Figures 3A to 3C). A portion of this was likely committed for secretion because wild-type proEP-B was not completely secreted by 3 hr (Figure 2B, cells) and each of constructs 1 through 3 secreted some proenzyme. However, the remainder of this proprotein did not leave the ER, as shown by the recovery of a 43-kD protein in the ER/Golgi fraction even after a 5-hr chase period (see below; Figure 4B, pellet). This proprotein was probably retained in the ER because it was folded incorrectly. Incorrectly folded or assembled proteins are known to be retained in the ER, and some of these are degraded at variable rates (Lippincott-Schwartz et al., 1988; Hurtley and Helenius, 1989; Pelham, 1989).

We predicted that proEP-B chimeric proteins would undergo some form of proteolytic processing and/or degradation if they were targeted to the vacuole because this organelle/pathway contains a number of proteolytic processing activities (Holwerda et al., 1990). Because wild-type proEP-B was not converted to a smaller mature form when it was secreted by tobacco cells (Figure 2B), the accumulation of 39- and 33-kD products was consistent with targeting of these proEP-B derivatives to a proteolytic compartment such as the vacuole.

To show that the 39- and 33-kD proteins were not the result of degradation in the ER, we treated protoplasts with brefeldin A before pulse labeling. When transport beyond the ER/Golgi interchange was inhibited with brefeldin A, cells expressing construct 2 accumulated a 43-kD protein (Figure 4A), but in contrast to untreated cells they did not secrete or convert any of this proEP-B derivative to either 39- or 33-kD derivatives,

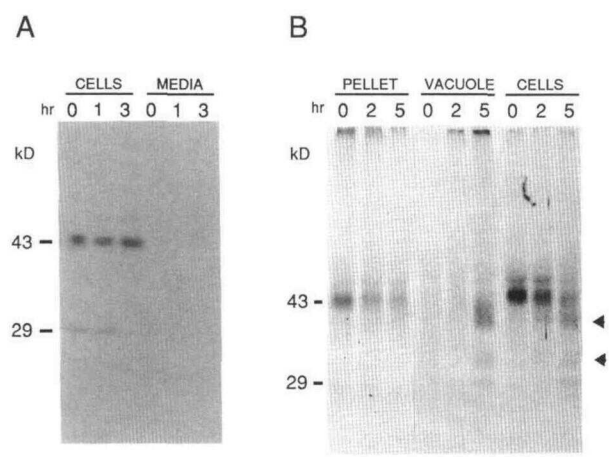

Figure 4. Brefeldin A Prevents Sorting of ProEP-B Derivatives to the Vacuole.

(A) Incubation with brefeldin A. Tobacco protoplasts expressing construct 2 were preincubated in 20 μg/mL of brefeldin A before pulse-labeling and chasing with unlabeled amino acids for the indicated times. Immunoprecipitates were made using anti–EP-B antibodies and analyzed by SDS-PAGE and fluorography.
(B) ER/Golgi fraction. Tobacco protoplasts expressing construct 2 were pulse-labeled and chased with unlabeled amino acids for the indicated times. At each time point, cells were fractionated and separated into vacuole and ER plus Golgi-containing fractions in addition to making extracts from unfractionated cells as indicated. Immunoprecipitates were made using anti–EP-B antibodies and analyzed by SDS-PAGE and fluorography. Arrowheads indicate proteolyzed vacuolar 33- and 39-kD EP-B derivatives.

as seen in Figure 3B. Similar results were observed when protoplasts expressing constructs 1 or 3 were treated with brefeldin A (data not shown). The inhibition of proteolytic processing/degradation by brefeldin A indicated that this event(s) must occur downstream of the ER/Golgi interchange, most likely in a proteolytic compartment such as a vacuole.

To show that the 39- and 33-kD products from constructs 1, 2, and 3 were localized in vacuoles, we analyzed subcellular fractions prepared from pulse-labeled tobacco protoplasts (Figure 4B). The 43-kD proEP-B derivative from construct 2 was the predominant protein recovered from the ER/Golgi-containing pellet fractions in amounts that decreased over a 5-hr chase period (Figure 4B, pellet). Parallel to the loss of the 43-kD proprotein was accumulation of the 39- and 33-kD derivatives in the vacuole fractions (Figure 4B, vacuole). Similar results were obtained for constructs 1 and 3 (data not shown). These results indicated that products of constructs 1, 2, and 3 are targeted to vacuoles. As a consequence of this targeting, they undergo proteolytic processing/degradation forming a 33-kD derivative and, in the case of constructs 1 and 2, an additional 39-kD derivative.

These results defined a sufficient vacuolar targeting determinant SSSSFADSNPIR (construct 2) that can be divided into two smaller, but still sufficient, determinants, SSSSFADS (construct 1) and SNPIR (construct 3). The fact that only smaller proteolyzed forms accumulated in vacuoles allowed us to estimate targeting efficiency by measuring the amounts of 39- and 33-kD proteins. This was determined after a 3-hr chase period because wild-type aleurain, our standard targeted protein, was completely sorted after 3 hr (Figure 2A). Quantitation of the 39- and 33-kD proteins from construct 2 (SSSSFADSNPIRP-EP-B) showed that 52% (n = 3) of the 43-kD proprotein (at 0 hr) was targeted to vacuoles by 3 hr. However, both of the constructs with smaller parts of this determinant targeted with lower efficiency; construct 1 (SSSSFADS-EP-B) targeted with 25% (n = 2) efficiency and construct 3 (SNPIR-EP-B) targeted with 7.2% (n = 2) efficiency based on the amount of the 33-kD protein. The fact that both smaller determinants sort at lower efficiency when separated indicates that they interact in a positive manner when they are combined in the larger determinant.

Alterations in the Sorting Determinants Decrease the Targeting Efficiency of Proaleurain

Having established that SSSSFADSNPIR is sufficient for vacuolar targeting of proEP-B, it was important to perform the converse experiment and alter this determinant and/or neighboring residues in proaleurain. If a determinant is necessary, a change in it should lower targeting efficiency. Because wild-type aleurain was targeted with 100% efficiency in tobacco protoplasts (Figure 2A), recovery of proaleurain in the media served as a sensitive indicator of aberrations in targeting. We did not use the amount of proaleurain recovered from the media to calculate targeting efficiency because of variable losses due to nonspecific degradation, as described for proEP-B (above). Instead, we estimated targeting efficiency as the ratio of 32-kD mature aleurain recovered after a 3-hr chase period over the amount of 42-kD proaleurain derivative at 0 hr.

The results in Figure 5 show that both parts of the SSSSFADSNPIR determinant (i.e., SSSSFAD and SNPIR) and the adjacent residues (VTDRAAST) are necessary for efficient vacuolar targeting of proaleurain. Construct 4 tested the contribution of SSSSFADS by deleting SSSSFAD from the propeptide of aleurain (Figure 5, top). Tobacco protoplasts expressing construct 4 targeted most (90%; n = 2) of the mutant proaleurain to the vacuole, as evidenced by the accumulation of mature 32-kD aleurain (Figure 5A). However, in contrast to wild-type aleurain, detectable amounts of the 42-kD proprotein were secreted (Figure 5A, media), indicating that the targeting determinant had been altered in a negative manner. In construct 5, the SNPIR determinant was disrupted by replacing NPIR with similarly positioned residues of proEP-B (Figure 5, top). The targeting fate of construct 5 was similar to that for construct 4, but less was targeted to the vacuole (24%; n = 3; Figure 5B).

Both of these results (constructs 4 and 5) and results for construct 1 (SSSSFADS-EP-B) and construct 3 (SNPIR-EP-B) demonstrate that SSSSFADS and SNPIR each make important contributions to the targeting determinant. It is not possible, however, to compare the targeting efficiencies of each sequence between the positive (i.e., sorting of proEP-B) and negative (i.e., loss of targeting of proaleurain) experiments. First, comparisons of targeting efficiencies need to account for differences in transport competence from the ER. For example, almost all construct 4 proprotein left the ER after a 3-hr chase period (as evidenced by the lack of a 43-kD band; Figure 5), whereas ~50% of construct 5 proprotein remained in the ER after the same chase period. Of the 50% of construct 5 proprotein that left the ER, ~48% (i.e., 24% ÷ 50%) was targeted. Second, the presentation of the targeting determinant to a putative receptor will depend upon the overall conformation of the protein. Again, with construct 5, replacement of NPIR not only changed the SNPIR determinant, but it may have disrupted the context of the remaining SSSSFADS determinant and thereby led to a greater decrease in efficiency than might be caused by the loss of one determinant alone. A chimeric protein with both parts of the determinant altered or removed, pre-(deleted SSSSFADS)SCSAI-aleurain, did not express well and was not studied further.

We then tested the role of amino acids immediately proximal to the combined determinant. Construct 6 is the same as wild-type aleurain, except that the residues adjacent to the combined determinant VTDRAAST (Figure 1; residues −106 to −99) were replaced by similarly positioned residues (MEDKD) from proEP-B. This exchange was based on alignment of the shared LES residues (Figure 1; residues −98 to −96) and deleted three residues from the respective aleurain derivatives. Although construct 6 has the combined determinant, only 62% (n = 3) of the product was targeted to the vacuole (Figure 5C). This result is consistent with two

			Vacuoles
Construct 4	pre-	[delete SSSSFAD]SNPIRPVTDRAASTLES -aleurain	90%
Construct 5	pre-	SSSSFADScsaipVTDRAASTLES -aleurain	24%
Construct 6	pre-	SSSSFADSNPIRPmedkd...LES -aleurain	62%
Construct 7	pre-	[delete SSSSFAD]SNPIRPmedkd...LES -aleurain	3.3%
Construct 8	pre-	SSSSFADScsaipmedkd...LES -aleurain	13%

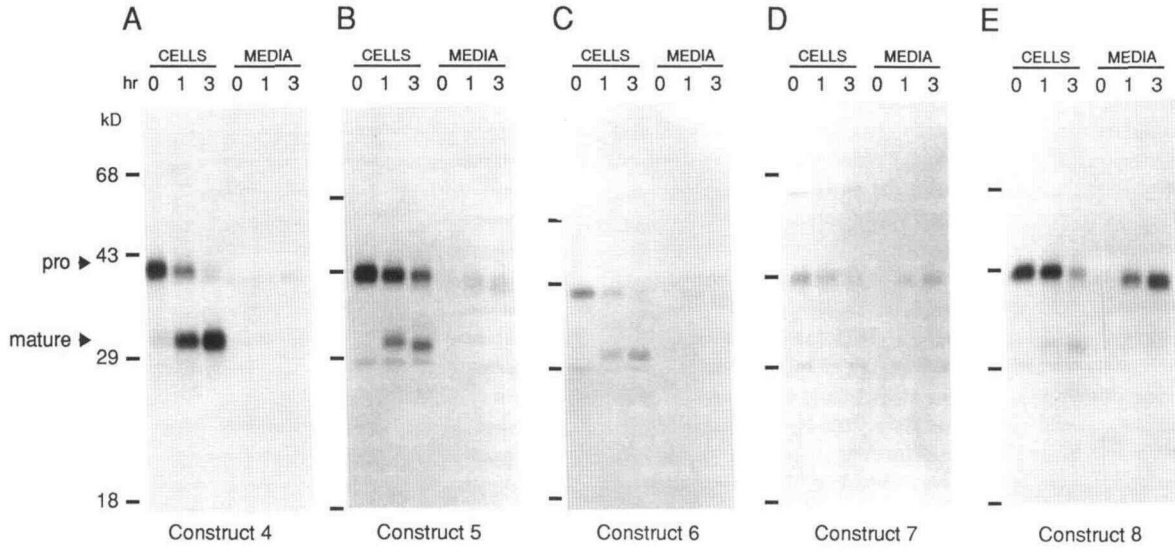

Figure 5. Changes to the Aleurain Targeting Determinants Cause a Loss of Vacuolar Targeting Efficiency.

At the top, the deduced amino acid sequences of constructs 4 through 8 are shown with aleurain residues in uppercase letters and EP-B residues in lowercase letters. The signal peptide (pre) for these constructs is from aleurain and continues toward the N terminus, precisely as shown in Figure 1. With the exclusion of the indicated amino acids from EP-B, the remainder of each construct is derived from aleurain. The column on the right indicates the vacuolar targeting efficiency (see text).

(A) Protoplast treatment. Tobacco protoplasts expressing construct 4 were pulse-labeled and chased for the indicated times. Immunoprecipitates were made using aleurain antibodies and analyzed by SDS-PAGE and fluorography.

(B) Tobacco protoplasts expressing construct 5 treated as in **(A)**.

(C) Tobacco protoplasts expressing construct 6 treated as in **(A)**.

(D) Tobacco protoplasts expressing construct 7 treated as in **(A)**.

(E) Tobacco protoplasts expressing construct 8 treated as in **(A)**.

Arrowheads at 42 and 32 kD indicate the position of the proaleurain derivatives and mature aleurain, respectively.

possible explanations; either VTDRAAST is a targeting determinant, and/or the combined targeting determinant was presented in a less optimal context. We could not determine if this sequence was sufficient for targeting because constructs that incorporated VTDRAAST into proEP-B made proteins that were seriously impaired in their ability to leave the ER (see below).

We next examined the effect of replacing VTDRAAST (with MEDKD) in combination with each of the smaller determinant parts. Construct 7, which incorporates SNPIR adjacent to MEDKD (Figure 5, top), produced a protein of which only 3.3% (n = 2) was targeted to the vacuole (Figure 5D). The drastically reduced targeting efficiency of construct 7 provides further evidence that VTDRAAST itself is a targeting determinant, but there probably are additional confounding effects due to a change in context on the SNPIR determinant. These ideas are strengthened by comparison with construct 4, which has VTDRAAST adjacent to SNPIR and targeted with 90% efficiency. Construct 8 has the SSSSFADS determinant positioned five residues away from MEDKD (Figure 5, top) and targeted with 13% (n = 1) efficiency (Figure 5E). In comparison, construct 5, which has the same determinant (SSSSFADS)

positioned five residues away from VTDRAAST, targeted with almost twice the efficiency (24%) as construct 8 (13%). The consistent increase in targeting efficiency with constructs having VTDRAAST (versus MEDKD) strongly suggests that this sequence is acting as a targeting determinant. However, we cannot exclude the possibility that this positive effect resulted from a more optimal context for the other determinants.

Small Changes in the Propeptide Affect the Transport Competence of ProEP-B

In our efforts to test whether VTDRAAST could target EP-B to the vacuole, we made the three constructs shown in Figure 6. The common feature of these proEP-B derivatives is that they all have the aleurain residues VTDRAAST replacing MEDKD. Constructs 9 and 10 have the entire determinant (SSSSFADSNPIR); construct 9 links aleurain at residue −92 to EP-B at −80 (Figure 1); construct 10 links aleurain and EP-B at the shared LES residues, whereas construct 11 is similar to construct 10, but has only the SNPIR determinant. Only the results for construct 9 are presented in Figure 6; similar results were found for constructs 10 and 11 (data not shown). When these constructs were expressed in tobacco protoplasts, they produced two proteins of 43 kD and 37 kD that were stably maintained within the cells (Figure 6A). The more abundant 43-kD protein is consistent with the predicted size of the chimeric proenzyme; the source of the less abundant 37-kD protein is not clear. The relative amounts of the two proteins did not change over a 3-hr chase period, suggesting there was no precursor–product relationship. Subcellular fractionation showed that the 43-kD protein was not in the vacuoles, but was localized in the ER and/or Golgi because it was recovered in the pellet fraction (Figure 6B). Blocking transport beyond the ER/Golgi with brefeldin A made no difference in the relative amounts of either protein (Figure 6C), indicating that the 37-kD protein is not the product of some vacuolar processing event that could not be recovered in immunoprecipitates of the vacuole fractions. The 37-kD protein may be an anomalous cytoplasmic product of these constructs, but we did not investigate it further. The fact that these three constructs each produce a major 43-kD product that does not leave the ER suggests that MEDKD is essential to the development of transport-competent proEP-B molecules, perhaps by facilitating proper folding. These results emphasize that small changes in the sequence of a secreted protein may have unexpected effects on its behavior in the endomembrane system of plant cells.

DISCUSSION

The goal of our research is to gain an understanding of the mechanisms by which particular proteins are recognized in the plant secretory pathway and targeted to vacuoles. As part of this aim, we designed experiments to identify amino acid sequences in proaleurain that are necessary and sufficient for targeting to vacuoles. Our experiments involved following the targeting fate of structurally altered proteins in tobacco protoplasts. Although this approach has been used in a number of other plant vacuolar targeting studies (Bednarek et al., 1990; Wilkins et al., 1990; Bednarek and Raikhel, 1991; Chrispeels,

Construct 9 pre- SSSSFADSNPIRPUTDRAASTLESAULGhruvr -EP-B

Construct 10 pre- SSSSFADSNPIRPUTDRAASTleseeal -EP-B

Construct 11 pre- [delete SSSSFAD]SNPIRPUTDRAASTleseeal -EP-B

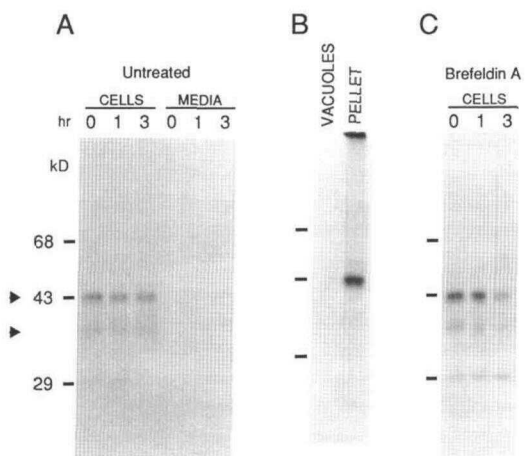

Figure 6. Replacement of MEDKD with VTDRAAST Causes Retention of ProEP-B Derivatives in the ER.

At the top, the deduced amino acid sequences of constructs 9, 10, and 11 are shown with aleurain residues in uppercase letters and EP-B residues in lowercase letters. The signal peptide (pre) for these constructs is from aleurain and continues toward the N terminus as shown in Figure 1. With the exclusion of the indicated amino acids from aleurain, the remainder of each construct is derived from EP-B.

(A) Pulse chase analysis. Tobacco protoplasts expressing construct 10 were pulse-labeled and chased with unlabeled amino acids for the indicated times. Immunoprecipitates were made using anti–EP-B antibodies and analyzed by SDS-PAGE and fluorography. Arrowheads denote the locations of the 43-kD proEP-B derivative and the minor 37-kD product (see text).

(B) Subcellular fractionation. Tobacco protoplasts expressing construct 10 were subjected to subcellular fractionation immediately after labeling for 1 hr. Immunoprecipitates made with anti–EP-B antibodies from vacuole and pellet fractions were analyzed by SDS-PAGE and fluorography.

(C) Preincubation with brefeldin A. Tobacco protoplasts expressing construct 10 were preincubated in 20 μg/mL of brefeldin A before pulse-labeling and chasing for the indicated times. Immunoprecipitates were made using anti–EP-B antibodies and analyzed by SDS-PAGE and fluorography.

1991; Neuhaus et al., 1991; Saalbach et al., 1991), it has a potential drawback; making structural alterations to any protein may cause unpredictable and possibly adverse changes in its conformation. To minimize secondary structural effects, we made chimeric molecules from two structurally related thiol proteases. Our approach was to transfer sequences from aleurain into another member of this group in an attempt to minimize changes in the overall three-dimensional structure of the resulting chimeric molecule. The availability of a well-characterized thiol protease (EP-B) that is secreted by the same cells (aleurone) made it an ideal candidate for this purpose. Transient expression in tobacco protoplasts using electroporation served as a reliable method to assay targeting efficiency of the test constructs.

We identified sequences in the propeptide of aleurain that are necessary and sufficient for targeting to vacuoles in plant cells. A major result of this study is that the proaleurain targeting determinant is comprised of smaller positively interacting parts; SSSSFADSNPIR can be divided into two smaller parts, SSSSFADS or SNPIR, each of which is able to redirect a normally secreted protein to the plant vacuole. These targeting determinants were defined on the basis of two criteria. First, their incorporation into the propeptide of a normally secreted protein (EP-B) resulted in the vacuolar targeting of the novel proEP-B derivatives, and second, the disruption of these determinants in proaleurain resulted in a decrease in targeting efficiency. In addition to the SSSSFADSNPIR determinant, the adjacent residues VTDRAAST are also necessary for efficient sorting of proaleurain. This leads us to conclude that the entire sequence SSSSFADSNPIRPVTDRAAST in the propeptide of aleurain is necessary and sufficient for efficient delivery of this protein to a plant vacuole. We could not evaluate whether VTDRAAST alone was sufficient for targeting because substitution of this sequence for MEDKD in proEP-B produced transport-incompetent molecules; the fact that replacement of MEDKD consistently led to this result suggests that this sequence is important to proper folding of proEP-B. In mammalian cells, folding aberrations in a single domain of an otherwise correctly folded protein can cause ER retention (Hurtley and Helenius, 1989).

The proaleurain vacuolar targeting determinant was identified by both positive and negative sorting criteria. The earliest reports identifying sorting determinants relied on detecting secretion of vacuolar proteins after removal of amino acid sequences thought to be recognized for targeting (Bednarek et al., 1990; Matsuoka and Nakamura, 1991). Although this approach can only identify necessary sequences, it has proved to be an important complement to further studies aimed at establishing targeting sufficiency of a determinant. This was shown by Bednarek and Raikhel (1991), who reported that the C-terminal extension of barley lectin is sufficient for targeting cucumber chitinase (a normally secreted protein) to the vacuole; an earlier study showed that removal of this peptide from barley lectin resulted in secretion (Bednarek et al., 1990). In a different study, the C-terminal extension of tobacco chitinase A was also shown to be necessary and sufficient for vacuolar targeting using positive and negative sorting criteria (Neuhaus et al., 1991). Although we identified our determinants as both necessary and sufficient, we cannot rule out the possibility that a particular association with some conserved motif in a thiol protease contributes to their recognition as a targeting determinant.

The concept of multiple targeting domains was first proposed by Tague et al. (1990) in their study of phytohemagglutinin (PHA) targeting in yeast. The targeting of PHA–invertase fusions to yeast vacuoles using a short sequence of the PHA propeptide showed that the determinant LQRD was sufficient for vacuolar targeting. However, in the context of a larger stretch of PHA a different determinant appeared to be functioning because disruption of LQRD caused little change in the amount of PHA–invertase fusion protein targeted to yeast vacuoles. Further evidence for the existence of multiple targeting determinants has come from a study of bean legumin sorting in yeast (Saalbach et al., 1991). By utilizing legumin–invertase fusion proteins, it was found that long segments from either the N-terminal or C-terminal of legumin (281- and 76-amino acid residues, respectively) were sufficient for vacuolar targeting in yeast. Because different parts of legumin directed invertase to the vacuole, it was concluded that multiple vacuolar targeting determinants exist in legumin. The concept of multiple targeting determinants was developed from experiments using yeast cells, and although plant and yeast vacuoles share many analogous functions, it is not clear that they share the same mechanisms for vacuolar sorting. Chrispeels (1991) reported that plant and yeast vacuolar targeting determinants are different because PHA–invertase fusions that were efficiently targeted in yeast cells were secreted by plant (Arabidopsis) cells.

Carboxypeptidase (CPY) and proteinase A (PrA) have served as model proteins to identify yeast vacuolar sorting determinants. A stretch of 11 to 13 amino acids near the N terminus of proCPY is necessary and sufficient for vacuolar targeting (Johnson et al., 1987; Valls et al., 1987), and four of these residues, QRPL, are known to be required for efficient targeting (Valls et al., 1990). Part of the PrA propeptide also targets invertase–fusion proteins to the vacuole, but it has no homology to the primary sequence of the CPY propeptide (Klionsky et al., 1988). The proaleurain targeting determinants (SSSSFADSNPIR or VTDRAAST) show no homology to the propeptides of CPY or PrA. If there are similarities between yeast and plant vacuolar targeting mechanisms, they appear not to exist in the recognition of sorting determinants on the respective vacuolar proteins. Interestingly, the aleurain targeting determinants are arranged at the N terminus of the propeptide immediately adjacent to the signal peptide; the same arrangement is found for the QRPL targeting determinant of yeast CPY (Rothman et al., 1989; Valls et al., 1990). Perhaps this region of both propeptides is most accessible to the sorting machinery.

Comparison of the aleurain SNPIR determinant to the sporamin propeptide (Matsuoka et al., 1990) shows identity of four residues, NPIR. The 16–amino acid sporamin propeptide was

shown to be necessary for vacuolar targeting in tobacco cells (Matsuoka and Nakamura, 1991), but it has not been reported whether the sporamin propeptide is composed of more than one independently acting determinant in addition to the apparent NPIR determinant. There are no sequence homologies between the aleurain targeting determinants and the C-terminal extension of barley lectin (Bednarek et al., 1990). The C-terminal extension of barley lectin is predicted to form an α-helical secondary structure (Wilkins and Raikhel, 1989), whereas the parameters of Chou and Fasman (1978) predict that the combined determinants of aleurain have little propensity to form any α-helical structure. The only general consensus among the known plant targeting determinants is that they contain a number of residues with hydroxyl groups (S and T residues) interspersed with both negatively and positively charged residues. It is interesting that the cDNA sequence for the rice homolog of barley aleurain, oryzain γ (Watanabe et al., 1991), shows that most of the targeting determinants we identified are conserved (i.e., ASSGFDDSNPIRSVTDHAASA; underlined residues are identical to aleurain). Although it remains to be demonstrated that oryzain γ is targeted to vacuoles, the conserved residues most likely include those crucial to the sorting process.

Another major conclusion from this work is that the determinants interact in a positive manner. The amount of EP-B targeted to the vacuole by the combined determinant (SSSSFADSNPIR) was more than the sum of either separate determinant alone. A portion of this cooperative interaction is probably due to the determinants providing an optimal context for each other. To date, a vacuolar targeting receptor has not been described in either plants or yeast. If a single receptor is responsible for targeting aleurain, it may have an extensive binding site that interacts with each part of the proaleurain determinant. In such a model, the positive interaction would be explained by the binding of part of the determinant that brings the remainder of the determinants into close proximity, resulting in higher binding affinity. Alternatively, there may be a finite number of low-affinity receptors that each binds a specific part of the determinant. Efficient vacuolar targeting could then rely on the interaction of a number of these receptors with an end result similar to that expected for a single high-affinity receptor. In this case, proaleurain would be predicted to interact with at least two or possibly three of these putative receptors during the sorting process. Whichever model is correct, a general mechanism for plant vacuolar protein sorting will have to account for the large number of different vacuolar proteins that appear to have no primary sequence identities.

METHODS

DNA Constructions

All the constructs were derived by combining parts of the aleurain cDNA (Rogers et al., 1985) and the endoproteinase B (EP-B) cDNA (pHVEP4) (Koehler and Ho, 1990a). The constructs were assembled in pUC18 (Yanisch-Perron et al., 1985) to facilitate screening and DNA sequencing; all polymerase chain reaction (PCR)–derived fragments and ligation sites were sequenced entirely. The inserts encoding the aleurain–EP-B chimeric protein were then excised from pUC18 and used to replace the β-glucuronidase sequence in pBI221 (Jefferson, 1987). In this manner, each construct was expressed under the control of the cauliflower mosaic virus 35S promoter and a nopaline synthase 3′-terminating sequence. Insertion of the aleurain cDNA into this expression vector yielded pJR258 and with the EP-B cDNA yielded pJR257.

Construct 1 (pJR292) was made by ligating a PCR-derived fragment from the aleurain cDNA 5′ end encoding the signal peptide and the first eight residues (SSSSFADS) of the propeptide with EP-B at a unique PstI site in the respective propeptide (see Figure 3, top). The amplified product was made using the universal reverse sequencing primer and a primer (5′-ACCTGCAGGAGTCGGCGAAGGA-3′) that added a novel PstI site to the 3′ end of the aleurain-derived product.

Construct 9 (pJR279) was made by ligating a PCR-derived fragment from the 5′ end of the aleurain cDNA to position 186 (Rogers et al., 1985) with a novel DraIII site at its 3′ end to the first DraIII site in EP-B (position 157 in pHVEP4) (Koehler and Ho, 1990a). The primers were the universal reverse sequencing primer and 5′-CGCACGCGGTGGCCGAGGACGGC-3′.

All other constructs were made by the splicing overlap extension technique (Horton et al., 1989) using aleurain and EP-B cDNAs as templates. Separate PCR-derived fragments were made from the aleurain and EP-B cDNAs using primers that produced overlapping ends that precisely defined the fusion point in the final construct. These first-round PCR-derived fragments were combined in a second PCR reaction using only the distal primers from the first reactions to generate a precise fusion between aleurain and EP-B. The distal primers were the universal reverse or forward sequencing primers and 5′-GCTCTCGGAGAAGATCC-3′ for aleurain fragments and 5′-TTGTTGTGGGAGTGGAT-3′ for EP-B fragments. Some of the constructs were derived from others by using the latter as templates in the first round of PCR reactions: constructs 3 and 11 required construct 4 as a template, constructs 5 and 8 required construct 1, construct 6 required construct 2, and construct 7 required construct 3 as a template. The following primers were used in these PCR reactions to make overlapping ends that defined the fusion junctions:

construct 2 (pJR309), construct 3 (pJR311), construct 6 (pJR331), and construct 7 (pJR333)

```
          5′-CCAACCCGATCCGGCCGATGGAGGACAAG-3′
          3′-GGTTGGGCTAGGCCGGCTACCTCCTGTTC-5′
amino acids      N  P  I  R  P  M  E  D  K
```

construct 4 (pJR291)

```
          5′-GCCGTCGCCGTCGCCTCCAACCCGATCCGG-3′
            3′-GCAGCGGCAGCGGAGGTTGGGCTAGGCC-5′
amino acids        V  A  V  A  S  N  P  I  R
```

construct 5 (pJR313)

```
          5′-GCAGCGCCATCCCGGTCACCGACCGCGC-3′
          3′-CGTCGCGGTAGGGCCAGTGGCTGGCGCG-5′
amino acids      S  A  I  P  V  T  D  R
```

construct 8 (pJR321)

```
          5′-GATGGAGGACAAGGACCTCGAGTCCGCCGT-3′
          3′-CTACCTCCTGTTCCTGGAGCTCAGGCGGCA-5′
amino acids    M  E  D  K  D  L  E  S  A
```

construct 10 (pJR317) and construct 11 (pJR319)

```
          5′-TCGACGCTCGAGTCCGAGGAGGCGCTATGG-3′
          3′-AGCTGCGAGCTCAGGCTCCTCCGCGATACC-5′
amino acids    S  T  L  E  S  E  E  A  L  W
```

PCR reactions were run for 35 to 40 cycles using a program of denaturation at 94°C for 1 min, annealing for 3 min at 36°C to 52°C, and polymerization at 72°C for 2 min. The annealing temperatures were selected on the basis of the predicted melting temperature of the primers. The PCR reactions were run using *Taq* DNA polymerase and buffer as specified by Promega. Other DNA manipulations including subcloning and plasmid preparation for transient expression were done by standard protocols (Maniatis et al., 1982).

Antibodies

The preparation of affinity-purified antibodies to aleurain raised to an *Escherichia coli* TrpE-aleurain fusion protein has been described previously (Holwerda et al., 1990). The anti–EP-B-specific antibodies were also raised to a TrpE-fusion protein with pHV14 (Chandler et al., 1984). pHV14 is a partial cDNA clone isolated from barley that is >90% identical to the protease domain of pHVEP4 (Koehler and Ho, 1990a). A 650-bp PvuII-PstI fragment from pHV14 was subcloned into SmaI and PstI sites, respectively, of pUC19 (Yanisch-Perron et al., 1985) to make pJR235. pJR235 was digested within the pHV14-derived insert with RsrII and made blunt-ended with Klenow fragment of *E. coli* DNA polymerase I plus deoxynucleotide triphosphates before completing excision with HindIII. To put the pJR235 insert into the correct reading frame with TrpE, pATH3 (Dieckmann and Tzagoloff, 1985) was digested with BamHI and blunt-ended using the Klenow fragment before digestion with HindIII. Ligation of the insert into pATH3 yielded pJR245. *E. coli* bearing pJR245 produced substantial quantities of a correctly sized, 53-kD TrpE-pHV14 fusion protein (data not shown). This fusion protein was used to immunize rabbits and make an affinity column for antibody purification as described previously (Holwerda et al., 1990). The purified antibody reacted only with the TrpE-pHV14 fusion protein on immunoblots and native EP-B (data not shown).

Tobacco Cell Culture and Transient Expression

A liquid suspension culture of *Nicotiana tabacum* cv Xanthi called tobacco-Xanthi-diploid was maintained at room temperature with constant agitation and constant fluorescent lighting in a medium composed of MS salts (Murashige and Skoog, 1962), B_5 vitamins (Gamborg et al., 1968), 3% sucrose, 4 mg/liter *p*-chlorophenoxyacetic acid, and 50 μg/L kinetin. This cell line was initiated by S. Dellaporta (Department of Biology, Yale University, New Haven, CT) from stem explants and selected for a finely divided, rapidly growing diploid line in a liquid culture containing 2,4-dichlorophenoxyacetic acid (generously provided by R. Horsch, Monsanto Co., St. Louis, MO).

Protoplasts were prepared by incubating the cells in a solution containing 2% Cellulysin (Calbiochem, San Diego, CA), 0.5 mg/mL pectinase (Worthington, Freehold, NJ), 2 mg/mL Driselase (Sigma), 9% mannitol, 7 mM $CaCl_2$ in 3 mM 2-(*N*-morpholino)ethanesulfonic acid buffer, pH 5.7, for 6 hr at room temperature before washing three times in electroporation buffer (128 mM NaCl, 200 mM mannitol, and 4 mM $CaCl_2$ in 0.147 mM KH_2PO_4, 0.81 mM Na_2HPO_4, pH 7.2) (Fromm et al., 1987), and resuspension of the final cell pellet was in an equal volume of electroporation buffer. Protoplasts (0.5 mL, 1 to 2 × 10^6) were transferred to an electroporation chamber held in an ice bath before adding 20 μg of construct DNA and 5 μg of pBI221 (Jefferson, 1987). The latter construct, pBI221, is a β-glucuronidase expression cassette that was used to monitor the efficiency of transfection. The electroporation chambers were constructed by affixing aluminum foil electrodes on the inside of a 1-mL disposable spectrophotometric cuvette (Sarstedt, Newton, NC) with a spacing of 3.5 mm as described by Potter et al. (1984). The contents were mixed gently before discharging a 450-μF capacitor charged to 140 V through the chamber. The protoplasts were transferred to culture media supplemented with 3% mannitol and allowed to recover for 16 to 24 hr at room temperature before pulse-labeling experiments were performed.

Sorting Assay and Subcellular Fractionation

Immediately prior to a pulse-labeling, transfected protoplasts were washed three times in electroporation buffer and resuspended at a final density of 1 × 10^6 protoplasts per milliliter in culture medium supplemented with 3% mannitol. Exclusion of methylene blue dye was used to monitor the fraction of viable cells throughout the assay. On average, 50% of the cells excluded the dye; this proportion did not change over the course of the experiment. Ten to twenty microliters of TranS^{35}-label (70% ^{35}S-Met and 15% ^{35}S-Cys; >1000 Ci/mmol; ICN Biomedicals, Costa Mesa, CA) was added to 1 to 1.5 mL of protoplast suspension, and after a 1-hr incubation at room temperature, an equal volume of culture medium supplemented with 3% mannitol, 10 mM Met, and 5 mM Cys was added to start the chase. Equal-sized aliquots (0.6 to 1.0 mL, constant for a given experiment) of the protoplast suspension were removed at indicated times and put on ice before separating media and cells. The media was removed from the cells after centrifugation at 50 *g*; the cells were washed once with 1.0 mL of electroporation buffer, and this was combined with the first supernatant to make the media fraction. The media fraction was adjusted to 1 × Tris-buffered saline (TBS; 50 mM Tris-HCl, pH 7.9, and 150 mM NaCl), 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, and 5 μg/mL leupeptin. The remaining cell pellet was homogenized in 0.5 mL of this same TBS-buffer mixture by sonication, and insoluble debris was removed by centrifugation. Aleurain, EP-B, and their derivatives were immunoprecipitated and analyzed by SDS-PAGE as described previously (Holwerda et al., 1990). To quantitate a particular protein, the corresponding portion of the dried gel was excised and counted by liquid scintillation. The targeting efficiency was calculated as the ratio of protein in vacuoles at 3 hr (recovered as proteolytic processed products) over proprotein (42- or 43-kD) recovered from cells at 0 hr (see Results). In some experiments, the protoplasts were preincubated for 1 hr in 20 μg/mL of brefeldin A (Epicenter Technologies, Madison, WI) before the addition of radioactive amino acids.

Vacuoles were prepared from pulse-labeled tobacco protoplasts following a protocol adapted from Guy et al. (1979). Labeled protoplasts were washed and resuspended in electroporation buffer and layered

on a step gradient of 12 and 15% Ficoll (M_r 400,000) in 0.6 M mannitol and 20 mM Hepes, pH 7.7. After centrifugation at 42500 rpm for 2 hr at 4°C in an SW-50 rotor, the vacuoles were removed from the top of the 12% Ficoll step gradient. This fraction was adjusted to a final concentration of 1 × TBS, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, and 5 μg/mL leupeptin before homogenizing by sonication. The dense pellet from the high-speed spin was homogenized in 0.4 mL of the same buffer. The vacuole and pellet homogenates were clarified by centrifugation before immunoprecipitation as described (Holwerda et al., 1990). α-Mannosidase activity using *p*-nitrophenol-α-mannopyranoside as substrate was assayed as described by Boller and Kende (1979), and total protein was assayed according to Bradford (1976) using BSA as a standard.

ACKNOWLEDGMENTS

This research was supported by Grant No. DE-FG02-87ER12704 from the United States Department of Energy. In addition, B.C.H. was supported by a postdoctoral fellowship from the Medical Research Council of Canada.

Received November 19, 1991; accepted January 6, 1992.

REFERENCES

Bednarek, S.Y., and Raikhel, N.V. (1991). The barley lectin carboxyl-terminal propeptide is a vacuolar sorting determinant in plants. Plant Cell **3,** 1195–1206.

Bednarek, S.Y., Wilkins, T.A., Dombrowski, J.E., and Raikhel, N.V. (1990). A carboxyl-terminal propeptide is necessary for proper sorting of barley lectin to vacuoles of tobacco. Plant Cell **2,** 1145–1155.

Boller, T., and Kende, H. (1979). Hydrolytic enzymes in the central vacuole of plant cells. Plant Physiol. **63,** 1123–1132.

Bradford, M.M. (1976). A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72,** 248–254.

Chandler, P.M., Zwar, J.A., Jacobsen, J.A., Higgins, T.J.V., and Inglis, A.S. (1984). The effects of gibberellic acid and abscisic acid on α-amylase mRNA levels in barley aleurone layers: Studies using an α-amylase clone. Plant Mol. Biol. **3,** 407–418.

Chou, P.Y., and Fasman, G.D. (1978). Empirical predictions of protein conformation. Annu. Rev. Biochem. **47,** 251–276.

Chrispeels, M.J. (1991). Sorting of proteins in the secretory system. Annu. Rev. Plant Physiol. Plant Mol. Biol. **42,** 21–53.

Dayhoff, M.O., Barker, W.C., and Hunt, L.T. (1983). Establishing homologies in protein sequences. Methods Enzymol. **91,** 524–545.

Denecke, J., Botterman, J., and Deblaere, R. (1990). Protein secretion in plant cells can occur via a default pathway. Plant Cell **2,** 51–59.

Dieckmann, C.L., and Tzagoloff, A. (1985). Assembly of the mitochondrial membrane system—*CBP6*, a yeast nuclear gene necessary for synthesis of cytochrome *b*. J. Biol. Chem. **260,** 1513–1520.

Dorel, C., Voelker, T.A., Herman, E.M., and Chrispeels, M.J. (1989). Transport of proteins to the plant vacuole is not by bulk flow through the secretory system, and requires positive sorting information. J. Cell Biol. **108,** 327–337.

Folz, R.J., and Gordon, J.I. (1987). Computer-assisted predictions of signal peptidase processing sites. Biochem. Biophys. Res. Commun. **146,** 870–877.

Fromm, M., Callis, J., Taylor, L.P., and Walbot, V. (1987). Electroporation of DNA and RNA into plant protoplasts. Methods Enzymol. **153,** 351–366.

Gamborg, O.L., Miller, R.A., and Ojima, K. (1968). Nutrient requirements of suspension cultures of soybean root cells. Exp. Cell Res. **50,** 151–158.

Guy, M., Reinhold, L., and Michaeli, D. (1979). Direct evidence for a sugar transport mechanism in isolated vacuoles. Plant Physiol. **64,** 61–64.

Holwerda, B.C., Galvin, N.J., Baranski, T.J., and Rogers, J.C. (1990). In vitro processing of aleurain, a barley vacuolar thiol protease. Plant Cell **2,** 1091–1106.

Horton, R.M., Hunt, H.D., Ho, S.N., Pullen, J.K., and Pease, L.R. (1989). Engineering hybrid genes without the use of restriction enzymes: Gene splicing by overlap extension. Gene **77,** 61–68.

Hurtley, S.M., and Helenius, A. (1989). Protein oligomerization in the endoplasmic reticulum. Annu. Rev. Cell Biol. **5,** 277–307.

Iturriaga, G., Jefferson, R.A., and Bevan, M.W. (1989). Endoplasmic reticulum targeting and glycosylation of hybrid proteins in transgenic tobacco. Plant Cell **1,** 381–390.

Jefferson, R.A. (1987). Assaying chimeric genes in plants: The GUS gene fusion system. Plant Mol. Biol. Rep. **5,** 387–405.

Johnson, L.M., Bankaitis, V.A., and Emr, S.D. (1987). Distinct sequence determinants direct intracellular sorting and modification of a yeast vacuolar protease. Cell **48,** 875–885.

Klionsky, D.J., Banta, L.M., and Emr, S.D. (1988). Intracellular sorting and processing of a yeast vacuolar hydrolase: Proteinase A propeptide contains vacuolar targeting information. Mol. Cell. Biol. **8,** 2105–2116.

Koehler, S.M., and Ho., T.-H.D. (1990a). Hormonal regulation, processing, and secretion of cysteine proteinases in barley aleurone layers. Plant Cell **2,** 769–783.

Koehler, S., and Ho., T.-H.D. (1990b). A major gibberellic acid–induced barley aleurone cysteine proteinase which digests hordein: Purification and characterization. Plant Physiol. **94,** 251–258.

Kornfeld, S. (1987). Trafficking of lysosomal enzymes. FASEB J. **1,** 462–468.

Kornfeld, S., and Mellman, I. (1989). The biogenesis of lysosomes. Annu. Rev. Cell Biol. **5,** 483–525.

Lippincott-Schwartz, J., Bonifacino, J.S., Yuan, L.C., and Klausner, R.D. (1988). Degradation from the endoplasmic reticulum: Disposing of newly synthesized proteins. Cell **54,** 209–220.

Lippincott-Schwartz, J., Yuan, L.C., Bonifacino, J.S., and Klausner, R.D. (1989). Rapid redistribution of Golgi proteins into the ER in cells treated with brefeldin-A: Evidence for membrane cycling from Golgi to ER. Cell **56,** 801–813.

Maniatis, T., Fritsch, E.F., and Sambrook, J. (1982). Molecular Cloning: A Laboratory Manual. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory).

Matile, P. (1976). Vacuoles. In Plant Biochemistry, J. Bonner and J.E. Varner, eds (New York: Academic Press), pp. 189–224.

Matile, P. (1978). Biochemistry and function of vacuoles. Annu. Rev. Plant Physiol. **29,** 193–213.

Matsuoka, K., and Nakamura, K. (1991). Propeptide of a precursor to a plant vacuolar protein required for vacuolar targeting. Proc. Natl. Acad. Sci. USA **88,** 834–838.

Matsuoka, K., Matsumoto, S., Hattori, T., Machida, Y., and Nakamura, K. (1990). Vacuolar targeting and posttranslational processing of the precursor to the sweet potato tuberous root storage protein in heterologous plant cells. J. Biol. Chem. **265,** 19750–19757.

Murashige, T., and Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol. Plant. **15,** 473–497.

Neuhaus, J.M., Sticher, L., Meins, F., Jr., and Boller, T. (1991). A short C-terminal sequence is necessary and sufficient for the targeting of chitinase to the plant vacuole. Proc. Natl. Acad. Sci. USA, **88,** 10362–10366.

Pelham, H.R.B. (1989). Control of protein exit from the endoplasmic reticulum. Annu. Rev. Cell Biol. **5,** 1–23.

Potter, H., Weir, L., and Leder, P. (1984). Enhancer-dependent expression of human κ immunoglobulin genes introduced into mouse pre-B lymphocytes by electroporation. Proc. Natl. Acad. Sci. USA **81,** 7161–7165.

Rogers, J.C., Dean, D., and Heck, G.R. (1985). Aleurain: A barley thiol protease closely related to mammalian cathepsin H. Proc. Natl. Acad. Sci. USA **82,** 6512–6516.

Rothman, J.H., Yamashiro, C.T., Kane, P.M., and Steven, T.H. (1989). Protein targeting to the yeast vacuole. Trends Biochem. Sci. **14,** 347–350.

Saalbach, G., Jung, R., Kunze, G., Saalbach, I., Adler, K., and Müntz, K. (1991). Different legumin protein domains act as vacuolar targeting signals. Plant Cell **3,** 695–708.

Sonnewald, U., von Schaewen, A., and Willmitzer, L. (1990). Expression of mutant patatin protein in transgenic tobacco plants: Role of glycans and intracellular location. Plant Cell **2,** 345–355.

Tague, B.W., Dickinson, C.D., and Chrispeels, M.J. (1990). A short domain of the plant vacuolar protein phytohemagglutinin targets invertase to the yeast vacuole. Plant Cell **2,** 533–546.

Valls, L.A., Hunter, C.P., Rothman, J.H., and Stevens, T.H. (1987). Protein sorting in yeast: The localization determinants of yeast vacuolar carboxypeptidase Y resides in the propeptide. Cell **48,** 887–897.

Valls, L.A., Winther, J.R., and Stevens, T.H. (1990). Yeast carboxypeptidase Y vacuolar targeting signal is defined by four propeptide amino acids. J. Cell Biol. **111,** 361–368.

Voelker, T.A., Herman, E.M., and Chrispeels, M.J. (1989). In vitro mutated phytohemagglutinin genes expressed in tobacco seeds: Role of glycans in protein targeting and stability. Plant Cell **1,** 95–104.

von Figura, K., and Hasilik, A. (1986). Lysosomal enzymes and their receptors. Annu. Rev. Biochem. **55,** 167–193.

von Heijne, G. (1983). Patterns of amino acids near signal-sequence cleavage sites. Eur. J. Biochem. **133,** 17–21.

von Heijne, G. (1986). A new method for predicting signal sequence cleavage sites. Nucl. Acids Res. **14,** 4683–4690.

Watanabe, H., Abe, K., Emori, Y., Hosoyama, H., and Arai, S. (1991). Molecular cloning and gibberellin-induced expression of multiple cysteine proteinases of rice seeds (Oryzains). J. Biol. Chem. **266,** 16897–16902.

Wilkins, T.A., and Raikhel, N.V. (1989). Expression of rice lectin is governed by two temporally and spatially regulated mRNAs in developing embryos. Plant Cell **1,** 541–549.

Wilkins, T.A., Bednarek, S.Y., and Raikhel, N.V. (1990). Role of propeptide glycan in post-translational processing and transport of barley lectin to vacuoles in transgenic tobacco. Plant Cell **2,** 301–313.

Yanisch-Perron, C., Vieira, J., and Messing, J. (1985). Improved M13 phage cloning vectors and host strains: Nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33,** 103–119.