Vacuolar Targeting and Posttranslational Processing of the Precursor to the Sweet Potato Tuberous Root Storage Protein in Heterologous Plant Cells*

Ken Matsuoka†, Shogo Matsumoto‡, Tsukahiro Hattori‡, Yasunori Machida‡, and Kenzo Nakamura‡†

From the †Laboratory of Biochemistry, School of Agriculture, and the ‡Department of Biology, School of Science, Nagoya University, Chikusa, Nagoya 464-01, Japan

Sporamin, the tuberous root storage protein of the sweet potato, which is localized in vacuoles, is synthesized as a prepro-precursor with an N-terminal sequence of amino acids that includes a signal peptide and an additional pro-segment of 16 amino acids. A full-length cDNA for sporamin was placed downstream of the 35S promoter of cauliflower mosaic virus and introduced into tobacco and sunflower genomes by Ti plasmid-mediated transformation. A polypeptide of nearly the same size as mature sporamin from the sweet potato was detected in transformed calli of tobacco and sunflower, as well as in the leaves, stems, and roots of regenerated, transgenic tobacco plants. Amino acid sequence analysis of the nearly mature-sized form of sporamin from the transformed tobacco cells revealed that it is actually longer by three amino acids at its N terminus than authentic sporamin purified from the sweet potato. By pulse labeling of suspension-cultured tobacco cells with [35S]methionine, the pro-form of the precursor to sporamin, but not the prepro-precursor, was detected. The 35S-labeled pro-form was chased to the nearly mature-sized form via an intermediate form which is slightly larger than the nearly mature-sized form. Analysis by Edman degradation of the intermediate form that was labeled in vivo with [3H]histidine suggested that it is longer by two amino acids at its N terminus than the nearly mature-sized form of sporamin. These results suggest that at least two steps of posttranslational processing of the pro-form occurs sequentially in tobacco cells.

The posttranslational processing of the pro-form of the precursor to sporamin was inhibited by monensin, suggesting that this step takes place in the acidic compartment, probably in the vacuole. All of the sporamin polypeptides synthesized in transformed tobacco cells were retained inside the cell and sporamin was localized in the vacuole, as judged from results of subcellular fractionation. These results indicate that sporamin is appropriately targeted to the vacuole in tobacco cells.

and maintenance of the organization and function of eukaryotic cells. Recently, signals required for targeting proteins to specific organelles have been identified for many proteins, such as those found in mitochondria (1), chloroplasts (2), endoplasmic reticulum (3), lysosomes (4), and nuclei (5). In many cases, these targeting signals are encoded in the N-terminal amino acid sequence of the precursor, which is removed during maturation of the protein by proteolytic processing. In some cases, at least two signals are required for the correct targeting of a protein to specific organelles and to a specific intraorganellar space. Some nuclear-encoded chloroplast proteins require two signals in the transit peptide segment of the precursor, one for transport into the chloroplast and the other for translocation to the appropriate location inside of the organelle (2). Vacuolar hydrolases of the yeast Saccharomyces cerevisiae also require two signals for their correct targeting (6). In addition to the N-terminal signal peptide required for entry into the lumen of the endoplasmic reticulum, a second signal is required for directing this protein into the vacuole. The latter signal is located in the prepeptide region of the precursor and is cleaved off after entry into the vacuole (6). However, the mechanism of signal recognition for the translocation of proteins and the proteases responsible for the maturation of precursor polypeptides have not yet been well characterized in most cases. It is obvious, however, that the presence of such mechanisms is a prerequisite for the functional expression of genes that encode organelar proteins.

In many plant cells, the vacuole is the largest organelle and it has diverse functions, such as the intracellular digestion of materials, the accumulation and storage of organic and inorganic nutrients and metabolites, and the generation of turgor, with specific function depending on the type of cell and on the stage of development of the cell (7). Vacuoles in certain cells that have differentiated to become storage cells may, themselves, differentiate into protein storage organelles or protein bodies by accumulating large amounts of storage proteins. Plant vacuolar matrix proteins are synthesized by membrane-bound polyosomes as precursors with N-terminal signal peptides, and many of them are also subject to posttranslational processing prior to maturation (8). However, the exact nature of their vacuole-targeting signals and the cellular mechanisms required for their targeting and maturation have not yet been determined.

Systems for transgenic gene expression have proved useful for the analysis of the mechanisms of protein targeting in various systems. Several reports have previously described the expression of precursors to plant vacuolar proteins in heterologous systems. Bean phytohemagglutinin, a vacuolar storage protein in seeds, has been expressed in yeast cells and

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†To whom correspondence should be addressed.
targeted to the vacuole (9), suggesting that some common mechanisms may be operating in the targeting of proteins to the vacuole in these two organisms. The precursor to phytohemagglutinin was also expressed in transgenic tobacco (10), and the correct glycosylation, Golgi processing, and targeting to the vacuole were observed. Furthermore, it was shown that glycan side chains are not required for the correct targeting of phytohemagglutinin to the vacuole in transgenic tobacco seeds (11). Expression of other plant vacuolar proteins, $\beta$-conglycinin from soybean seeds (12), lectin from barley seeds (13), 2S albumin from Arabidopsis seeds (14), and patatin from potato tubers (15), in heterologous plant tissues has also been examined.

Sporamin is the most abundant protein in the tuberous root of the sweet potato, accounting for about 80% of the total soluble protein (16), and it is localized in the vacuole (17). It is encoded by a nuclear multigene family of more than 10 different genes and consists of a mixture of closely related polypeptides with molecular weights of approximately 20 kDa (16, 18). Unlike many other vacuolar storage proteins in plants, sporamin is not glycosylated (16). It is synthesized by membrane-bound polysomes as a larger precursor (19) with an extra N-terminal sequence of 35 or 37 amino acids, the number depending on the specific gene (18, 20). The structure of the N-terminal region of the precursor to sporamin can be divided into two segments: the N-terminal, hydrophobic signal peptide and an additional segment of 16 amino acids which contains an unusually high proportion of charged amino acids (18, 20). We showed previously that only the N-terminal signal peptide segment of the precursor is removed by cotranslational processing in vitro with microsomal membranes (21), a result that strongly suggests the posttranslational removal of the additional segment of 16 amino acids. Here we report the processing and targeting to vacuoles of a precursor to sporamin expressed in transformed tobacco and sunflower cells.

**EXPERIMENTAL PROCEDURES**

**Construction of Plasmids for Agrobacterium-mediated Transformation**—A fusion gene, consisting of the 35S promoter from cauliflower mosaic virus (CaMV) and sporamin cDNA for the expression of the precursor to sporamin in plant cells, was constructed as follows. The poly(dC) tail and 17 base pairs of the 5'-noncoding region were deleted from the 5' terminus of a full length sporamin cDNA insert in pMO23 (19) by Bal31. They were attached to the Sau3A site at the 3' end of the CaMV 35S promoter fragment from pCaMV CAT (22) by BamHI linker, and the resulting 35S promoter-sporamin fusion gene fragment was cloned into the BamHI-HindIII sites of pUC18 to yield pCSAD. The 35S promoter-sporamin fusion gene fragment from pCSAD, together with the NPT-II kanamycin-resistance gene fragment from pUC18 (23), was placed between BamHI B2 and B19 fragments of the Ti plasmid pTiB6-806 (24) to generate an intermediate plasmid. This intermediate plasmid was transferred into gene fragment from pKC7 (23), was placed between BamHI B2 fragment from pCSAD, together with the NPT-II kanamycin-resistance gene fragment from pCSAD (27) and the resulting plasmid, pVSAD (Fig. 1C), was introduced into A. tumefaciens that harbored pTiS161F, and transformed calli were selected by growth on phytohormone-free Murashige and Skoog agar medium (28). Kanamycin-resistant shoots regeneraded from leaf discs after transformation with A. tumefaciens that harbored pVSAD were transferred to rooting medium and cultivated axenically on the same medium. The sunflower (Helianthus annuus) tissue was achieved with A. tumefaciens that harbored pTiS161F, and calli that showed phytohormone-independent growth were selected (29).

**Immunoblot Analysis of Sporamin-related Polypeptides in Tissues of Transformed Plants**—Tissue was homogenized with a mortar and pestle in two volumes of extraction buffer (50 mM Tris-acetate (pH 7.5), 1 mM EDTA, 1% sodium ascorbate, 0.5 mM sucrose) at 4°C. The homogenate was centrifuged at 15,000 × g for 10 min and the supernatant was used as the soluble protein fraction. Sporamin-related polypeptides in protein fractions were detected by immunoblotting with rabbit antisera raised against native sporamin (25), or denatured sporamin, and 25S-protein A as described previously (17).

A whole cell extract of S. cerevisiae pep4 mutant cells (30) that express the precursor to sporamin under the control of the Gal10 promoter, was used to generate standards for the prepro-precursor and the pro-form of sporamin for use in these immunoblot analyses. In S. cerevisiae pep4, two forms of sporamin-related polypeptides are detected by immunoblotting and these two polypeptides migrate with the same electrophoretic mobilities as the prepro-precursor and the pro-form of sporamin obtained by the translation and processing in vitro of sporamin mRNA using the SP6 transcript of pIM023 cDNA (21). Expression of the precursor to sporamin in S. cerevisiae will be described elsewhere.

**Immunofluorescence Purification of the Nearly Mature-sized Form of Sporamin in Transformed Tobacco Cells, and Its N-terminal Amino Acid Sequence Analysis**—Suspension cultures were generated from the transformed tobacco cells. These cultures were grown at 28°C on a rotary shaker (130 rpm) in Murashige and Skoog medium and they were subcultured at weekly intervals at a dilution ratio of 1:15. Cells from 3-day-old cultures were collected by centrifugation. The soluble protein fraction (500 μg) prepared from the cells was applied to an immunoaffinity column for the purification of the nearly mature-sized form of sporamin. The immunoaffinity column was prepared by covalent linking of IgG fraction of sporamin-specific antisera to Affi-Gel by using the Affi-Gel hydrazine immunofluorescence kit (Bio-Rad). Bound proteins were eluted from the column by 0.2 M glycine-HCl (pH 2.5). After neutralization of the solution, proteins were concentrated by ultrafiltration, separated by SDS-polyacrylamide gel electrophoresis and transferred to Immobilon membrane (Millipore). The band corresponding to the nearly mature-sized form of sporamin, after visualization with Coomassie Brilliant Blue, was excised from the gel and the N-terminal amino acid sequence was determined with a protein sequencer (model 470A, Applied Biosystems, Inc., Foster City, CA).

**Labeling in Vivo of Sporamin-related Polypeptides in Transformed Tobacco Cells**—3-day-old suspension cultures of transformed tobacco cells were harvested by centrifugation at 3000 × g for 1 min, resuspended in the culture medium, and incubated with a concentration of 0.3 μl of packed cell volume per ml. For each 0.5 ml of the suspension of cells, 75 μCi of [35S]methionine were added and the cells were then incubated for an appropriate period of time. For chase experiments, the incorporation of [35S]methionine was stopped by addition of 50 μl of 10 mM L-methionine to the culture medium. Cells were harvested by centrifugation, washed once with ice-cold Tris-buffered saline (50 mM Tris-HCl (pH 7.5), 0.15 M NaCl) and resuspended in ice-cold extraction buffer (50 mM Tris-HCl (pH 7.5), 0.15 M NaCl, 1% sodium ascorbate, 0.05% Triton X-100, 0.05% SDS, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 100 μM leupeptin, 100 μM pepstatin, and 100 μM antipain). Cells were sonicated with an Ohtake Sonicator (Ohtake Industries, Sendai, Japan) at 50 watt output (27). Insoluble debris was removed by centrifugation at 15,000 × g for 10 min and the supernatant was used for immunoprecipitation with sporamin-specific antiserum. Alternatively, washed cells were resuspended in sample buffer with SDS and sonicated. Sonicated cells were heated at 95°C for 10 min and centrifuged to remove insoluble debris. The resultant supernatant for immunoprecipitation was immunoprecipitated with sporamin-specific antisera immobilized by the use of protein A-Sepharose, and the precipitates were analyzed by SDS-polyacrylamide gel electrophoresis with subsequent fluorography. The SP6 transcript of pIM023 cDNA was translated in the presence of [35S]methionine and a limited amount of dog pancreatic microsomal membranes at 30°C for 20 min and immunoprecipitated with sporamin-specific antiserum for the prepro-precursor and the pro-form of sporamin, since

1 The abbreviations used are: CaMV, cauliflower mosaic virus; MES, 2(N-morpholino)ethanesulfonic acid; SDS, sodium dodecyl sulfate.

2 K. Matsuoka and K. Nakamura, unpublished results.
both of these forms are obtained under these conditions (21).

Edman Degradation Analysis Suspenison cultured tobacco cells (0.5 ml) were labeled with 200 Ci of L-[2,5-3H]histidine for 1 h with a subsequent chase with 1 mM L-histidine for 1 h. Sporamin-related polypeptides were immuno precipitation from the cell lysate with sporamin-specific antisera and protein A-Sepharose, and then they were eluted by boiling in a mixture of 2% SDS, 1% β-

mercaptoethanol, and 20 mM ammonium carbonate (pH 9.0). Polypeptides in the supernatant were concentrated by ultrafiltration, mixed with bovine serum albumin, and subjected to sequential Edman degradation as described previously (21).

Preparation of the Vacuole Fraction—4-day-old, suspension-cul-
tured, sporamin-expressing cells were harvested by centrifugation and washed once with 0.6 M mannitol. The packed cells were resus-
pended in 4 volumes of a mixture of 2% Cellulase Y-C, 0.5% Macer-
zyme R-10, 0.1% Pectolyase Y-23, 0.6 M mannitol (pH 5.2 with HCl), and incubated at 32 °C for 2 h, with occasional gentle pipeting, to convert most of the cells to protoplasts. Protoplasts were harvested by centrifugation and washed twice with 0.6 M mannitol.

15 ml of the suspension of protoplasts (106 protoplasts/ml) were centrifuged at 1500 X g for 10 min on a gradient that was composed, from bottom to top, of 5 ml of 10% Ficoll, 0.1% dextran sulfate, 7.5 ml of 5% Ficoll, 0.1% dextran sulfate, and 20 ml of 2.5% Ficoll, 0.6% DEAE-dextran. All of these Ficoll-dextran solutions were prepared in 10 mM MIES, 1% (pH 6.9) and 0.6 M mannitol. Lysed cells at the interface between layers of 5 and 10% Ficoll were collected and resuspended in 5 volumes of 5% Ficoll, 0.1% dextran sulfate, 10 ml of 5% Ficoll, 0.1% dextran sulfate, and 5 ml of 0.1% dextran sulfate were overlaid on the suspension, and the entire preparation was centrifuged at 1500 X g for 10 min. Vacuoles and some surviving protoplasts that floated to the interface between 5 and 0% Ficoll were resuspended in 10% Ficoll to a volume of 12 ml, overlaid with 2 ml of 2.5% Ficoll, 0.1% dextran sulfate, and 1 ml of 0.1% dextran sulfate and centrifuged at 1500 X g for 10 min. The material at the interface between layers of 2.5 and 0% Ficoll, enriched with vacuoles, was collected and used as the vacuole fraction.

Enzymatic and Chemical Assays—Activities of phosphodiesterase and glucan synthase 1 were assayed by the methods of Boller and Kende (31) and Green (32), respectively. Other enzymatic activities and concentrations of protein were assayed as described previously (17).

Chemicals—L-[35S]Methionine (1000 Ci/mmol), L-[2,5-3H]histidine (50 Ci/mmol), L-[3,4,5-3H]proline (100 Ci/mmol), and [152] tyrosine A (30 Ci/mg) were purchased from Amersham-Japan Co. Cellulase Y-C and Pectolyase Y-23 were from Seishin Pharmaceutical Co. (Tokyo, Japan) and Macerozyme R-10 was from Yakuruto Honsha Co. (Tokyo, Japan). Ficoll 400 and dextran sulfate were used as purchased from Becton Dickinson LKB Biotechnology Inc. and DEAE-dextran from Sigma. All other enzymes and chemicals were from standard commercial sources.

RESULTS

Expression of the Precursor to Sporamin in Transformed Tobacco and Sunflower Callus Tissues—A full length sporamin cDNA in pIMO23 (19) encodes a precursor to sporamin of 230 amino acids. Processing of this precursor in vitro with dog pancreas microsomal membranes (21) removes only the N-terminal signal peptide segment to yield a pro-form of the precursor which still retains an extra 16 amino acids at the N terminus of the mature form (Fig. 1A). A polypeptide with the same electrophoretic mobility as the pro-form of the precursor to sporamin in E. coli (21), or in S. cereuisiae pIMO23 was fused to the downstream end of the 35 S promoter from CaMV. We used a derivative of pIMO23 in which the 5' poly(dG) tail and part of the 5'-noncoding region has been removed from the cDNA insert, for the construction of 35 S promoter-sporamin fusion gene, since the presence of the 5' poly(dG) tail inhibited the expression of the precursor to sporamin in yeast cells.2 The 35 S promoter-sporamin fusion gene was integrated into the T-DNA region of the T' plasmid pTiB6S3tra' (19), as described under "Experimental Procedures." A. tumefaciens harboring pTISA16F was used to generate crown gall-type transformed calli of tobacco and sunflower.

The tobacco and sunflower calli transformed with Agrobacterium that harbored pTISA16F contained polypeptides that cross-reacted with sporamin-specific antisera (Fig. 2A, lanes 3 and 4). No immunoreactive polypeptides were detected in calli transformed with Agrobacterium that harbored pTiB6S3tra' alone (Fig. 2A, lanes 5 and 6). Although the level of expression of sporamin-related polypeptides varied considerably among individual transformants (data not shown), a polypeptide band of almost identical size was detected. The size of the material in the immunoreactive band was smaller than the size of the pro-form of the precursor to sporamin (Fig. 2A, lane 1), but it was slightly larger than the average size of a mixture of sporamin isoproteins isolated from the sweet potato (Fig. 2A, lane 2).

FIG. 1. The primary structure of the precursor to sporamin (A) and the structure of the chimeric T' plasmid (B) and the binary plasmid (C) used for the expression of the precursor to sporamin in heterologous plant cells. A, the primary structure of the N-terminal part of the precursor to sporamin encoded by pIMO23 (19). Hydrophobic and charged amino acid residues are indicated by circles and hexagons, respectively. Thick underlining and dotted underlining indicate the mature part and the pro-segment, respectively. B, the structure of the T-DNA region of pTiB6-806 (upper, Ref. 24) and the critical part of pTISA16F (lower). T1 and T2, two T-DNA regions of pTiB6-806; filled arrowheads, the positions of 25-base pair border sequences. 35S, CaMV 35 S promoter, SPO, sporamin cDNA, derived from pIMO23; NTP-II, neomycin phosphotransferase gene. C, the structure of the binary plasmid pVSAD. The EcoRI-HindIII fragment carrying the 35 S sporamin-promoter fusion gene from pVSAD was inserted into the EcoRI-HindIII sites of pGA469 (27). Nos t and nos t (nopaline synthase)-promoter and nos-terminator, respectively. Filled arrowheads indicate the positions of 20-base pair border sequences. E, EcoRI; B, BamHI; H, HindIII.
Sporamin migrates anomalously on SDS-polyacrylamide gels during electrophoresis, with its mobility depending on the concentration of acrylamide (19). Sporamin isolated from the sweet potato migrates as a broad band, reflecting the microheterogeneity of the preparation, which has a peak size of 19.95 kDa determined by analysis of a Ferguson plot or molecular masses of the pro-form and the protein in the immunoreactive band from tobacco cells was nearly mature-sized form of sporamin. The mobility of the more slowly migrating band was similar to that of the band that could not be unambiguously identified, corresponds to the amino acid sequence of the precursor to sporamin from positions Glu5 to Asp18 (19; see Fig. 1A). These results indicate that the nearly mature-sized form of sporamin in tobacco cells is actually longer by three amino acids than authentic sporamin purified from the sweet potato.

Labeling in Vivo of the Precursor to Sporamin in Transformed Tobacco Cells—When transformed tobacco cells in suspension culture were labeled with [35S]methionine for 6 h and [35S]-labeled cellular polypeptides were subjected to immunoprecipitation with sporamin-specific antiserum, a polypeptide with the same electrophoretic mobility as the nearly mature-sized sporamin was precipitated (data not shown). By contrast, when the cells were labeled with [35S]methionine for 1 h, two labeled polypeptides were precipitated (Fig. 3A, lane 2). The mobility of the more slowly migrating band was identical to the mobility of the pro-form of the precursor to sporamin which was obtained by processing in vitro of the precursor sporamin with dog pancreatic microsomal membranes (Fig. 3A, lane 1, lower band), and the mobility of the more rapidly migrating band was similar to that of the band detected by immunoblotting. After a 2-h chase, the upper band disappeared and only the more rapidly migrating band was observed (Fig. 3A, lane 3). The time-course of the conversion of the pro-form to the more rapidly migrating band during the chase (Fig. 3B) indicated that an increase in the amount of material in the more rapidly migrating bands occurs concomitant with a decrease in the amount of the pro-form.

Expression of the Precursor to Sporamin in Various Organs of Transgenic Tobacco Plants—The 35 S promoter-sporamin fusion gene was introduced into the binary vector pGA469 (27) to yield pVSAD (Fig. 1C), and A. tumefaciens harboring both pTiB6S3traA and pVSAD was used to obtain regenerated, transformed tobacco plants. A polypeptide with the same electrophoretic mobility as that of polypeptides present in transformed calli was detected as the major sporamin-related polypeptide in extracts from the leaves, stems, and roots of axenically grown, transgenic tobacco plants (Fig. 2B) indicating that the precursor to sporamin is processed in these organs in a similar manner to that in callus tissue.

Posttranslational Processing of the Pro-form of the Precursor to Sporamin—The more rapidly migrating band which appeared after the chase of the [35S]-labeled pro-form was some-
Fig. 3. Conversion of the pro-form of the precursor to sporamin to the nearly mature-sized form in transformed tobacco cells in suspension culture. A, a suspension culture of cells, derived from tobacco callus transformed with pTiSAlGF, that expressed the precursor to sporamin (lanes 2 and 3) or from callus transformed with the control pTiBSStxmt (lanes 4 and 5) were labeled with \[^{35}S\]methionine for 1 h (lanes 2 and 4) and chased with excess cold methionine for 2 h (lanes 3 and 5). Sporamin-related \[^{35}S\]-labeled polypeptides were precipitated with sporamin-specific antiserum and analyzed by SDS-polyacrylamide gel electrophoresis. Lane 1, precursor to sporamin partially processed in vitro with dog pancreatic microsomal membranes (see "Experimental Procedures"); pp, precursor; p, pro-form; m, mature sporamin. B, tobacco cells in suspension culture that expressed the precursor to sporamin were labeled with \[^{35}S\]Methionine for 30 min (lane 1) and chased for 15 (lane 2), 30 (lane 3), 60 (lane 4), 120 (lane 5), and 720 min (lane 6). pp, p, and m, same as in A.

In order to examine further the nature of the posttranslational processing of the precursor to sporamin in tobacco cells, we labeled cells with \[^{3}H\]histidine for 1 h and chased with unlabeled histidine for 1 h. Under these conditions, the \[^{3}H\]-labeled pro-form of the precursor was almost completely processed to the intermediate form, and the nearly mature-sized form represented only a minor proportion of the labeled protein (data not shown; see Fig. 3B, lane 4). The \[^{3}H\]-labeled sporamin-related polypeptides were immunoprecipitated and analyzed by the manual, sequential Edman degradation. A peak of radioactivity appeared in the second cycle (Fig. 4), indicating that the N-terminal amino acid of the 24.3-kDa intermediate form is Thr\[^{33}\] (see Fig. 1A). That conversion of the 24.3-kDa band to the 24.0-kDa nearly mature-sized form occurs by removal of the Thr\[^{33}\]-His\[^{34}\] dipeptide was supported by Edman degradation analysis of a mixture of the 24.3-kDa band and the 24.0-kDa band that had been obtained by pulse labeling with \[^{3}H\]proline for 1 h and subsequent chase with unlabeled proline for 2 h. In this case, peaks of radioactivity appeared at the second and fourth cycles (data not shown).

Effect of Monensin on Posttranslational Processing of the Precursor to Sporamin—We examined the effects of monensin on the posttranslational processing of the precursor to sporamin in tobacco cells. Monensin has been reported to inhibit posttranslational processing of precursors to several vacuole-localized seed proteins (33, 34), and it is suggested that monensin causes alkalinization of the vacuolar matrix, which in turn inhibits the vacuole-localized processing enzyme(s) (33, 35). As shown in Fig. 5, the posttranslational processing of the pro-form of the precursor to sporamin to the nearly mature-sized form was almost completely inhibited by 5 \(\mu\)M monensin.

Vacuolar Localization of Sporamin Expressed in Transformed Tobacco Cells—Sporamin-related polypeptides could not be detected in the culture medium of the transformed tobacco cells not only by immunoblotting of an SDS gel, but also by immunoprecipitation after long term labeling with \[^{35}S\]methionine (data not shown). Moreover, no sporamin-related polypeptides were detected in the culture medium after 30 min of pulse labeling and after a subsequent 2-h chase (Fig. 6). These results suggest that all of the sporamin-related polypeptides synthesized in tobacco cells are retained inside of the cell and none of them are secreted to the medium.

In order to examine the intracellular site of localization of sporamin in tobacco cells, we prepared the vacuole fraction from protoplasts of tobacco cells grown in suspension culture, as described under "Experimental Procedures." The isolated vacuole fraction was found to be contaminated by less than 0.1% protoplasts when examined under the light microscope, and it was enriched with round vesicles which could be stained with Neutral Red. The average size of vacuolar vesicles was similar to that of protoplasts (a diameter of about 20 \(\mu\)m) suggesting that most of the vacuoles in this fraction were of...
the central vacuole type. Specific activities of vacuolar marker enzymes, namely, phosphodiesterase and \( \alpha \)-mannosidase, were about 11- and 17-fold greater, respectively, in the vacuole fraction than in the protoplasts (Table I). By contrast, the specific activities of marker enzymes for other organelles (NADPH-cytochrome c reductase for the endoplasmic reticulum, glucan synthase I for Golgi, cytochrome c oxidase for mitochondria, catalase for microbodies, glucose-6-phosphate dehydrogenase for plastids and cytoplasm) in the vacuole fraction were lower than those in protoplasts (Table I).

The relative amount of the nearly mature-sized sporamin in the vacuole fraction was analyzed by immunoblotting of an SDS-polyacrylamide gel (Fig. 7). The amount of sporamin detected in a total of 5 \( \mu \)g of protein from the vacuole fraction was similar to that in 75 \( \mu \)g of protein from the protoplasts (Fig. 7, lanes 1 and 4). This difference indicates that sporamin was concentrated to about 15-fold during the purification of the vacuole fraction. This ratio of the concentration of sporamin in the vacuole fraction to that in the protoplast was similar to those for the vacuolar marker enzymes described above. These results strongly suggest that the intracellular site of localization of sporamin in transformed tobacco cells is the interior of the vacuole, as is the case in the tuberous root of the sweet potato (17).

**DISCUSSION**

Structural analyses (19, 20) and processing in vitro by microsomal membranes (21) of the precursor to sporamin suggest that, in addition to cotranslational removal of a signal peptide, posttranslational removal of a pro-segment of 16 amino acids is required for the maturation of sporamin in the sweet potato (Fig. 1A). The subcellular site and the nature of the posttranslational processing of the precursor in the sweet potato are not known. In this paper, we described our analysis of the expression of a single precursor polypeptide for sporamin in heterologous plant cells under control of the 35 S promoter from CaMV. This approach is particularly appropriate for studies of sporamin, especially for the analysis of posttranslational processing at the molecular level, since sporamin consists of a mixture of various isoproteins encoded by a multigene family (16, 18). Comparison of amino acid sequences of six precursors for sporamin, as deduced from the nucleotide sequences, show sequence homologies of 77-98% between them, and the total number of genes for sporamin in the sweet potato genome is estimated to be about 60 (18). In order to avoid complexities due to the microheterogeneity at the sequence level, it is desirable to analyze the behavior of a single polypeptide precursor to sporamin. However, to date, transformation of the sweet potato has not been achieved.

In crown gall-type calli of tobacco and sunflower and in the leaves, stems, and roots of axenically cultured, transgenic tobacco plants the precursor to sporamin was processed to a nearly mature-sized polypeptide (Fig. 2). Amino acid sequence analysis of this nearly mature-sized form of sporamin in tobacco cells indicated that it is actually longer by three amino acids at its N terminus than the authentic sporamin purified from the sweet potato. The occurrence of the posttranslational processing of the precursor to sporamin in tobacco cell is clearly indicated by the pulse-chase labeling experiments using the suspension-cultured cells. The signal peptide is very rapidly removed from the precursor and the resulting proform of the precursor is further processed in two sequential steps (Fig. 3). It is not known whether two sequential posttranslational processes also occurs in the sweet potato or not. However, the difference in the N-terminal structures between them, and the total number of genes for sporamin in the sweet potato is due to the difference in plant species or to the presence of a particular protease in the tuberous root in which the expression of sporamin genes is specifically activated.

Only limited information is available at present concerning the proteases involved in the posttranslational processing of plant vacuolar proteins. In the case of yeast vacuolar proteins, vacuolar proteases catalyze the cleavage of the pro-segment from pro-proteins after their arrival in the vacuole. Thus, in *S. cerevisiae* *pep4*, a mutant deficient in vacuolar proteinase A (30), precursor forms of several vacuolar proteins accumulate in the vacuole (36). Many storage proteins and lectins in seeds, which accumulate in vacuoles or protein bodies, also undergo posttranslational proteolytic cleavage (8). Endoproteolytic activities for the processing of precursors to ricin and agglutinin in castor bean endosperm (37), lectin in rice embryo (33), and 11 S globulin in pumpkin cotyledon (35) have acidic

**Table 1**

**Specific activities of marker enzymes for various organelles in protoplasts and in the vacuole fraction**

The specific activities are expressed as mmol/mg of protein/min.

| Enzyme                     | Protoplasts (I) | Vacuole fraction (II) | Ratio (II/I) |
|----------------------------|-----------------|-----------------------|--------------|
| NADPH-cytochrome c reductase| 0.0717          | 0.0208                | 0.290        |
| Cytochrome c oxidase        | 0.416           | 0.114                 | 0.270        |
| Catalase                   | 15.6            | 15.6                  | 1.000        |
| Glucose-6-phosphate dehydrogenase | 0.122       | 0.0379                | 0.311        |
| Glucan synthase I          | 7.80 \( \times \) \(10^{-2}\) | ND*                   |              |
| Phosphodiesterase          | 0.952           | 11.0                  | 11.6         |
| \( \alpha \)-Mannosidase    | 0.0268          | 0.470                 | 17.5         |

* ND, not detected.
Heterologous Plant Vacuolar Protein Expression

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19756 Heterologous Plant Vacuolar Protein Expression catalyzes the posttranslational processing of pro-globulin and pro-trypsin inhibitor. The enzyme seems to catalyze the precursor to sporamin is expressed in yeast, the pep4 mutation causes the appearance of the pro-form of the precursor (Fig. 2A, lane 1). Furthermore, posttranslational processing of the pro-form of the precursor in transformed tobacco cells (Fig. 3) takes place in the vacuole, or at a late stage of its transport to the vacuole. When the precursor to sporamin is expressed in yeast, the pep4 mutation causes the appearance of the pro-form of the precursor (Fig. 2A, lane 1). Monensin has been reported to inhibit posttranslational processing of the precursor to lectin in rice embryos (33) and to 11 S globulin in pumpkin cotyledons (34). In the case of pumpkin cotyledons, monensin does not inhibit the transport of pro-globulin to the vacuole (34), and it has been suggested that monensin causes alkalinization of the vacuolar matrix, which in turn inhibits the activity of the processing enzyme (33, 35). In addition, posttranslational processing is a much slower process, with a half time of 21 min, than the very rapid cotranslational processing of the precursor to sporamin in transformed tobacco cells (Fig. 3). This time difference may be required for the transport of the pro-form by endomembrane transport systems from the lumen of the endoplasmic reticulum to the site of processing.

We did not detect any sporamin-related polypeptides in the culture medium of the suspension-cultured tobacco cells (Fig. 6) and sporamin polypeptides were localized in the vacuole fraction (Fig. 7). These results suggest that the precursor to sporamin can be appropriately transported to the vacuole in the heterologous tobacco cells. We have not examined the subcellular localization of sporamin-related polypeptides in various organs of transgenic tobacco plants. However, if the posttranslational processing of the precursor takes place in the vacuole, the fact that the sizes of sporamins detected in the leaves, stems, and roots of transgenic tobacco plants were almost identical to that of the sporamin in suspension-cultured, transformed tobacco cells (Fig. 2) suggests that precursors to sporamin in these organs are also appropriately targeted to the vacuole.

The targeting of proteins to lysosomes in animal cells and to vacuoles in yeast cells requires, in addition to a signal peptide, a second positive signal, namely, mannose 6-phosphate groups in the former case (4) and the polypeptide of the pro-segment in the latter case (6, 41–43). Sporamin is a simple protein which does not contain glycans (16). If the targeting of sporamin to vacuoles in plant cells also requires a second positive signal, it is most likely to be contained in the structure of the pro-form of the precursor. Glycan side chains on the precursors to several plant vacuolar proteins are not required for the correct targeting to the vacuole (11, 13, 44). Heterologous expression of the precursor to sporamin in transformed tobacco cells, as described in this paper, should provide a useful experimental system with which to study mechanisms for targeting of proteins to plant vacuoles, and should help in identifying the vacuolar targeting signal in the protein. Our recent studies indicate that the pro-segment of the precursor to sporamin is required for correct targeting of sporamin to the vacuole.

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