Chapter 3

Propeptide is Necessary for Proper Sorting of Sporamin to the Vacuole of Tobacco Cells

INTRODUCTION

Sporamin is synthesized by membrane-bound polysomes as a prepro-precursor (Hattori et al. 1985; Hattori et al. 1987). The most N-terminal part of the precursor is a signal peptide, and the adjoined propeptide of 16 amino acid residues is removed post-translationally In Chapter 2, I showed that the precursor to sporamin expressed in transformed tobacco calli is correctly targeted to the vacuole. To analyze the function of the propeptide of the precuresor to sporamin about the transport of this protein to to the vacuole, a derivative of sporamin cDNA which encodes a precursor without the propeptide was constructed. I report here that deletion of the propeptide from the precursor results in secretion of sporamin into the culture medium of transformed tobacco cells .

MATERIALS AND METHODS

Materials.

Tran^{35}S-label (43 TBq/mmol, a mixture of [^{35}S]Met and [^{35}S]Cys), and potassium dextran sulfate were obtained from ICN, Amersham, and Meito Sangyo (Nagoya), respectively. Oligo nucleotides were synthesized on a DNA synthesizer (Model 381A, Applied Biosystems). Other reagents were obtained and used as in Chapter 2.

Construction of the Δpro-muant of sporamin cDNA.

The *Bam*HI-*Hin*dIII fragment from pCSAD (Chapter 2) containing cDNA for sporamin A (pIMO23; Harrori et al., 1985) was cloned into the *Bam*HI and *Sma*I sites of pNUT7 with a *Hin*dIII linker to generate pMAT103. pNUT7 is derived from the pNUT4 vector (Kimura et al., 1990) that contains *tac*-SP6 promoters by the insertion of the M13 intergenic region and the *lacI* gene, which makes it possible to obtain single-stranded DNA with a helper phage and an RNA copy of the cDNA using SP6 polymerase (Ohshima, A. et al., unpublished results).

Using single-stranded DNA from pMAT103, I introduced an unique *Pvu*II site near the C-terminus of the coding sequence for the signal peptide by site-directed mutagenesis (Kunkel et al., 1987), using an oligonucleotide CAATCCAGCTGATTCCAGGTTCA, to yield pMAT104. A *Stu*I site was introduced near the C-terminus of the coding sequence for the propeptide, using an oligonucleotide CCACACATGAACCCGCCT-CCTCTCCGAA, to generate pMAT105. The *Eco*RI/*Pvu*II fragment of pMAT104, which contained the coding sequence for the signal peptide was introduced into the *Eco*RI and *Stu*I sites of pMAT105 to yield a derivative of the cDNA for sporamin in which the 48-bp sequence coding for the propeptide was deleted (Δpro mutant; pMAT107).

Binary plasmids for the expression of wild-type and Δpro cDNAs for sporamin in tobacco cells.

A plant binary expression vector pMAT037 [Fig. 14 B] was constructed from pCSAD, which contained the 35S promoter of CaMV , by tandem duplication of the enhancer sequence of the 35S promoter (Kay et al.,1987). The *Bam*HI-*Hin*dIII fragment of pMAT103 and pMAT107 were cloned into the *Bgl*II and *Hin*dIII sites of pMAT037 to yield the plasmids pMAT110 and pMAT108, respectively. These plasmids were transferred to *A. tumefaciens* EHA101 (Hood et al., 1986), and then they were used to transform suspension-cultured cells of tobacco (*N. tabacum*) line BY-2 (Ikeda, et al.,1976) essentially as described by An (An, 1987). Approximately 3×10^3 kanamycin-resistant colonies were pooled, reintroduced into suspension-cultures and maintained as BY-2 cells (Nagata et al., 1981). Cells transformed with pMAT110 and pMAT108 are referred to below as wild-type transformants and Δpro transformants, respectively.

Fractionation of the culture.

The medium, and the cell, protoplast, and vacuole fractions were prepared from 4-day-old cultures. Culture medium was separated from cells by filtration and concentrated by centrifugal ultra-filtration unit (10,000 N.M W.L., Centricell™, Polyscience Inc., PA). The concentrated medium was buffered with 50 mM Tris-HCl (pH 7.5)/1 mM EDTA and used as the medium fraction. Cells harvested by filtration were suspended in two volumes of extraction buffer (Chapter 2) and lysed by sonication. After centrifugation at 1,000 × *g* for 10 min, the supernatant was used as the cell fraction.

Protoplasts were prepared from the cells by the method of Nagata et al. (1981). The protoplast pellet was homogenized with

two volumes of extraction buffer (Chapter 2) in a Teflon homogenizer. The homogenate was centrifuged at 1,000 × *g* for 10 min, and the supernatant was used as the protoplast fraction. For the preparation of vacuoles, the following buffers were used: buffer A, 5 mM MES (2-(N-Morpholino)ethanesulfonic acid)-Tris (pH 6.9)/0.4 M mannitol; buffer B, 0.6% (w/v) DEAE-dextran/2.5% (w/v) Ficoll 400 in buffer A; buffer C, 0.1% (w/v) dextran sulfate/10% Ficoll in buffer A; buffer D, 0.025% dextran sulfate/2.5% Ficoll in buffer A. Protoplasts were suspended in 0.4 M mannitol at a density of 0.4 ml packed protoplasts per ml, and then they were chilled on ice for 30 min. To 12 ml of the suspension of protoplasts, 20 ml of buffer B were added and mixed gently for 1.5 min. One hundred ml of buffer A and then 50 ml of buffer C were added and the mixture was further incubated on ice for 20 min to lyse the protoplasts. Aliquotes of 40 ml each of this suspension were transferred to centrifuge tubes and 7.5 ml of buffer D and 5 ml of buffer A were overlaid on the mixture. After centrifugation at 3,300 rpm for 10 min, the material floating between buffer D and buffer A was collected. This fraction (7.5 ml) was mixed with 2.5 ml of buffer C, overlaid with 2 ml of buffer D and 1 ml of buffer A, and centrifuged at 3,000 rpm for 10 min. Material floating between buffer D and buffer A was collected and used as the vacuole fraction. The purity of the vacuoles in this fraction was checked under the light microscope and by the assay of specific marker enzymes, as described previously (Chapter 2).

Labeling in vivo.

An aliquot of 0.5 ml of a 3-day-old culture of cells was incubated with 2.8 MBq of Tran^{35}S-label for 15 min, and chased by the addition of 50 µl of 50 mM methionine and 50 mM cystein for indicated time periods. The cell and medium fractions were

prepared and sporamin-related polypeptides were precipitated with sporamin-specific antiserum and analysed by SDS-PAGE (Chapter 2). Standards of precursors with and without the signal peptide were prepared by translation *in vitro* of SP6 transcripts of the wild-type and Δpro cDNAs in a rabbit reticulocyte lysate in the presence of canine pancreas membranes (Hattori et al. 1989).

SDS-PAGE and immunoblotting.

SDS-PAGE on 12.5% polyacrylamide gels, and immunoblotting with antiserum against SDS-denatured sporamin and ^{125}I-labeled protein A were performed as described in Chapter 2.

The secreted form of sporamin.

The secreted form of sporamin in the medium fraction from a culture of the Δpro transformants was prepared as follows. Culture medium of the 7-day old culture of the Δpro transformants was separated from the cells by filtration through a layer of filter paper. 300 ml of the medium was buffered to 10 mM Tris-HCl (pH 7.5 at 4˚C), 1mM EDTA, 10 w/v% sodium ascorbate and mixed with 15 *g* of Polyclar AT (polyvinyl-pyrolydon, Meito Sangyo Co., Nagoya) and stirred for 10 min. After filtration to remove Polyclar AT particles, the medium was centrifuged at 10,000 x *g* and the supernatant was applied to DEAE-cellulose column (ϕ=20 mm x 50mm bed height; Whatmann DE-52). After washing the column by 100 ml of separation buffer (10 mM Tris-HCl) containing 0.5 mM EDTA, the bound proteins were eluted with separation buffer containing 200 mM NaCl. The fractions containing eluted proteins were pooled, and proteins were separated by ammonium sulfate precipitation. The precipitant of the 30%-50% saturation of ammonium sulfate were dissolved in and dialyzed to the separation buffer, and applied to FPLC monoQ column. Column

bound proteins were eluted by 0-100 mM linear gradient of KCl in separation buffer. Proteins in the peak fraction of sporamin were concentrated and separated by SDS-PAGE and transferred to PVDF membrane. The protein band correspond to the secreted sporamin, which were detected by coomassie staining of proteins on the membrane were excised and the N-terminal sequence of the protein were determined by protein sequencer (Model 470A, Applied Biosystems).

Estimation of the parameters for the kinetics of secretion

Halftime and lagtime of secretion of proteins from Δpro transformant cells were calculated as follows.. Relative amount of radioactivity in each band of the fluorogram was determined by densitmetric sacnning of the X-ray film. Parameters of secretion, the lagtime and halfime, of each protein was determined assuming that secretion occurred following the first-order kinetics under the following conditons,

$$t_i > 0,$$

where t_i is a chase time of time point i, and

$$S_{ti} > S_{tj}, \text{ when } t_i > t_j,$$

where S_{ti} is a density of band at time t_i.

Halftime and lagtime for the secretion of a prtein were calculated by computer-assisted best-fit analysis using the following equation,.

$$S_t = S[1-(1/2)^{\{(t-t_0)/t_{1/2}\}}]$$

where S is a suppositional total density of of target protein on assay, S_t is a density of fluorographic band of respective protein at time t, t_0 is the lagtime , and $t_{1/2}$ is the halftime.

RESULTS

Construction of plasmids and transformation of suspension-cultured tobacco cells.

A cDNA from plasmid pIMO23, encoding a precursor to A-type sporamin (Hattori et al. 1985), was modified by deletion of a 48-bp nucleotide sequence that corresponds to the propeptide of the precursor to sporamin. The resulting Δpro mutant of sporamin cDNA codes for a precursor polypeptide in which the mature part of sporamin is immediately adjacent to the signal peptide [Fig. 14(A)]. In order to obtain high-level expression of the precursors to sporamin in tobacco cells, I constructed a binary Ti-plasmid vector pMAT037 [Fig. 14(B)] which contains the 35S promoter of the CaMV with a tandemly linked enhancer region (Kay et al. 1987).

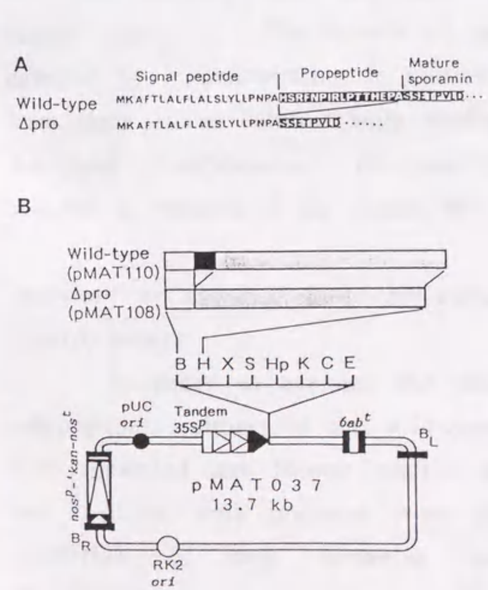

Fig. 14 The Δpro mutant of the precursor to sporamin. (A) Amino acid sequence of the N-terminal part of the wild-type and Δpro pre-cursors to sporamin deduced from the nucleotide sequence. (B) Structure of binary Ti-plasmids. Tandem $35S^P$, the 35S promoter from CaMV with a tandemly duplicated enchancer; BR and BL, T-DNA right and left borders, respectively; $6ab^t$, transcription terminator region of T-DNA transcripts 6a and 6b; *nosp-'kan-nost*, promoter of the nopaline synthase of the T-DNA of the Ti plasmid driven kanamycin-resistance gene; B, *Bgl*II; H, *Hin*dIII; X, *Xba*I; S, *Sac*II; Hp, *Hpa*I; K, *Kpn*I; C, *Cla*I; E, *Eco*RI.

The wild type and the Δpro mutant of sporamin cDNA were cloned into the multiple cloning site of pMAT037 to generate plasmids pMAT110 and pMAT108, respectively [Fig. 14(B)]. These plasmids were introduced into *Agrobacterium* and used to transform suspension-cultured tobacco cells of line BY-2 (Ikeda et al., 1976). About 3 x 10^3 kanamycin-resistant colonies were obtained 3 weeks after co-culture of 2 x 10^6 BY-2 cells with *Agrobacterium*. Expression of polypeptides that were immuno-reactive with sporamin-specific antibodies was detected in each of the 48 independent colonies analyzed for each transformant, although the level of expression varied significantly among individual transformants. To eliminate the possible effect of differences in the level of expression, mixtures of about 3,000 colonies were re-introduced to suspension culture in the case of both the wild-type and the Δpro transformants, and used for further analyses. The levels of sporamin-related polypeptides detected by immunoblotting in cultures of equal numbers of cells from these mixed cultures were similar between the wild-type and the Δpro transformants. No immunoreactive polypeptides were detected in cultures of the control BY-2 cells.

Sporamin is secreted into the culture medium from the Δpro transformants.

In order to examine the distribution of sporamin-related polypeptides, cultures of the wild-type and the Δpro transformants were separated into culture medium and cells. Then, protoplasts and vacuoles were prepared from the cells [Fig.15)]. Specific activities of two vacuolar marker enzymes, namely, phosphodiesterase and α-mannosidase, in the vacuole fraction were 32- to 37-fold higher than those in the protoplast fraction [Table 2]. By contrast, specific activities of marker enzymes for

other organelles were significantly lower in the vacuole fraction than those in the protoplasts[Table 2] indicating that the purity of the vacuoles in this fraction was fairly high. On average, one ml of the 4-day-old culture contained about 13 mg of medium proteins and 300 μg of cellular proteins, of which about 8.7 μg were recovered in the vacuole fraction.

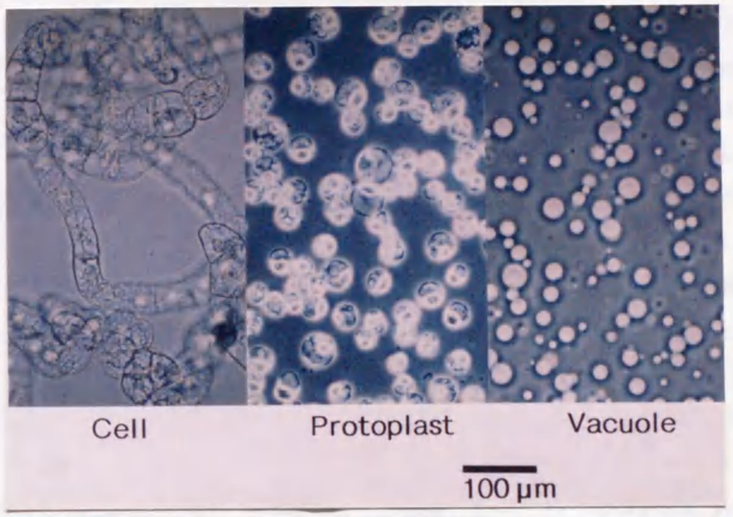

Fig. 15 Light photomicrographs of the BY-2 cells, protoplasts and vacuoles prepared from BY-2.

TABLE II

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

The avarage activities of the six preparations of non-transformed and transformed BY-2 cells were shown. The specific activities are expressed as μmol/mg protein/min.

Enzyme	Protoplasts (I)	Vacuole fraction (II)	Ratio (I)/(II)
	μmoles / mg of protein / min		
NADPH-cyt *c* reductase	0.0681	0.0047	0.085
Cyt *c* oxidase	0.850	0.0196	0.023
Catalase	1.06	0.0593	0.056
Glucose-6-phosphate dehydrogenase	0.122	0.0705	0.063
Phosphodiesterase	0.928	32.0	34.4
α-Mannosidase	0.0128	0.417	32.6

The relative levels of sporamin-related polypeptides in these fractions were examined by immunoblotting of an equal amounts of proteins (2 μg) separated by SDS-PAGE [Fig. 16]. In the cultures of the wild-type transformants, sporamin was concentrated in the vacuole fraction of the cells and could not be detected in the culture medium. A band of sporamin in the cell and in the protoplast fractions could be detected only in autoradiographs exposed for a relatively long time (360 h). A comparison of the intensities of bands revealed that sporamin is concentrated about 40-fold in the vacuole fraction as compared to the protoplast fraction, indicating that sporamin is almost exclusively localized in the vacuoles. These results are consistent with the results obtained with the transformed tobacco calli described previously (Chapter 2). By contrast, a heavy band of sporamin-related polypeptide was detected in the case of the culture medium and only a very faint band was detected in the vacuole fraction, when cultures of the Δpro transformants were analyzed [Fig. 16]. Although longer exposure of the filter revealed a clear band of sporamin in the vacuole fraction, the concentration of sporamin was estimated

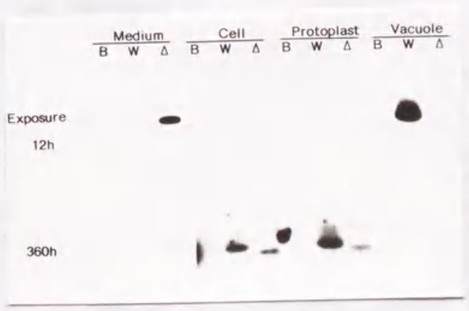

Fig. 16. Secretion of sporamin from the Δpro transformant cells. Two μg of protein in various fractions from cultures of non-transformed BY-2 cells (B), the wild-type transformant (W) and the Δpro transformant (Δ)were separated by SDS-PAGE and sporamin-related polypeptides were detected by immunoblotting with sporamin-specific antiserum and ^{125}I-protein A.

to be more than 20-fold lower than that in the vacuole of the wild-type transformants. It is calculated that more than 90% of the sporamin-related polypeptides expressed in the Δpro transformants were present in the culture medium.

The Secreted Form of Sporamin.

The sporamin-related polypeptide detected by immunoblotting of proteins in both the culture medium and the cell fractions of cultures of the Δpro transformants migrated with an apparent molecular weight of 23.8 kDa, which is slightly smaller than that of the sporamin-related polypeptide detected in the vacuole of the wild-type transformants. I have previously shown that sporamin accumulated in the vacuoles of tobacco callus in which the wild-type precursor to sporamin is expressed, is longer by 3 amino acids at its N-terminus than authentic sporamin purified from the sweet potato (Chapter 2).

A band of 23.8-kDa protein was detected as one of the major proteins in the culture medium of the Δpro transformants by staining of SDS-polyacrylamide gels with Coomassie Brilliant Blue [Fig. 17A]. This 23.8-kDa protein which was absent from the culture medium of

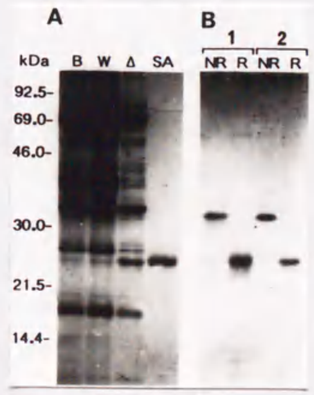

Fig. 17. The secreted form of sporamin. (A) Staining with Coomassie Brilliant Blue of proteins (20 μg) in the culture medium of non-transformed BY-2 cells (B), the wild-type transformant (W) and the wpro transformant (Δ). Lane SA, 2 μg of sporamin A purified from sweet potato. (B) SDS-PAGE of authentic sporamin A (1) and the secreted form of sporamin (2) after heating in a 1% solution of SDS in the absence (NR) or presence (R) of β-mercaptoethanol.

non-transformed BY-2 cells and of the wild-type transformants [Fig. 3A), suggesting that this protein is the secreted form of sporamin. The 23.8-kDa protein in the culture medium of the Δpro transformants was purified and its N-terminal amino acid sequence was determined. The sequence,

EYPVLDINGDEVRAGGNYYMVSAIXGAG

shown with the one-letter code with X representing unidentified amino acids, corresponds to the amino acid sequence of the prepro prerursor to sporamin from positions 40 to 67 (Hattori et al. 1985; see Fig. 1A). From these results, the 23.8-kDa protein in the culture medium of the Δpro transformants was identified as the secreted form of sporamin, which is shorter by two amino acids than authentic sporamin at its N-terminus.

Sporamin A treated in a solution of SDS without a reducing reagent migrates on SDS-polyacrylamide gels more slowly than the reduced form (Maeshima et al. 1985). This anomalous migration is probably due to intramolecular disulfide bridges, since the amino acid sequence of sporamin shows homologies to the Kunitz-type trypsin inhibitor from soybean, and the four Cys residues involved in the formation of two intramolecular disulfide bridges in the inhibitor are conserved in sporamin (Hattori et al. 1989; Fig. 1). When treated with a solution of SDS in the absence of a reducing reagent, the secreted form of sporamin migrated at position that corresponded to an apparent molecular mass of 30.0 kDa, which is almost identical to the position to which authentic sporamin A migrates under the same conditions [Fig. 17(B)]. These results suggest that appropriate intramolecular disulfide bridges are formed in the absence of the propeptide.

Kinetics of secretion of sporamin into the culture medium.

The kinetics of the processing and transport of precursors to sporamin in the wild-type and the Δpro transformants were examined by pulse-labelling the cultures with [^{35}S]Met and [^{35}S]Cys for 15 min, with a subsequent chase with excess unlabelled Met and Cys for various periods of time. Sporamin-related polypeptides in the cell and medium fractions of the culture were precipitated with sporamin-specific antiserum and analyzed by SDS-PAGE

In the case of the wild-type transformants, pulse-labeled cells gave a major band that corresponded to the pro-form of the precursor to sporamin [Fig. 18(A)]. The pro-form was converted into the apparently mature-sized form of sporamin during the chase by at least two processing steps. In transformed tobacco calli, the propeptide is first cleaved between Thr^{32}-Thr^{33}and then the dipeptide Thr^{33}-His^{34} is removed (Chapter 2). The nature of the minor bands is not clear. The half-time for the first step of post-translational processing was 21 min, which is similar to the value obtained with the callus (Chapter 2). No ^{35}S-labeled sporamin was detected in the culture medium even after a chase for 4 h [Fig. 18(A)].

In the case of the pulse-labeled Δpro transformants, a single band was found which migrated slightly faster than the Δpro precursor to sporamin processed *in vitro* by canine microsomal mambrane [Fig. 18(B)]. The amount of ^{35}S-labeled sporamin in the cell decreased during the chase, and the secreted form of sporamin started to appear in the medium after 30 min of chase [Fig. 18(B]) About 95% of the sporamin labeled during the pulse was secreated into the medium

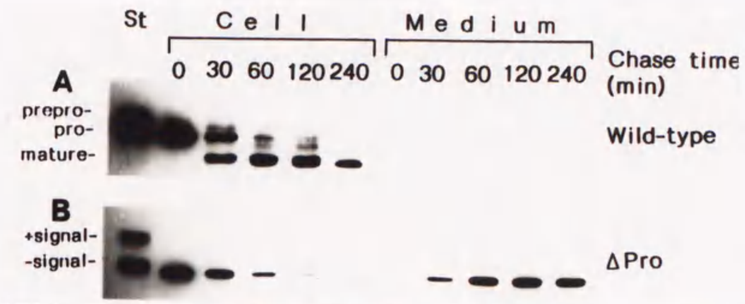

Fig. 18 Pulse-chase analysis of secretion of sporamin. The wild-type (A) and the Δpro (B) transformants labeled with Tran^{35}S-label (see Materials and Methods) for 15 min were chased for the indicated periods of time and sporamin-related polypeptides in the cell and medium fractions from equal volumes of cultures were immunoprecipitated and analyzed by SDS-PAGE. Lane St shows size standards for the precursors; SP6 transcripts of the wild-type (A) and the Δpro mutant (B) cDNA were translated *in vitro* in the presence of canine microsomal membrane and immunoprecipitated.

within 4 h during the chase period.

During the chase, no changes in the size of pulse-labeled, sporamin-related polypeptides in the Δpro transformants were observed. Thus, it appears that the Δpro precursor is co-translationally cleaved at Ser^{39}-Glu^{40} in tobacco cells. The site of cotranslational cleavage by the canine membranes has not been examined.

The culture medium of the Δpro transformants, after a 60-min chase, contained at least fourteen ^{35}S-labeled bands of polypeptides [Fig. 19(A)]. Among these was the 23.8-kDa band which was identified as the secreted form of sporamin since this band was absent from the culture medium of non-transformed cells and of the wild-type transformants. Furthermore, the polypeptide could be specifically precipitated with sporamin-specific antiserum (data not shown).

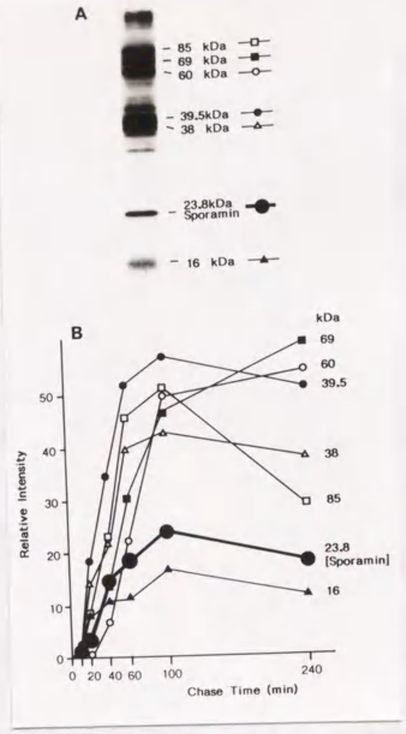

Fig. 19 Kinetics of secretion of sporamin. (A) Seven major ^{35}S-labeled polypeptides in the culture medium of the Δpro transformants were analyzed in terms of their kinetics of secretion (see text). (B) Changes in relative radioactivities of polypeptides in equal volumes of culture medium after the chase.

The rate of secretion of sporamin from the Δpro transformants was compared with rates of secretion of endogenous secretory proteins from the host tobacco cells, ^{35}S-labeled polypeptides in identical volumes of culture medium at various times during the chase were separated by SDS-PAGE, and changes in relative radioactivities of seven major polypeptides, indicated in Figure 19(A), were determined by densitometric scanning of the fluorogram [Table III; Fig. 19(B).] The kinetics of secretion of the various polypeptides differed slightly from one another. However, the lag-period to the appearance of labeled polypeptides in the medium in the case of six endogenous polypeptides fell within a range from 2 to 33 min, and the half-time for the increase in the radioactivity ranged between 20 and 37 min (Table 3).

TABLE III

The half time and lag time of the sectetion of seven major extrapeptides of the Δpro transformants.

Proteins analyzed in this table are indicated in Fig. 19 (A).

Protein		Lag Time (min)	Half Time (min)
85 kDa		12	23
69 k Da		15	32
60 k Da		33	37
39.5 k Da		8	29
38 k Da		8	22
23.8 k Da	Sporamin	15	21
16 k Da		2	20

The lag-time and the half-time for the secreted form of sporamin were 15 and 23 min, respectively, indicating that the kinetics of secretion of sporamin from the Δpro transformant cells are similar to those of the secretion of endogenous proteins.

The radioactivity of some of the secreted polypeptides decreased after 100 min of chase. Degradation or processing of polypeptides after secretion may explain the difference between the profiles of ^{35}S-labeled polypeptides [Fig. 19(A)] and the staining pattern of proteins [Fig. 17(A)] in the culture medium.

DISCUSSION

The absence of glycan side chains in the precursor to sporamin and the presence of a short propeptide between the signal peptide and the mature region in the precursor, which is probably removed after the arrival of the polypeptide in the vacuole (Chapter 2), prompted me to search for the vacuolar targeting signal in the sporamin precursor. The use of suspension-cultured cells of the BY-2 line of tobacco (Ikeda et al., 1976) facilitated our analysis, since BY-2 cells grow rapidly, can be transformed with *Agrobacterium* very efficiently, and are well suited for the preparation of pure vacuoles.

In sharp contrast to the efficient transport of sporamin to the vacuole in the wild-type transformants, almost all the sporamin synthesized was secreted into the culture medium in the case of the Δpro transformants [Fig. 16]. Furthermore, the kinetics of secretion of sporamin from the Δpro transformant cells were similar to those of the endogenous proteins secreted by the host BY-2 cells [Fig. 19], suggesting that the secretion of sporamin follows the general pathway for secretion of polypeptide from these cells. From these results, I conclude that the propeptide of the precursor to sporamin contains the information required for targeting of sporamin to the vacuole in tobacco cells. However, the results presented here do not preclude the possibility that other regions of prosporamin are important for vacuolar targeting, and it is not known at present whether the propeptide of the sporamin precursor, together with the signal peptide, is sufficient for the targeting of alien proteins to the vacuole. The results presented in this paper, together with results reported previously (Dorel et al., 1989, Lund et al., 1989, Iturriaga et al., 1989, Denecke et al., 1990), also suggest that proteins can be secreted from the plant

cells by a bulk flow default pathway in the absence of positive topogenic information, as in the case of animal and yeast cells (Pfeffer and Rothman 1987, Rothman et al. 1989).

The propeptides in two precursors to yeast vacuolar proteins, namely, carboxypeptidase Y (Johnson et al. 1987; Valls et al. 1987) and proteinase A (Klinoski et al. 1988), have been shown to contain vacuolar targeting signals, and the propeptide of rat prepro-somatostatin (Savarino et al. 1989) has been shown to contain the information necessary to target the somatostatin to storage secretory vesicles for subsequent participation in the regulated secretory pathway. Propeptides of these precursors are much longer than the propeptide of the precursor to sporamin. The propeptides of precursors for yeast vacuolar and animal lysosomal hydrolytic enzymes are also required to keep the enzyme in a latent form (Valls et al. 1987; Klinoski et al. 1988). Some of the propeptides may play a role in the folding of polypeptides (Zhu et al. 1989). The secreted form of sporamin seems to contain intramolecular disulfide bridges similar to those in authentic sporamin from the sweet potato [Fig. 17B], suggesting that the correct folding of the sporamin polypeptide takes place in the absence of the propeptide. The short propeptide of the precursor to sporamin may function primarily in vacuolar targeting.

The propeptide of the precursor to sporamin does not contain any sequence similar to the vacuoler-targeting domain of the propeptide of the precursor to carboxypeptidase Y (Q-R-P-L; ref. Rothman et al. 1989). The propeptide of the precursor to yeast proteinase A also does not contain this sequence (Rothman et al. 1989). It is not known whether the two yeast vacuolar proteins are sorted by different recognition systems, or whether some unrecognized structural feature shared by the two propeptides is recognized by a common "receptor". It also remains to be

determined whether topogenic signals in proteins for transport to acidic compartments of the cell are evolutionaly conserved between yeast and plants.

Vacuolar-targeting information in precursors to sporamin is likely to be conserved among the members of the sporamin multigene family. A comparison of the nucleotide sequences of six members of the family (Hattori et al. 1989) indicates that the amino acid sequence of the propeptide of the precursor to sporamin differs at five positions between the A and B subfamilies, and most of the substitutions are located in the C-terminal half of the propeptide. Further analyses with short deletions and point mutations should reveal the minimum structure of the propeptide that is required for vacuolar targeting.

Chapter 4

Mutations in the Propeptide of the Precursor to sporamin Cause Alterations in the Site of Cleavage of the Signal Peptide and Transport of the Protein in Transformed Tobacco Cells

INTRODUCTION

Precursors to proteins that enter the secretory pathway in eukaryotic cells usually contain a signal peptide (Blobel and Dobberstein, 1975), which is removed by subsequent proteolytic cleavage, at their N-terminus. This peptide functions as a signal for the translocation of the protein through the membrane of the rER. Mechanisms for the signal peptide-mediated translocation of newly synthesized polypeptides through the membrane have been studied extensively with canine pancreatic microsomal membranes *in vitro* and in *Escherichia coli* cells, and the basic mechanisms seem to have been conserved in various evolutionarily divergent organisms.

Primary structures of signal peptides from various organisms show little homology if any. However, several general features of the structure of the signal peptide have been identified (Inouye and Halegoua 1980; von Heijne 1983; Perlman and Halvorson 1983). The signal peptide consists of three structurally distinct regions: a basic N-terminal region (n-region); a central hydrophobic region

(h-region); and a polar C-terminal region (c-region) (von Heijne, 1985). Signal peptides are cleaved by signal peptidases located on the luminal side of the rER during or immediately after translocation of newly synthesized polypeptides (Pugsley 1989), and the c-region defines the site of cleavage of the signal peptide (von Heijne 1985, 1986). Specific amino acid residues in the c-region, especially those at positions -1 and -3 relative to the site of cleavage have been proposed to be crucial for the specificity of the signal peptidases. However, mutational analyses of signal peptides have suggested that, in addition to the primary sequence of the c-region, other factors, such as secondary structure around the site of cleavage and the amino acid sequence outside the c-region, also affect the cleavage of the signal peptide (Folz and Gordon 1986; Andrews et al 1988; Folz et al 1988; Cioffi et al. 1989; Nothwehr and Gordon 1989, 1990; Nothwehr et al. 1990; and those reviewed in Pugsley 1989). Despite these conserved features, some species-specific variations in the structure of the signal peptide have been noted (von Heijne and Abrahmsen 1989) and signal peptides from one organism are not always cleaved efficiently or correctly when the polypeptides are expressed in foreign hosts.

The signal peptides of plant proteins can direct the translocation of proteins through membranes and they are cleaved from the mature proteins by membranes of various other organisms, such as *E. coli* (Hattori et al, 1987), yeast (Tague and Chrispeels 1987; Utsumi et al. 1988; K. Matsuoka and K. Nakamura submitted for publication) and animal (Vitale et al 1986; Voelker et al. 1986; Hattori et al. 1987; DellaPenna and Bennett 1988). The signal peptides of bacterial proteins (Lund et al. 1989; Denecke et al. 1990) can function in plant cells, and the signal peptides of bacterial and animal proteins can be cotranslationally

processed by wheat germ microsomal membranes (Prehn et al. 1987). However, the cleavage of signal peptide in plant cells has not yet been characterized in detail.

In chapter 3, I have also shown that deletion of the propeptide from the precursor results in secretion of sporamin into the culture medium, indicating that the propeptide is essential for the targeting of sporamin to the vacuole. In order to analyze the minimum sequence of the propeptide required for vacuolar targeting, I introduced several mutations into the propeptide. Unexpectedly, some of these mutations resulted in a change in the site of cleavage of the signal peptide to a cryptic site located downstream from the propeptide and the resultant processed polypeptides were secreted into the culture medium in transformed tobacco cells. However, the same mutations did not cause the cleavage of the signal peptide at this cryptic site in a processing system *in vitro* with canine microsomal membranes, the results suggesting that the signal-peptide processing machinery of plant cells functions differently from that in animal cells.

MATERIALS AND METHODS

Construction of mutants

The wild-type and the Δpro mutant form of sporamin cDNA were described in Chapter 3). In H22D, the nucleotide sequence of cDNA corresponding to the N-terminal part of the propeptide was modified to include a PvuII restriction site by mutagenesis of sporamin cDNA in plasmid pMAT103, using the oligonucleotide CAATCCAGCTGA-TTCCAGGTTCA (Kunkel et al. 1987). A StuI restriction site was created in the cDNA that corresponds to the C-terminal region of the propeptide to produce mutant P36Q by using the oligonucleotide CCACACATGAACCCGCCTCCTCTCCGAA. The H22QQIC mutant was constructed by combining appropriate 5'- and 3'-deletion derivatives of the sporamin cDNA by use of a BglII linker (GCAGATCTGC). Three mutants with deletions of four consecutive amino acids in the propeptide, namely, Δ23-26, Δ27-30, and Δ35-38, were generated by oligonucleotide-directed mutagenesis using CAATCCAGCCCATCCCATCCGCCT, ATTCCAGG-TTCAACCCCACCACACACGA, and CACCACACACTCTGAAACTCCAGT, respectively. These mutant cDNAs were placed downstream from the 35S promoter from Cauliflower Mosaic Virus with tandemly duplicated enhancers in a Ti-binary expression vector pMAT037 (Chapter 3), for the expression of mutant precursors in tobacco cells.

Transformation of suspension-cultured cells of tobacco

Transformation of suspension-cultured cells of tobacco

line BY-2 and the culture of transformed cells were carried out essentially as described in Chapter 3. Cells transformed with binary plasmids that contained mutant cDNA are referred to below by the name of the mutation (e.g. H22D transformant).

Labeling of transformed tobacco cells

A 0.5-ml sample of a 3-day-old culture of cells was incubated with 2.8 MBq of Tran^{35}S-label (ICN) for 15 min, and then 50 µl of 50 mM methionine and 50 mM cysteine were added and cells were further incubated for indicated time periods. The cells and the medium were separated and sporamin-related polypeptides were precipitated with sporamin-specific antiserum and analyzed by SDS-polyacrylamide gel electrophoresis (Chapter 2; Chapter 3). In the case of labeling for only short periods of time, 0.5 ml of culture was incubated with 3.7 MBq of Tran^{35}S-label for the indicated time periods and the culture was quickly frozen in liquid nitrogen. After the addition of an equal volume of sonication buffer, which contained SDS and protease inhibitors (Matsuoka et al, 1990) for the frozen culture, cells were lysed by sonication and sporamin-related polypeptides were immunoprecipitated.

Translation-processing assay in vitro with canine microsomal membranes

The plasmid pMAT103, which was used to generate mutants of sporamin cDNA, contains the SP6 promoter immediately upstream from the cDNA (Chapter 3). SP6 transcripts of the sporamin cDNA were translated *in vitro* in a rabbit reticulocyte lysate in the absence and in the presence of canine microsomal membranes (Chapter 2).

N-Terminal amino acid sequence of the secreted form of sporamin

Sporamin secreted into the culture medium was purified from 7-day-old cultures of transformants and the N-terminal amino acid sequence of each form of sporamin was determined as described previously (Chapter 3).

RESULTS

Construction of mutants of a precursor to sporamin

My initial purpose in constructing and expressing precursors to sporamin, with various mutations in the propeptide, in tobacco cells was to determine the minimum sequence of the propeptide that is required for targeting sporamin to the vacuole. In the H22D and P36Q, His^{22} and Pro^{36}, as numbered from the initiator Met residue (Hattori et al. 1985), were changed to Asp and Gln, respectively. The mutation in H22QQIC changed His^{22} to a Gln-Gln-Ile-Cys (QQIC) sequence. In addition to these substitution mutants, three mutants with deletions of four consecutive amino acids in the propeptide, namely, Δ23-26, Δ27-30 and Δ35-38, were also constructed. The nucleotide sequences of the mutant cDNAs were verified by nucleotide sequencing, and the deduced amino acid sequences of the mutant precursors to sporamin are shown in Figure 20.

Secretion of sporamin from tobacco cells by various mutations in the propeptide

The mutant cDNAs were inserted downstream from the 35S promoter of CaMV with the tandemly duplicated enhancer sequence of the 35S promoter in the binary expression vector pMAT037 (Chapter 3). The resultant plasmids were transferred to Agro-acterium and used to transform suspension-cultured BY-2 tobacco cells (Ikeda et al, 1976). I used a mixture of several thousand kanamycin-resistant colonies to eliminate the differences in the level of expression among various transformants.

To determine whether or not mutations in the propeptide affected the vacuolar targeting of sporamin in tobacco cells, the

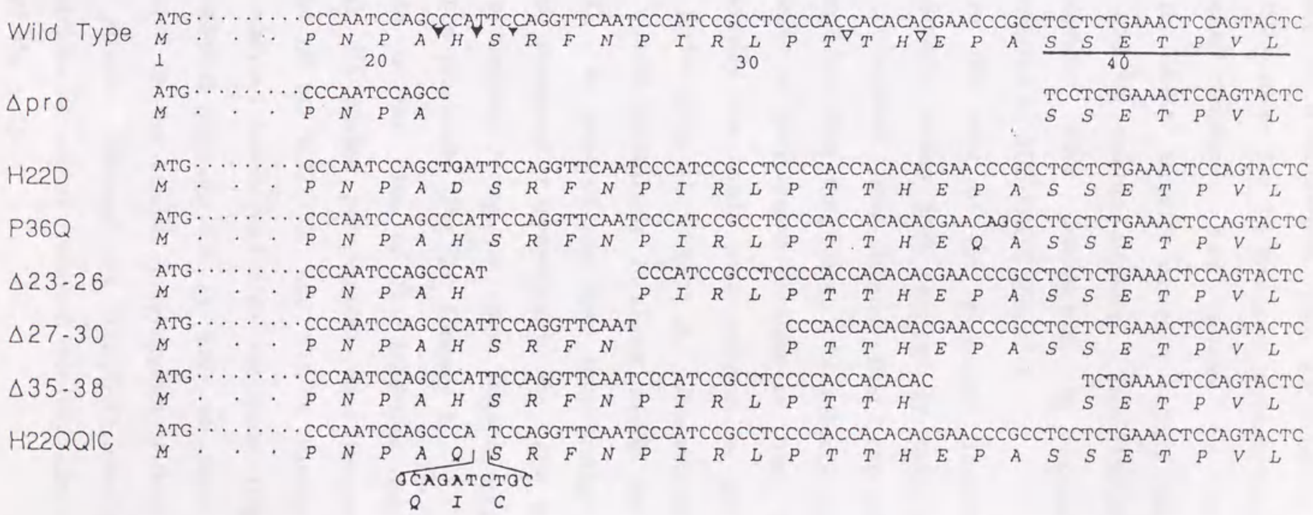

Fig. 20. Mutations in the propeptide of the precursor to sporamin. Nucleotide sequence and deduced amino acid sequence of the critical regions of the wild-type and mutant cDNAs for sporamin. The structures of the cDNAs for the wild-type (Hattori et al, 1985) and the Δpro mutant form (Matsuoka Nakamura, 1991) of the precursor to sporamin were described previously. The filled arrowhead indicates N-terminal amino acid residues of prosporamin detected after processing *in vitro* of the precursor to sporamin with canine microsomal membranes (Hattori et al, 1987). The open arrowhead shows the site of post-translational processing of the precursor to sporamin in tobacco cells (Chapter 2). The N-terminal structure of sporamin purified from the sweet potato roots is underlined. Boldface letters indi-cate the substituted nucleotides and amino acids.

cultures of transformants were pulse-labeled with ^{35}S-methionine and ^{35}S-cysteine for 15 min and subsequently chased with excess unlabelled methionine and cysteine for 120 min. After the pulse and the chase, aliquots of each culture were separated into the cells and the culture medium. The ^{35}S-labeled, sporamin-related polypeptides were precipitated by sporamin-specific antiserum and analyzed by SDS-PAGE [Fig. 21].

In the case of the wild-type transformant, the band of prosporamin, which was detected by pulse-labeling of the cells was converted into bands that represented two smaller polypeptides after the chase. In tobacco cells, post-translational processing of prosporamin involves at least two sequential steps and usually two bands were detected on gels after a 120-min of chase period [Fig. 21; Chapter 2]. The processed forms of sporamin were almost exclusively localized within the vacuole (Chapter 2 and 3). In case of the Δpro transformant, most of the pulse-labeled, processed polypeptides in the cells were secreted into the culture medium during the chase without any change in size, as described previously [Fig. 21; Chapter 3].

As in the case of the wild-type transformant, sporamin-related, ^{35}S-labeled polypeptides were detected only in the cell fraction of the H22QQIC and Δ35-38 transformants when cultures were analyzed before and after the chase [Fig. 21]. Furthermore, pulse-labeled polypeptides in both of these transformants were converted to the smaller polypeptides within the cell during the chase period. Although the H22QQIC transformant contained two polypeptides of almost identical mobility after the chase, as in the case of the wild-

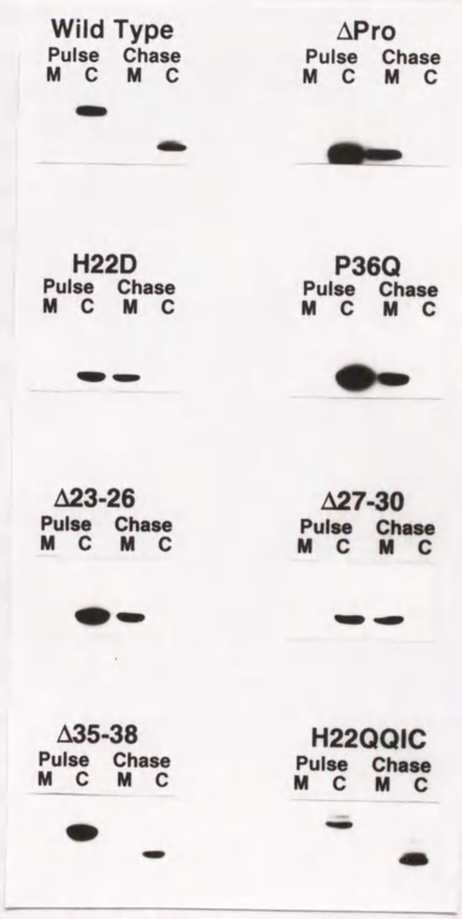

Fig. 21. Analysis by pulse-chase labeling of sporamin-related polypeptides express-ed in transformed tobacco cells. Cells in suspension culture were labeled for 15 min with Tran^{35}S-label (Pulse; see Materials and Methods) and subsequently chased with excess amounts of unlabeled methionine and cysteine for 120 min (Chase). The cells (C) and culture mediums (M) were separated and sporamin-related polypeptides were precipitated with sporamin-specific antiserum and analy-zed by SDS-polyacrylamide gel electrophoresis and fluorography. Aliquots corres-ponding to the equal volume of the culture were loaded on the gel.

type transformant, only a single band of ^{35}S-labeled polypeptide was detected in the case of the Δ35-38 transformant. The first and second steps in the post-translational cleavage of prosporamin in tobacco cells occur at Thr^{32}-Thr^{33} and His^{34}-Glu^{35}, respectively (Chapter 2), and Glu^{35} is absent, as a result of a deletion, in the Δ35-38 mutant [Fig. 20]..

In contrast to the mutants described above, almost all of the pulse-labeled polypeptides in the H22D, P36Q, Δ23-26 and Δ27-30 transformant cells were secreted into the culture medium after the chase [Fig. 21]. Furthermore, the size of the labeled polypeptides did not change during the pulse and chase periods in these cells, suggesting the absence of post-translational processing of these mutant precursors. When proteins in the culture medium of these transformants were separated by electrophoresis on SDS-polyacrylamide gels, a band of sporamin could be detected by staining of gels with Coomassie Brilliant Blue (data not shown; see Chapter 3).

Size of the pulse-labeled polypeptides

The mutant precursors described in this report differed in terms of the structure of the propeptide from the wild-type precursor by a maximum size difference of only plus or minus four amino acid residues [Fig. 20]. However, a comparison of the sizes of pulse-labeled polypeptides indicated that pulse-labeled polypeptides in all of the four mutant transformants that secreted sporamin migrated more rapidly than the band of prosporamin detected in analysis of the wild-type transformant [Fig. 22]. Furthermore, to my surprise, the sizes of all of these polypeptides were not only identical to one another but they were also identical to the size of the pulse-labeled polypeptide detected in the analysis of the Δpro transformant.

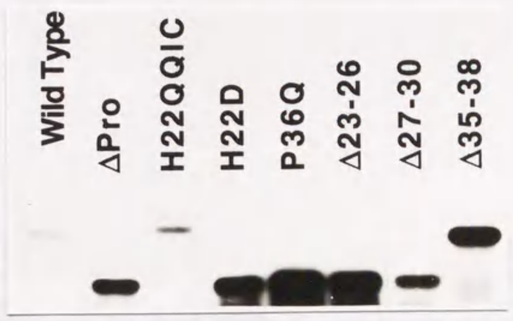

Fig. 22. Comparison of the sizes of the sporamin-related polypeptides detected in transformed tobacco cells after pulse-labeling for 15 min. Amounts of samples applied to the gel were not equalized in this experiment.

By contrast, the size of the major bands of the pulse-labeled polypeptides isolated from H22QQIC and Δ35-38 transformants migrated to positions similar to that of prosporamin. In the H22QQIC transformant, a minor band with lower mobility was also detected.

Short-term labeling of sporamin

To eliminate the possibility that post-translational processing of the mutant precursors occured more rapidly than that of the wild-type precursor, such that pulse-labeled polypeptides detected in the analysis of the H22D, P36Q, Δ23-26 and Δ27-30 transformants were the products of post-translational processing, the cultures of the H22D and Δpro transformants were labaled with ^{35}S-methionine and ^{35}S-cysteine for shorter periods of time and

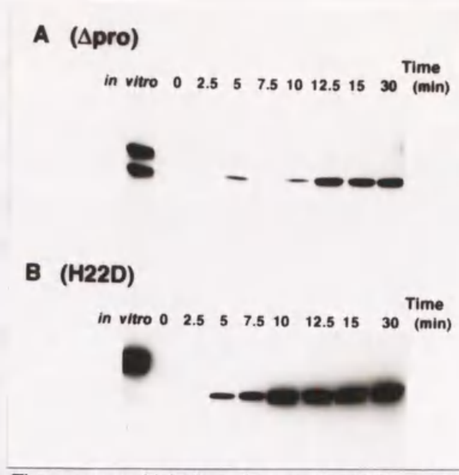

Fig. 23. Time-course of labeling of sporamin-related polypeptides in cultures of the Δpro and H22D transformants. Experimental conditions are described in Materials and Methods. As standards for the precursor with and without the signal peptide, the Δpro mutant precursor and H22D mutant precursor were translated in a rabbit reticulocyte lysate in the presence of limiting amounts of canine microsomal membranes (*in vitro*).

the ^{35}S-labeled, sporamin-related polypeptides in the whole culture were analyzed after immunoprecipitation.

In the case of both H22D and Δpro transformants, a single band of ^{35}S-labeled polypeptide, identical in mobility to the processed form of the Δpro mutant precursor, could be detected as early as 2.5 min after the onset of labelling with ^{35}S-amino acids [Fig. 23]. Precursors with the signal peptide still attached could not be detected even with longer exposure of the film (data not shown). The electrophoretic pattern of the ^{35}S-labeled polypeptide did not change when the labelling time was extended to as much as 30 min. These results strongly suggest that the ^{35}S-labeled polypeptide detected in the pulse-labeled H22D transformant cells and, most likely, in the P36Q, Δ23-26 and Δ27-30 transformants as well, was the product of a signal-peptide processing reaction that occured during or immediately after the translation of the mutant precursor.

N-Terminal amino acid sequence of the secreted form of sporamin

The pulse-labeled polypeptides in the H22D, P36Q, Δ23-26 and Δ27-30 transformants were secreted into the culture medium without being subjected to any further processing [Fig. 21]. I purified the secreted form of sporamin from the culture medium of the P36Q and Δ27-30 transformants and the N-terminal amino acid sequence of these proteins were determined. The sequence of the secreted form of sporamin from the P36Q transformant,

E-T-P-V-L-D-I-N-G-X-E-V-R-A-G-,

shown in the one-letter code with X representing an unidentified amino acid, suggested that cleavage of the P36Q mutant precursor occurs between Ser^{39} and Glu^{40}, namely, 18 amino acid residues downstream from the major site of cotranslational cleavage of the wild-type precursor by canine microsomal membranes [Fig. 20). This site is identical to the site of processing of the Δpro mutant precursor in tobacco cells (Chapter 3).

The sequence of the secreted form of sporamin from the Δ27-30 transformant,

X-P-V-L-D-I-N-G-D-E-V-R-A-G-G-,

suggested that cleavage of the Δ27-30 mutant precursor occured at one residue downstream from the site of cleavage of the P36Q and Δpro mutant precursors. Since the sizes of the pulse-labeled polypeptides in the H22D and Δ23-26 transformants were also almost identical to that of the pulse-labeled polypeptide in the Δpro transformant [Fig. 22], it seems that cleavage of these mutant precursors also occured at the corresponding Ser-Glu or Glu-Thr bonds.

Processing of mutant precursors by mammalian membranes in vitro

As seen in Figure 23, the pulse-labeled polypeptide from the H22D transformant migrated more rapidly than the H22D mutant

precursor that had been partially processed *in vitro* by canine microsomal membranes, suggesting that cotranslational cleavage of the signal peptide of the H22D mutant precursor occurs at different sites in plant and animal systems. The pulse-labeled polypeptides in various transformants were compared with the respective precursors synthesized and processed *in vitro* in a rabbit reticulocyte lysate and by canine microsomal membranes, respectively [Fig. 24].

The pulse-labeled polypeptides from the wild-type and Δpro transformants migrated to positions almost identical to those of the respective precursors after processing *in vitro* by canine microsomal membranes, as described in Chapter 3. The pulse-labeled polypeptide from the H22QQIC transformant also migrated to the same position as the polypeptide that resulted from processing *in vitro* of the H22QQIC mutant precursor. The more slowly migrating, faint band was identical in mobility to the H22QQIC mutant precursor with the signal peptide still attached. The pulse-labeled polypeptide in the Δ35-38 transformant was identical in terms of mobility to the Δ35-38 mutant precursor with the signal peptide still attached, indicating that the Δ35-38 mutation severely diminished the efficiency of cotranslational cleavage in tobacco cells. However, this mutation did not affect the processing *in vitro* by canine membranes [Fig. 24]. In the case of each of the H22D, P36Q, Δ23-26 and Δ27-30 mutant

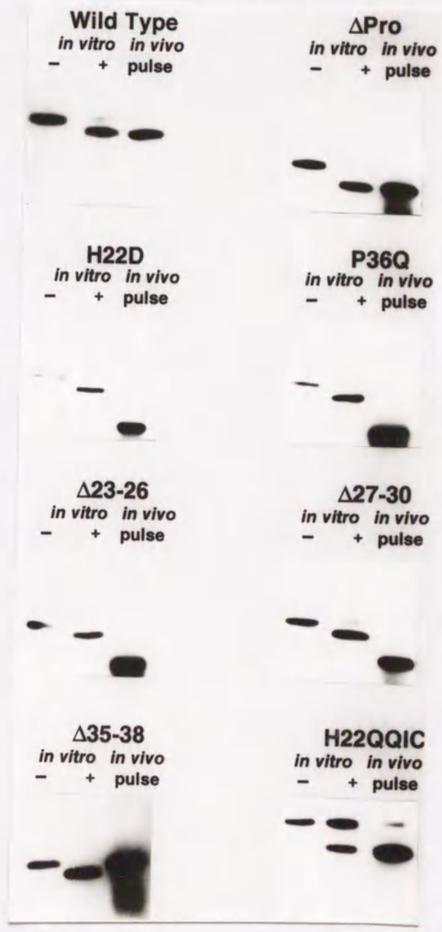

Fig. 24. Processing *in vitro* of mutant precursors to sporamin by canine microsomal membranes. The SP6 transcripts of the wild-type and mutant cDNAs for sporamin were trans-lated *in vitro* in a rabbit reticulocyte lysate in the absence (-) and in the presence (+) of canine microsomal membranes. The pulse-labeled, sporamin-related polypeptides in various transformed tobacco cells that had been prepared in experiments described in Fig. 22 were used.

precursors, processing *in vitro* with canine microsomal membranes produced polypeptides that were significantly larger than the pulse-labeled polypeptides in tobacco cells [Fig 24]. The sites of cotranslational cleavage of these mutant precursors *in vitro* have not been determined.

DISCUSSION

In cells expressing the precursor to sporamin with the H22D, P36Q, Δ23-26 and Δ27-30 mutations in the propeptide [Fig. 20], sporamin-related polypeptides detected after pulse-labeling for 15 min ("pulse-labeled polypeptides") were significantly smaller than wild-type prosporamin. The sizes of pulse-labeled polypeptides from these mutant transformants were not only identical to one another but they were also identical to the processed form of the Δpro mutant precursor [Fig. 22]. Furthermore, the N-terminal structures of the sporamin secreted by the P36Q and Δ27-30 transformants were identical to and different in only one amino acid residue from that of the sporamin secreted by the Δpro transformant, respectively. Several lines of evidence strongly suggest that these mutations caused an alteration in the site of cleavage of the signal peptide to an alternate cryptic site located downstream from the propeptide. First, processing of the respective mutant precursors to the pulse-labeled polypeptides occurred very rapidly [Fig. 23]. In case of the wild-type precursor, post-translational processing of prosporamin occurs slowly, with a half time of 21 min or longer (Chapter 3). Second, I did not detect any intermediate band of lower mobility than the pulse-labeled polypeptide, even under the short-term labelling conditions [Fig. 23] Third, sporamins were secreted into the medium by these mutant transformants [Fig. 21]. Secretion of sporamin from mutant transformants can be best explained by cleavage of the propeptide that carries the vacuolar-targeting information from the mutant precursor at an early stage of biosynthesis before the sorting of proteins takes place.

To determine the exact site of cotranslational cleavage of these mutant precursors in plants, I tried to examine the

processing of the precursor to sporamin *in vitro* using plant microsomal membranes (Higgins and Spencer 1981; Hattori et al. 1987; Prehn et al. 1987; DellaPenna and Bennett 1988). However, to date, my efforts to prepare a processing system *in vitro* with plant membranes that would be efficient enough to allow us to determine the site of cleavage have failed.

An effect of the mutation in the propeptide on the cleavage of the signal peptide that was different from effects observed with the above-mentioned four mutations was observed with the Δ35-38 mutant precursor. In the Δ35-38 transformants, pulse-labelling of cells revealed a band of precursor with the signal peptide still attached [Fig. 24]. This ^{35}S-labeled precursor was chased to a polypeptide that was similar in mobility to the apparently mature-sized form of sporamin detected in the wild-type transformant after the chase [Fig. 21]. It seems that the signal peptide of this mutant precursor is uncleavable during the cotranslational translocation step and is cleaved off, together with the propeptide segment, at a later time by a post-translational mechanism. This mutation did not allow secretion of sporamin into the medium and further analyses on the behavior of the Δ35-38 mutant precursor in tobacco cells are now in progress.

Statistical analyses of the structure of many signal peptides has revealed that there are some regularities in the amino acid residues around the site of cleavage of the signal peptide. Amino acid residues at positions -3 and -1 relative to the cleavage site probably plays the most important role (von Heijne 1983; Perlman and Halvorson, 1983). According to this (-3,-1)-rule, the residue in position -1 must be small (Ala, Ser, Gly, Cys, Thr or Gln) and the residue in position -3 must not be aromatic, charged, or large and polar (von Heijne, 1986). Furthermore, Pro

is only rarely present between positions -3 through +1 even though it is frequently found at positions that surround this region (von Heijne, 1986). Examination of the sequence of the precursor to sporamin indicates that there are two sequences that conform well to these general rules [Fig. 25]. Site I is Pro-Ala-His-Ser-Arg^{24} and site II is Pro-Ala-Ser-Ser-Asp^{40}, with the last amino acid residue corresponding to the +1 position. Both His and Ser are found at position -2 in many signal peptides, and both Arg and Asp are relatively favoured amino acids at position +1 in eukaryotic signal peptides (von Heijne, 1986).

Site I is located at the boundary between the signal peptide and the propeptide, and the general rule predicts the most favoured site of cleavage to be between Ser^{23} and Arg^{24}. In a previous study *in vitro* with a translocation-processing system that contained canine microsomal membranes, the exact site of cleavage of the signal peptide in the precursor to sporamin could not be determined unambiguously (Hattori et al, 1987). Sequential Edman degradation of processed form of the precursor labeled with ^{3}H-leucine yielded a broad peak of radioactivity from the 7th through the 9th cycles of degradation with the maximum level of radioactivity appearing at the 8th and 9th cycles. The appearance of the ^{3}H-label at the 7th cycle suggests cleavage at the Ser^{23}-Arg^{24} bond.

The site II sequence is located at the boundary between the propeptide and mature sporamin. In the Δpro mutant precursor, the site I sequence has been eliminated while the site II sequence has been recreated [Fig. 25] The cleavage of signal peptide from this mutant precursor in tobacco cells occurs exactly at the site that is predicted from the sequence of site II (Chapter 3; Fig. 25). Since the pulse-labeled polypeptides detected in the analysis of the H22D, P36Q, Δ23-26 and Δ27-30 transformants were almost identical in mobility to that from the Δpro transformant [Fig. 22],

Fig. 25. Amino acid sequence of the N-terminal extra peptide of the wild-type and various mutant precursors to sporamin. The meanings of the closed arrowheads and open arrowheads are explained in legend to Fig. 20. The N-terminal amino acid sequences of the secreted forms of sporamin in the culture medium of the Δpro and P36Q transformants are included in this Figure. Sequences of two potential sites of cleavage of the signal peptide, site I and site II, described in the text, are indicated by boxes.

and the N-terminal structure of the secreted form of sporamin in the culture medium of the P36Q and Δ27-30 transformants were identical to and different in only one amino acid residue from that of the Δpro transformant, cleavage of the signal peptide in all of these mutant precursors seems to occur at site II.

In the H22D and Δ23-26 mutants, the sequence at site I is disrupted by mutation. By contrast, mutations in the P36Q and Δ27-30 precursors do not alter the sequence of site I itself. Thus, not only the disruption of the original sequence of site I but also mutations in the sequence downstream from it cause switching of the site of signal peptide cleavage to an alternate cryptic site (site II). A substitution of Pro to Gln at the -5 position of the site II sequence in the P36Q mutant seems to allow the cleavage, as predicted from the statistical data (von Heijne, 1986). By contrast, consider the Δ35-38 mutant. The site II sequence is disrupted in the Δ35-38 mutant. This mutation also had a severe negative effect on cleavage at the original site I but the absence of an alternate site II sequence seems to have rendered impossible the cleavage of the signal peptide of this mutant precursor.

The H22QQIC mutation is located in the site I sequence and it affected the efficiency of cleavage around this site: pulse-labeling of the H22QQIC transformant revealed not only the processed form but also the precursor with the signal peptide still attached (Figs. 20 & 21). However, this mutation does not provoke any cleavage at site II. The site of cleavage of the signal peptide in the H22QQIC mutant precursor was not determined.

Unlike the case in transformed tobacco cells, the H22D, P36Q, Δ23-26 and Δ27-30 mutations in the precursor to sporamin did not result in cleavage of the signal peptide at site II in the processing

system *in vitro* with canine microsomal membranes [Fig. 26]. When the wild-type precursor to sporamin is expressed in *E. coli* cells, the signal peptide is processed in a *SecY*-dependent manner and pro-sporamin is secreted into the periplasmic space (the unpublished results). Alternate cleavage at site II was also not observed when the mutant precursors described herein were expressed in *E. coli* cells, although the patterns of processing of the signal peptide were not identical to those observed *in vitro* after processing by canine membranes. The the signal peptide of the Δ35-38 mutant precursor, which was not cleaved in transformed tobacco cells, was processed by canine membranes *in vitro* [Fig. 24] and in *E. coli* cells (data not shown). The efficiency of cleavage of the signal peptide from another mutant precursor to sporamin also differed between plant and yeast cells (K. Matsuoka and K. Nakamura submitted for publication). These results suggest that the signal-peptide processing machinery of plant microsomal membranes recognizes mutant precursors to sporamin in a different manner from such recognition by the membranes from other organisms.

The cleavage of the signal peptide of the precursor to sporamin at either site I or site II has a decisive effect on the final localization of sporamin. Cleavage at site I results in transport of sporamin to the vacuole, while cleavage at site II results in secretion of sporamin to the extracellular milieu. Several plant proteins are known to have vacuolar and extracellular isoproteins. Since substitution of even a single amino acid can switch the site of cleavage, it is tempting to speculate that the localization of a protein such as sporamin may have been altered with respect to the vacuole and the extracellular milieu by

mutations of this kind during evolution. That switching of the site of cleavage of the signal peptide is dependent on membranes implies that transport of a protein such as sporamin can be regulated by changes in a cellular component that is responsible for the selection of such a site of cleavage, a mechanism that is somewhat analogous to the switching of localization of specific proteins by alternate splicing in IgM (Early et al, 1980) and by alternate translational initiation of invertase in yeast (Carlson and Botstein, 1982).

General Discussion and Conclusion

The vacuole plays many roles in the regulation and maintainance of the function of plant cells, that is essential for the growth and development of higher plant. Although many reserches about the mechanism of the delivery of proteins to the vacuole from the secretory pahtwayin plant cells have been published, none of them identified the dignals in the precursor polypeptide that play a sorting signal. To understand the mechanism, it is essential to char-acterize the nature of the sorting signal that deliver vacuolar proteins to their desitination. Based on this viewpoint, I started to analyze the signals and machinaries for transport of proteins to the vacuole in plant cells, using a precursor to sporamin as a model protein. However when I started these studies, the vacuolar localization of sporamin was only merely presurmed. Thus, I first examined the site of localization of sporamin in the sweet potato (Chapter 1). With the cell fractionation experiments, I showed that sporamin is accumulated in the vacuoles from the parenchyma cell of the tuberous root. I also found that β-amylase, the second major protein of the organ next to sporamin, was co-purified with sporamin in the vacuole fraction. This raised the possibility that some of the vacuolar matrix proteins are directly transported to vacuole from the cytosol, since the precurseor to β-amylase does not contain the transitory signal peptide at its N-terminus..

Based on the knowledge of the vacuolar localization of sporamin, I started to analyze mechanism of vaculolar localization of this protein by using heterologous gene expression systems with molecular biological approach. The following knowledges fasciliated the analyses. First sporamin is a simple monomeric protein without glycan side chains, it has similarities in the

primary sequence to Kunitz type trypsin inhibitor of soybean. Second,sporamin is synthesized as a prepro-precursor that contain N-terminal signal pepetide and the adjoind short propeptide segment (Hattori et al. 1985, 1987). This basic structure of the precrusor to sporamin was similar to those of the vacuolar proteases of yeast , and the vacuolar targeting informations had been localized in their pro regions (Johnson et al. 1987, Valls et al. 1987, Klinosky et al. 1988).

Since the transformation systems for the sweet potato were not avalable, and relatively high background levels of sporamin was expepcted in the sweet potato cells, I planed to use the heterologous expression systems in tobacco, the most commonly used transgenic system in higher plants, to analyze signals in the precursor to sporamin that target this protein to the vacuole. For this purpose, I first analyzed whether the precursor to sporamin is correctly transporated to the vacuoles in tobacco cells (Chapter 2). Sporamin expressed in crown gall-derived cells of tobacco was correctly transported to the vacuole, although the N-terminal structure of sporamin accumlated in tovacco vacuoles was different from that in the sweet potato. The processing of the precursor to spramin expressed in variou organs of re-generated tobacco plant was also examined. Processing pattern of the precursor in seeds was significantly different from that in other vagetative organs, such as in stems, in roots and in leaves. Expression of a precursor to β-conglycinin, a storage protein of the soybean cotyledons in transgenic petunia plant under non-regulated promoters has been reported previously (Lawton et al.,1987). It was shown in this paper that the activity which degrade β-conglycinin high in vegetative organs, while little activity was detected in embryonic tissue, i.e seeds. It is not known what kind of proteases are responsible for degradation of sporamin or β-

conglycinin expressed in each organs. However the transgenic approach reported here and the repot of the Lawton et al. indicate that the proteases present in the vacuole of the vegetative and embryoinc cells are different. The transgenic approach should provide us a novel method to monitor the tissue-specific variations in the vacuolar proteases.

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 (reviewed by von Figura and Hasilik 1986) and the polypeptide of the pro-segment in the latter case (Johnson et al. 1987; Valls et al. 1987; Klinoski et al. 1988). Many plant vacuolar proteins, including sporamin do not contain glycans, and glycan side chains on the precursors to several plant vacuolar proteins are not required for the correct targeting to the vacuole (Voelker et al. 1989; Wilkins et al. 1990; Sonnewald et al. 1990). It seems that the structure of the polypeptide chain determine the delivery of proteins to plant vacuole.

From this point of view, I analyzed whether the pro region of the precursor to sporamin play any role in the transport of this protein to the vacuole, by expressing a mutant of the precursor to sporamin, in which the entire pro-region was deleted in tobacco cells (Chapter 3). In contrast to the vacuolar localization of sporamin in the cells that expressed the wild-type precursor, sporamin was secreted into the culture medium from cells in which the Δpro precursor was expressed. The kinetics of secretion of sporamin from the cell were similar to those of proteins normally secreted by the host tobacco cells. These results indicate that the propeptide of the precursor to sporamin is required for correct targeting of sporamin to the vacuole, and that

proteins can be secreted from plant cells by a bulk-flow default pathway in the absence of a functional sorting signal. Similar result were also reported recently by Bednarek et al. (1990) with the precursor to barley lectin, which contains carboxytermainal propeptide in its precursor.

In manmalian cells, lysosomal enzymes that are tagged by mannose 6-phosphate residues thet interact with mannose 6-phosphate receptors in the *trans*-Golgi network they are segregated into vesicles that are destined for lysosomes (reviewed by Kornfield and Mellman, 1989). In yeast the vacuolar matrix protein carboxypeptidase Y is believed to be sorted in a late Golgi (Valls et al. 1987).

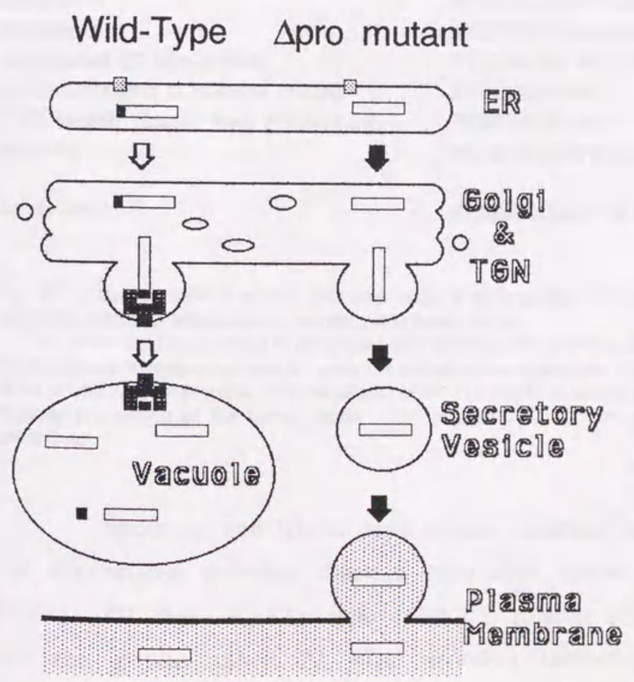

Fig.26 Model for the Sorting of Wild-Type and ΔPro Mutant Sporamin. TGN,*trans*-Golgi network, ▣,signal peptide; ■, propeptide; □, sporamin. ▬ putative receptor for vacuolar tartgeting signal.

Many plant vacuolar proteins pass through the Golgi apparatus. The post translational processing of prosporamin in tobacco cells was inhibited by moneinsin (Chapter 2,), an inhibitor which acidify the *trans*-Golgi network and vacuoles, and which may disrupt protein transport and sorting (Chrispeels 1983 ; Tartakoff 1983). Therefore, the sorting apparatus for the precursor to sporamin is presumably associated with the *trans*-Golgi compartment. By analogy with the machinaries of the receptor mediated sorting of manmalian lysosomal proteins, I propose that a domain structure of the prosporamin which includes the propeptide is recognized in the *trans*-Golgi network and prosporamin is segregated the sorting vesicles that are destinated for vacuoles [Fig. 26).

sporamin A	HSRFNPIRLPTTHEPASSETPVLDINGDE
sporamin B	HSKFNPIRLRPAHETASSETPVLDINGDE
potato tuber 22 kDa protein	FTSENPIVLPTTCHDD-DNLVLPEVYDQD
potato cathepsin D inhibitor (PCDI)	FTSQNLIDLPS---ESPLPKPVLDTNGKE
PCDI related protein from pKEN14-28	FTSKNPINLPS------DATPVLDVAGKE
Aleurein	FADSNPIRPVTDRAASTLESAVLGALGRT
Barley lectin	VFAEAIAANSTLVAE

Fig 27 Comparison of amino acid sequence of propeptides in precursor to of spramins, potato 22 kDa proteins, aleurein, and barley lectin.
The amino acid sequenced of pro-region and additional 12 amino acid residues of the precursors to sporaminA and B, were compared to the respective regions in area of the potato 22 k Da proteins, N-terminal part of the pro region of aleurein, and the C-terminal propeptide of the barley lectin. The mature part of each proteins are underlined.

Sporamin and Kunitz type trypsin inhibitor of soybean seeds, and the related proteins share a conserved amino acid sequences [Fig.1) Of these Kunitz type inhibitor related proteins, sporamin and the potato tuber 22 kDa proteins including cathepsin D inhibitors (Strukelj et al. 1990; Stiekema et al.1988; K.Ishikawa et al. unpublished result.) share a common feature of the length and

the primary sequence of the amino terminal extrapeptide of their precursors [Figs 1 and 27]. The intracellular localization sites of these potaot tuber proteins were not well characterized. However, the potato tuber 22 kDa proteins including cathepsin D inhibitor were recovered in the vacuole fraction isolated from the potato tubers (K. Matsuoka et al., unpublished results). It is intresting that the precursor to these vacuolar proteins contain similar (putative) propeptides. The similarity of the primary sequence of the pro-region was not restricted to the potato proteins and sporamins. The primary sequence of the pro-region of the precursor to sporamin resembles to the amino terminal part of the propeptide of Aleurein [Fig. 27]. Aleurein, a thiol protease of the aleuron layers of the barley seeds, is synthesized as a prepro precursor with its amino terminus propeptide (Rogers et al. 1985). It is localized in the non-protein body type vacuole of the aleuron layers (Holwersa et al., 1990). The aleuron layers of the barley seeds secrete other thiole proteases, upon inducible by gibberelin (Koehler and Ho 1990). These proteases are also synthesized as prepro precusors, and many of the primary structures of the precursors to these proteinases are similar to that of the aleurein. However, the pro-region of the precursor to aleurein is longer by 11 amino acid residues at its N-terminus, compared to those of other secretory thiol proteases. This 11 amino acid-long extranous sequence contains a sequence similar to the pro-region the precursor to sporamin [Fig. 27]. It is possible that the pro-region of the precursor to aleurein also plays an important role in its targeting to the vacuole. Interestingly, the pro-region of the precursor to sporamin has no significant homologies of the carboxyterminal propeptide of the precursor to barley lectin, which is required for proper sorting of this protein to the vacuole [Fig. 26, Bednarek et al. 1990). This raise the question whether the

different propeptide share a common receptor for the segregation of protein to the secretory pathway, or multiple receptors participate in the similar functions. If the receptor is only one, the higher structure rather than the primary sequence of these regions, may influence the recognition. To identify the minimum sequence that is required for vacuolar targeting and to analyze the fate of amino acid substitution mutants introduced in this region in tobacco cells makes to verify these two possibilities.

In order to determine the minimum sequence in the pro-region of the precursor to sporamin that is required for targeting of this protein to the vacuole, I introduced sevral amino acid substitution and deletion mutations in the propeptide, and the fate of the mutant precursors to sporamin in tobacco cells were analyzed (Chapter 4). Unexpectedly, some of the amino acid substitution and deletion mutations in the propeptide resulted in an alteration of the site of cotranslational cleavage of the signal peptide from the mutant precursor in transformed tobacco cells. In some of these mutants, the site of cotranslational cleavage jumped to a cryptic cleavage sites located downstream of the propeptide and resulted in secretion of processed polypeptide to the culture medium. Another mutation in the carboxyl-terminal part of the propeptide produced a mutant precursor with uncleavable signal peptide that is removed from the mature part only posttranslationally with the propeptide. These results indicate that the propeptide of the precursor to sporamin is important in defining the cotranslational cleavage of signal peptide in addition to target sporamin to the vacuole in transformed tobacco cells. However, none of these mutations in the propeptide did cause the cleavage of the signal peptide at the cryptic site in an in vitro processing system with canine microsomal membranes suggesting the evolutional

divergence in the function of signal peptide processing machinery between plants and animals.

The cleavage of the signal peptide of the precursor to sporamin at either original or cryptic sites has a decisive effect on the final localization of sporamin. Cleavage at original site leads sporamin to the vacuole, while cleavage at cryptic sites results in secretion of sporamin to the extracellular milieu. Several plant proteins such as bean β-1,3-glucanase (Mauch and Staehlin 1990) and barley thionin (Rheimann-Philipp et al. 1989) are known to have vacuolar and extracellular isoproteins. Since even a single amino acid substitution can switch the site of cleavage, it is tempting to speculate that the localization of a protein like sporamin may have altered between the vacuole and the extracellular milieu by this kind of mutations during the evolution. That switching of signal peptide cleavage site is dependent on membranes implies that transport of a protein like sporamin can be regulated by changes in a cellular component that is responsible for the selection of signal peptide cleavage site, a mechanism that is somewhat analogous to the switching of protein localization by alternate splicing in IgM (Rogers et al, 1980) and by alternate translational initiation in yeast invertase (Carlson and Botstein, 1982).

In conclusion, this work demonstrates that a pathway of protein transport to the vacuole in plant cellsis branched from the general secretory pathway. It is indicated that the propeptide of the precursor to sporamin plays an essential role in the sorting of sporamin to the vacuole. However, there remains many unresolved problems, and some new problems arose during the course of this reserch. One of the remaining problems as to the function of the propeptide of the precursor to sporamin is, whether this region is sufficient to target aline proteins to the vacuole. One of the newly developed problems is the nature of the signal peptide cleavge

machinery in plant cells. Obviously, much works are still needed to understand the whole nature of the molecular mechanisms of transport of proteins to the vacuole in plant cells. I hope that findings described in this thesis may become one of the basic standpoints for further reserch of these unresolved themes.

Acknowledgements

I wish to express my deepest sense of gratitude for this endeavor to Dr. Kenzo Nakamura, Associate Professor of Laboratory of Biochemistry, School of Agriculture, Nagoya University,for his consult guidance, penetrating discussions and criticism that made this work possible.

I am greatly indebted to Drs Tsukaho Hattori and Masayoshi Maeshima for their kind allowance to use cDNA and antibody against sporamin, since these materials made this work possible. I am also greatly indebted to Prof. Yasunori Machida, Dr Shogo Matsumoto and Mr. Yasuo Niwa of the Department of Biology, School of Science, Nagoya University for teaching me the technics about the transgenic plants, since the technics were the keys for completation of this work.

My gratitudes were also for Mr. Mikio Nakazono and Mr. Fujio Mukumoto for their helps and cooporations throughout this work.

My heartful thanks are for Prof. Tadashi Asahi, Mr. Atsushi Morikami and all other members of the Laboratory of Biochemistry, School of Agriculture, Nagoya University, where this work has been done, for their continuous encouregement, friendness and help.

I am thankful to Dr. Hiroyuki Hattori and Ms. Hiroko Kajiura of the National Institute for Basic Biology, Okazaki, for analysis of the N-terminal amino acid sequences.

The Fellowship for Japanese Junior Scientists of the Japan Society of Promotion of Science (Apr.1990-)is also greatfully acknoledged.

Lastly, I express my thanks to my mother for the encourgement and partience during my study at Nagoya.

February, 1991 The auther.

References

Allison D S & Young E T. *Mol. Cell Biol.* **8** 1915-1922 (1988).

Ammerer G, Hunter C P, Rothmann J H, Saari G C, Valls L A & Stevens T H. *Mol.Cell.Biol.*2490-2499 (1986).

An G. *Methods. Enzymol.* **153** 292-305 (1987).

An G, Watson B D, Stachel S & Nester E W. *EMBO J.* **4** 277-284 (1985).

Andrews D W, Perara E, Lesser C, & Lingappa V R J. Biol.*Chem.*, **263**, 15791-15798..(1988)

Baker. R K & Lively M O. *Biochemistry* **268** 561-8567. (1987).

Baranski T J, Faust P L & Kornfeld S. *Cell* **63** 281-291 (1990).

Bendnarek S Y, Wilkins T A, Dombrowski J E & Raikhel N V. *Plant Cell* **2** 1145-1155 (1990).

Bird P, Gething M-J & Sambrook J. *J. Cell Biol.* **105** 2905-2914 (1987).

Blobel G. in *International Cell Biology* (eds. Brinskey, B.R. & Porter, K.R.) 318-322 (Rockfeller University Press, New York, 1977).

Blobel G & Dobberstein B. *J. Cell Biol.* **67** 835-851. (1975).

Bohni P C, Dashaies R J & Shekman R W. *J. Cell Biol.* **106** 1035-1042 (1988).

Boller T & Kende H. *Plant Physiol.* **63** 1123-1132 (1979).

Boller T & Wiemken A. *Ann. Rev. Plant. Physiol.* **37** 137-164 (1986).

Borroto K & Dure III L. *Plant Mol. Biol.* **8** 113-131 (1987).

Bradshaw H D J Hollick J.B, Parsons T.P, Clarke H.R.G & Gordon M.P. *Plant Mol. Biol.* **14** 51-59 (1990).

Carlson M & Botstein D. *Cell.* **55** 683-692. (1982).

Chrispeels M J. *Planta* **158** 140-151 (1983).

Chrispeels M J. *Ann. Rev. Plant Physiol. Plany Mol. Biol.* **42** in press (1991).

Cioffi J, Allen K L, Lively M O & Kemper B. *J. Biol. Chem* **264** 15052-15058. (1989).

De Clercq V M, De Rycke R, J. V D, Van Montagu M, Krebbers E & Vandekerckhove J. *Plant Physiol.* **92** 899-907 (1990).

DellaPenna D & Bennett A B. *Plant Physiol.* **86** 1057-1063. (1988).

Denecke J, Botterman J & Deblaere R. *Plant Cell* **2** 51-59 (1990).

Dingwall C. **10** 64-66 (1985).

Dorel C, Voelker T A, Herman E M & Chrispeels M J. *J. Cell Biol.* **108** 327-337 (1989).

Duffaud G & Inouye M. *J. Biol. Chem.* **263** 10224-10228 (1988).

Early P, Rogers J, Davis M, Calame K, Bond M, Wall R & Hood L. *Cell,* **20** 313-319. (1980).

Eskridge E & Shields D. *J. Cell Biol.* **103** 2263-2272 (1986).

Evans E A, Gilmore R & Blobel G. *Proc. Natl. Acad. Sci. USA* .**83** 581-585. (1986).

Foltz R J & Gordon J I. *J. Biol. Chem.* **261** 14752-14759 (1986).

Foltz R J, Nothweha S F & Gordon J I. *J. Biol. Chem* **263** 2070-2078 (1988).

Franceschi V R, Wittenbach V A & Giaquinta R T. *Plant Physiol* 586-589 (1983).

Fromm M, Taylor L P & Walbot V. *Proc. Natl. Acad. Sci. USA* **82** 5824-5828 (1985).

Ganfikel D J, Simpson R B, Ream L W, White F F, Gordon M P & Nester E W. *Cell* **27** 143- 153 (1981).

Garcia P D, Gharayeb J, Inouye M & Walter P. *J. Biol. Chem.* **262** 9463-9468 (1987).

Ghrayeb J, Lunn C A, Inouye S & Inouye M. *J. Biol.Chem.* **260** 10961-10965 (1985).

Gieradch L M. **28** 923-930 (1989).

Green J R. in *Isolation of Membranes and Organelles from Plant Cells* (eds. Hall, .L.M., A.L.) 135-152 (Academic Press, London., 1983).

Greenwood J S & Chrispeels M J. *Plant Physiol.* **79** 65-71 (1985).

Haguenauer-Tsapsis R & Hinnen A. *Mol. Cell Biol.* 4 2668-2675 (1984).

Haguenauer-Tsapsis R, Nagy M & Ryer A. *Mol. Cell Biol.* **6** 723-729 (1986).

Hara-Nishimura I & Nishimura M. *Plant Physiol.* **85** 440-445 (1987).

Harley S M & and Lord J M. *Plant Sci.* **41** 111-116 (1985).

Hass G M & Ryan C A. *Meth. Enzymol.* **80** 778-791 (1983).

Hattori T, Ichihara S & Nakamura K. *Eur. J. Biochem.* **166** 533-538 (1987).

Hattori T, Nakagawa T, Maeshima M, Nakamura K & Asahi T. *Plant Mol. Biol.* **5** 313-320 (1985).

Hattori T, Yoshida N & Nakamura K. *Plant Molec. Biol.* **13** 563-572 (1989).

Hayashi M, Akazawa T, Nishimura M & Hara-Nishimura I. *FEBS Lett.* 197-200 (1988).

Hayashi S, Chang S Y, Chang S & Wu H C. *J. Biol. Chem.* **259** 10448-10454. (1984).

Hemmings B A, Zubenko G S, Hasilik A & Jones E W. *Proc. Natl. Acad. Sci. USA* **78** 435-439 (1981).

Herman E M & Shannon L M. *Plant Physiol.* **77** 886-890 (1985).

Hermann E M & Shannon L M. *Planta* **161** 97-104 (1984).

Higgins T J Å & Spencer D. *Plant Physiol.* **67** 205-211. (1981).

Hoffman L M, Sengupta-Gopalan C & Paaren H E. *Plant Mol. Biol.* **3** 111-117 (1984).

Holländer-Czytko H, Anderson J K & Ryan C A. *Plant Physiol.* **78** 76-79 (1985).

Holwada B C, N.J. G, Baranski T J & Rogers J C. *Plant Cell* **2** 1091-1106 (1990).

Hood E E, Halmer G I, Fraley R T & Chilton M-D. *J.Bacteriol.* **168** 1291-1301 (1986).

Hortin G & Boime I. *Cell* **24** 453-461 (1981).

Hurt E C & van Loon A P G M. *Trends Biochem. Sci.* **11** 204-207 (1986).

Ikeda T, Matsumoto T & Noguchi M. *Agric. Biol. Chem.* **40** 1765-1770 (1976).

Inouye M & Halegoua S. *CRC Crit. Rev. Biochem.* **7** 339-371 (1980).

Iturriaga G, Jefferson R A & Bevan M W. *Plant Cell* **1** 381-390. (1989).

Jackson R B & Blobel G. *Proc. Natl. Acad. Sci. USA* **74** 5598-5602 (1977).

Jany K D, Lederer G & . *Biol. Chem. Hoppe-Seyler,* **366** 807-808 (1985).

Jofuku K D & Goldberg R D. *Plant Cell* **1** 1079-1093 (1989).

Johnson L M, Bankaitis V A & Emr S D. *Cell* **48** 875-885 (1987).

Joubert F J, Heussen C & Dowdle B D. *J. Biol. Chem.* **260** 12948-12953 (1985).

Kaiser P A, Preuss D, Grisafi P & Bostatin D. **235** 312-317 (1987).

Kay R, Chan A, Dely M & McPherson J. *Science* **236** 1299-1302 (1987).

Keegstra K. *Cell* **56** 247-253 (1989).

Kim S-H, Hara S, Hase S, Ikenaka T, Toda H, Kitamura K & Kaizuma N. *J. Biochem.* **98** 435-448 (1985).

Kimura T, Shin T, Asahi T & Nakamura K. *J. Biol. Chem.* **265** 6079-6085 (1990).

Klinosky D J, Banta L M & Emr S D. *Mol. Cell Biol.* **8** 2015-2116 (1988).

Koehler S & Ho T-H D. *Plant Cell* **2** 769-783 (1990).

Kornfeld S & Mellman I. *Annu. Rev. Cell. Biol.* **5** 483-525 (1989).

Kott A A, Strike P M, Jersey J D E & . *Eur.J. Biochem.* **181** 403-408 (1989).

Kunkel T, Roberts J D & Zakour R A. *Methods Enzymol.* **154** 367-382 (1987).

Lamb F I, Roberts L M & Lord J M. *Eur. J. Biochem.* **148** 265-270 (1985).

Lawton M A, Tierney M A, Nakamura I, Anderson E, Komeda Y, Dube P, Hoffman N, Fraley R T & Beachy R N. *Plant Mol. Biol.* **9** 315-324. (1987).

Lazalow P B., *Curr. Opinion Cell.Biol.* **1**. 630-634

Leah R & Mundy J. *Plant Mol. Biol.* **12** 673-682 (1989).

Li H & Oba K. *Agr.Biol.Chem.* **49** 737-745 (1985).

Li P, Beckwith J & Inouye M. *Proc. Natl. Acad. Sci. USA* **85** 1988 (1988).

Lingappa V R, Thiery-Devillers A & Blobel G. *Proc. Natl.Acad. Sci. USA* **74** 2432-2436. (1977).

Lipp J & Dobberstain B. *J. Cell Biol.* **106** 1813-1820 (1988).

Lowry O H, Rosevbrough A L, Farr A L & Randall R J. *J.Biol. Chem.* **193** 265-275 (1951).

Lund P, Lee R & Dunsmure P. *Plant Physiol.* **91** 130-135 (1989).

Maeda K. *Biochem. Biophys. Acta* **871** 250-256 (1986).

Maeshima M, Sasaki T & Asahi T. **24** 1899-1902 (1985).

Matsudaira P. *J. Biol.Chem.* **262** 10035-10038 (1987).

Mauch F & Staehelin A. *Plant Cell* **2** 447-457 (1990).

Miyata T, Miyazawa S & Yasunaga T. *J. Mol. Evol.* **12** 219-236 (1979).

Muntz K, Bassuner R, Lichtenfeld C, Scholz G & Weber E. *Physiol. Veg.* **23** 75-94 (1985).

Murakami S, Hattori T & Naksamura K. *Plant Molec. Biol.* **7** 343-355 (1986).

Murashige T & Skoog F. *Physiol. Plant.* **15** 473-497 (1962).

Muto.S. & Uritani I. *Plant Cell Physiol.* **11** 767-776 (1970).

Nagata T, Okada K, Takebe I & Matsui C. *Mol.Gen.Genet.* **184** 161-165 (1981).

Nishimura M. *Plant Physiol.* **70** 742-744 (1982).

Nothwehr S F. & Gordon J I. *J. Biol. Chem.* **265** 17202-17208 (1990).

Nothwehr S F & Gordon J I. *J. Biol. Chem* **264** 3979-3987 (1989).

Nothwehr S F, Hoeltzli S D, Allen K L, Lively M O & Gordon J I. *J. Biol. Chem.* **265** 21797-21803. (1990).

Okamoto K & Akazawa T. *Plant Physiol.* 81 (1980).

Owles D J, Marcus S E, Pappin D J C, Findlay J B C, Eliopoulos E, Maycox P R &Burgess J. *J. Cell Biol.* 1284-1297 (1986).

Parade G. *Science* **189** 347-358 (1975).

Perlman D & Halverson H O. *J. Mol. Biol.* **167** 391-409 (1983).

Petit A & Tempe J. *Mol. Gen. Genet.* **167** 147-155 (1978).

Peyachoknagul S, Matsui T, Shibata H, Ikenaka T, Okada Y & Ohno S. *Plant Mol.Biol.* **12** 51-58 (1989).

Pfeffer S R & Rothman J E. *Annu. Rev. Biochem.* **56** 829-852. (1987).

Prehn S, Wiedeman M, Rapoport T & Zwieb C. *EMBO J.* **6** 2093-2097 (1987).

Pugsley A P. *Protein targeting* (Academic Press, San Diego, 1989).

Racusen D. *Can. J. Bot.* 1640-1644 (1984).

Rao R N & Rogers S G. *Gene.* **7** 79-82 (1979).

Reimann-Philipp U, Schrader G, Maetinoia F & Apel K. *J. Biol.Chem.* **264** 8978-8984 (1989).

Rogers J C, D. D & Heck G R. *Proc. Natl.Acad. Sci. USA* **82** 6512-6516 (1985).

Rothman J H, Yamamoto C T, Kane P M & Stevence T H. *Trends Biochem. Sci.* **14** 347-350 (1989).

Savarino K A, Stork P, Ventimiglia R, Mandel G & Goodman R H. *Cell* **57** 11-19 (1989).

Shauer I, Emr S, Gross C & Sheckmann R. *J. Cell Biol.* **100** 1664-1675 (1985).

Simon R, Altschuler Y, Rubin R & Galili G. *Plant Cell* **2** 941-950 (1990).

Singer S J, Maher P A & Yaffe M P. *Proc. Natl. Acad. Sci. USA* 84 1015-1019 (1987).

Sonnewald U, Sturm A, Chrispeels M J & and Willmitzer L. *Planta* **178** 171-180. (1989).

Sonnewald U, Von Schaewen A & and Willmitzer L. *Plant Cell* **2** 345-355 (1990).

Sonnewld U, Strum A, Chrispeels M J & Wilmitzer L. *Planta* **179** 171-180 (1989).

Stinissen H M, Peumans W J & Chrispeels M J. *Plant Physiol* **3** 495-498 (1985).

Strukelj B, Pungercar J, Ritonja A, Krizaj I, Gubensek F, Kregar I & Turk V. *Nuc. Acid. Res.* **18** 4605 (1990).

Sturm A, Voelker T A, Herman E M & Chrispeels M J. **175** 170-183. (1988).

Suh S-G, Peterson J E, Stiekema W J & Hannapel D J. *Plant Physiol.* 40-45 (1990).

Sweet R M, Wright H T, Chosia C H & Blow D M. **13** 4212-4228 (1974).

Tague B W, Dickinson C D & Chrispeels M J. *Plant Cell* **2** 533-546 (1990).

Tague B W & Chrispeels M J. *J. Cell Biol.* **105** 1971-1979. (1987).

Tartakoff A M. *Cell* **32** 1026-1028 (1983).

Theeraship S, Hitotsuya H, Nakajo S, Nakaya K, Nakamura Y & Kurihara Y. *J. Biol. Chem.* **264** 6655-6659 (1989).

Tronier B & Ory R L. *Cereal Chem.* **47** 464-471 (1970).

Utsumi S, Sato T, Kim C-S & Kito M. *FEBS Lett.* **233** 273-276. (1988).

Valls L A, Hunter C P, Rothman J H & Stevens T H. **48** 886-897 (1987).

Valls L A, Winter J R & Stevens T H. *J. Cell Biol.* **111** 361-368 (1990).

Van den Bulcke M, Bauw G, Castresana C, Van Montagu M & Vandekerckhove J. *Proc. Natl. Acad. Sci. USA* **86** 2673-2677 (1989).

Van Heute E, Joos H, Maes M, Warren G, Van Montagu M & Shell J. *EMBO J.* **2** 411-417 (1983).

Vitale A, Strum A & Bollini R. *Planta* **169** 108-116 (1986).

Voelker T A, Florkiewicz R Z & Chrispeels M J. *Eur. J. Cell Biol.* **141** 218-223 (1986).

Voelker T A, Herman E M & Chrispeels M J. *Plant Cell* 95-104 (1989).

von Figura K & Hasilik A. *Ann. Rev. Biochem.* **55** 167-193 (1986).

von Heijne G. *Nuc. Acid Res.* **14** 4683-4690 (1986).

von Heijne G. *Eur. J. Biochem.* **133** 17-21 (1983).

von Heijne G. *J. Mol. Biol.* **184** 99-105 (1985).

von Heijne G & Abrahmsen L. *FEBS Lett.* **244** 439-446 (1989).

Weisman L S, Krummel B M & Wilson A C. *J. Biol. Chem* **258** 12073-12080. (1986).

Wilkins T A, Bednarek S Y & Raikhel N V. *Plant Cell* **2** 301-313 (1990).

Woolford C A, Daniels L B, Park F J, Jones E W, Van Arsdell J N & Innis M A. *Mol. Cell Biol.* **6** 2500-2510. (1986).

Yamaki S & Uritani I. *Agric. Biol.Chem.* **37** 183-186 (1973).

Yamamoto M, Hara S & Ikenaka T. *J. Biochem.* **94** 849-863 (1983).

Yamamoto Y, Taniyama Y & Kikuchi M. *Biochemistry* **28** 2728-2732 (1989).

Yamamoto Y, Taniyama Y, Kikuchi M & Ikehara M. *Biochem. Biophys. Res. Commun.* **149** (1987).

Yoshida N & Nakamura K. *(submitted for publication)* (1991).

Zhu X, Ohta Y, Jordan F & Inouye M. *Nature* **339** 483-484 (1989).

Ziegler P & Beck E. *Plant Physiol.* **82** 1119-1121 (1986).

List of papers concerning this dissertation.

Tsukaho Hattori, Ken Matsuoka and Kenzo Nakamura
"Subcellular localizaion of the sweet potato tuberous root storage protein"

Agric. Biol. Chem. 52 1057-1059 (1988)

Ken Matsuoka, Shogo Matsumoto, Tsukaho Hattori, Yasunori Machida and Kenzo Nakamura
"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 (1990)

Ken Matsuoka and Kenzo Nakamura
"Propeptide of a precursor to a plant vacuolar protein required for vacuolar targeting"

Proc. Natl. Acad. Sci. USA in press

Ken Matsuoka, Fujio Mukumoto and Kenzo Nakamura
"Mutations in the propeptide of the precursor to a plant vacuolar protein cause alterations in the site of cleavage of the signal peptide and transport of the protein in transformed tobacco cells.

submitted to "The Plant Journal".