Original Paper Plant Biotechnology, **18** (3), 203-208 (2001)

Expression and Localization of a 36-kDa Peptide Derived from a 24-kDa Vacuolar Protein (VP24) Precursor in Anthocyanin-Producing Sweet Potato Cells in Suspension Culture

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Received 7 February 2001; accepted 27 April 2001

Abstract

Expression and localization of a 36-kDa peptide (VP36) derived from a 24-kDa vacuolar protein (VP24) precursor were investigated in anthocyanin-producing sweet potato cells (*Ipomoea batatas*). VP24 is one of the major proteins in the anthocyanin-containing vacuoles, and synthesized as a large precursor protein that contains a C-terminal region in addition to the mature domain [Xu *et al.*, Plant Physiol. (2001) 125: 447]. The accumulation patterns of both VP36 and VP24 were closely correlated with the accumulation of anthocyanin in the vacuoles. The immunocytochemical analysis using antibodies against the fusion protein containing a portion of the C-terminal peptide showed that VP36 was localized in intravacuolar pigmented globules (cyanoplasts) in a manner similar to VP24. These results further suggest that VP36 is a peptide derived from the VP24 protein precursor. Both VP36 and VP24 peptides are probably involved in formation of cyanoplasts in the anthocyanin-producing sweet potato cells.

Introduction

Biosynthesis of anthocyanins occurs in the cytosol (Hrazdina *et al.*, 1978; Jonsson *et al.*, 1983; Hrazdina *et al.*, 1987; Hrazdina and Jensen, 1992; Marrs *et al.*, 1995; Burbulis and Winkel-Shirley, 1999), but anthocyanins and their intermediates are never detected there. Only the end products, anthocyanins, are detected in the vacuoles. In our previous papers (Nozue and Yasuda, 1985; Nozue *et al.*, 1987; Nozue *et al.*, 1993), we demonstrated that cultured cells of sweet potato (*Ipomoea batatas* Lam.) produced large amounts of anthocyanin, and formed intensely pigmented globules called cyanoplasts (Politis, 1959) or anthocyanoplasts (Pecket and Small, 1980), within the anthocyanin-containing vacuoles. We also found that a 24-kD protein (VP24) accumulated as one of the major vacuolar proteins in the anthocyanin-containing vacuoles (Nozue *et al.*, 1995). Expression of VP24 was induced in the cultured cells upon exposure to light and closely paralleled the accumulation of anthocyanin. Immunocytochemical detection of VP24 showed that this protein was localized in cyanoplasts in the anthocyanin-containing vacuoles (Nozue *et al.*, 1997).

Recently, we have cloned and characterized a cDNA (accession number AB025531) encoding VP24 (Xu *et al.*, 2001). According to the deduced amino acid sequence of VP24 cDNA, VP24 is probably synthesized as a 96.3-kDa large precursor protein. The most salient feature of the deduced precursor sequence of VP24 cDNA was the presence of a hydrophobic region at the C-terminal propeptide containing multiple transmembrane domains. The above sequence analysis suggested the possibility that a functional transmembrane peptide was expressed in the vacuoles of anthocyanin-producing cells in addition to mature VP24. To

confirm the expression of the transmembrane domains in the anthocyanin-containing vacuoles of sweet potato cultured cells, we tried to obtain antibodies against the C-terminal propeptide using a fusion protein prepared by the pET-32 expression vector system (Novagen, Madison, WI, USA) as the antigen (Xu *et al.*, 2000). Although the exact position of the C-terminal propeptide in the VP24 precursor protein has not yet been identified, a 36-kD peptide (VP36) has been found to accumulate in anthocyanin-containing vacuoles in sweet potato cultured cells as shown by immunoblot analysis using antibodies against the fusion protein containing a portion of the C-terminal propeptide.

If VP36 is derived from the large VP24 protein precursor after transport to the vacuole, the accumulation pattern of VP36 is expected to be similar to that of VP24 during anthocyanin formation. Expression of VP24 has been induced in cultured cells exposed to light where it closely paralleled the accumulation of anthocyanin, and was found to localize in the cyanoplasts (Nozue *et al.*, 1997). It is not clear whether VP36 is a vacuolar membrane protein or a vacuolar matrix protein. In the present study, the accumulation pattern and the intravacuolar localization of VP36 in anthocyanin-producing sweet potato cells in suspension culture were examined by immunoblot analysis and immunocytochemical electron microscopic detection, respectively.

Materials and Methods

Cell cultures

Three lines of sweet potato (*Ipomoea batatas* Lam. cv. Kintoki) cells in culture (ALD, ALND and N) that produced anthocyanins at different rates (Nozue *et al.*, 1997), were used in the present experiments (**Fig. 1**). ALD and ALND were anthocyanin producing cell lines while N was a non-producing cell line. The ALD cell line produced large amounts of anthocyanin not only under continuous illumination but also in darkness. The ALND cell line produced large amounts of anthocyanin under continuous illumination but little anthocyanin in darkness. The N cell line produced little anthocyanin regardless of illumination conditions. Both the ALND and N cell lines were obtained by clonal selection from the same callus, which had been initiated from tuberous root tissue of sweet potato (Nozue *et al.*, 1987). The ALD cell line was also obtained by several rounds of clonal selection from the anthocyanin-producing cells that developed spontaneously in dark-grown cultures of ALND cells.

The callus of each cell line was maintained on 25 m*l* of PRL-4C (Gamborg, 1966) agar medium that contained 3% (w/v) sucrose and 0.1 mg l^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D) in a plastic dish in darkness. To induce VP24 and VP36 accumulation and anthocyanin synthesis, a 14-day-old callus (2 g wet weight) was transferred to 20 m*l* of 2,4-D-free liquid PRL-4C medium in a 100-m*l* Erlenmeyer flask and cultured at 25 ℃ under continuous illumination or in darkness on a rotary shaker at 120 cycles min^{-1} (Nozue *et al.*, 1993).

Extraction and estimation of VP36, VP24 and anthocyanin content

Suspension-cultured cells were harvested at various intervals by filtration on a suction funnel and their fresh weight was determined. Cells (500 mg fresh weight) were homogenized with a mortar and pestle in 0.75 m*l* of extraction buffer M (25 mM Tris-Mes, pH 6.8 containing 0.5 M NaCl, 0.3 M sucrose and 3 mM $MgCl_2$) with 50 mg of Polyclar VT (Wako Pure Chemical Industries, Osaka, Japan), 10 μl of protease inhibitor cocktail for plant cell extracts (Sigma Chemical Co., St. Louis, MO, USA) and 500 mg of quartz sand (Nacalai Tesque, Inc., Kyoto, Japan) at 0 ℃ . The homogenate was centrifuged at 300 g for 3 min at 4 ℃ , and the supernatant was stored at -40 ℃ prior to analysis. The supernatant was analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on 12.5% (w/v) gel with subsequent immunoblotting with polyclonal antibodies against the fusion protein containing the C-terminal region of the VP24 protein precursor (1 : 3,000 dilution) developed as described previously (Xu *et al.*, 2000) and against VP24 (1 : 1,000 dilution) (Nozue *et al.*, 1997). Only a 36-kDa protein (VP36) was detected as a major protein in vacuoles and protoplasts prepared from ALND cells by immunoblot analysis using the antibodies developed against the fusion protein (Xu *et al.*, 2000). Experiment using a peptide prepared by *in vitro* translation system also showed the C-terminal peptide of the VP24 protein precursor is recognized by the antibodies, but not the N-terminal His-tag and S-tag domains of the fusion protein (Xu *et al.*, 2000).

Protein content was determined as described by Bradford (1976) with bovine serum albumin as the standard.

Anthocyanin content was determined as described previously (Nozue *et al.*, 1993).

Immunocytochemical analysis

Fourteen-day-old suspension-cultured ALD cells that had been transferred to 2,4-D-free liquid

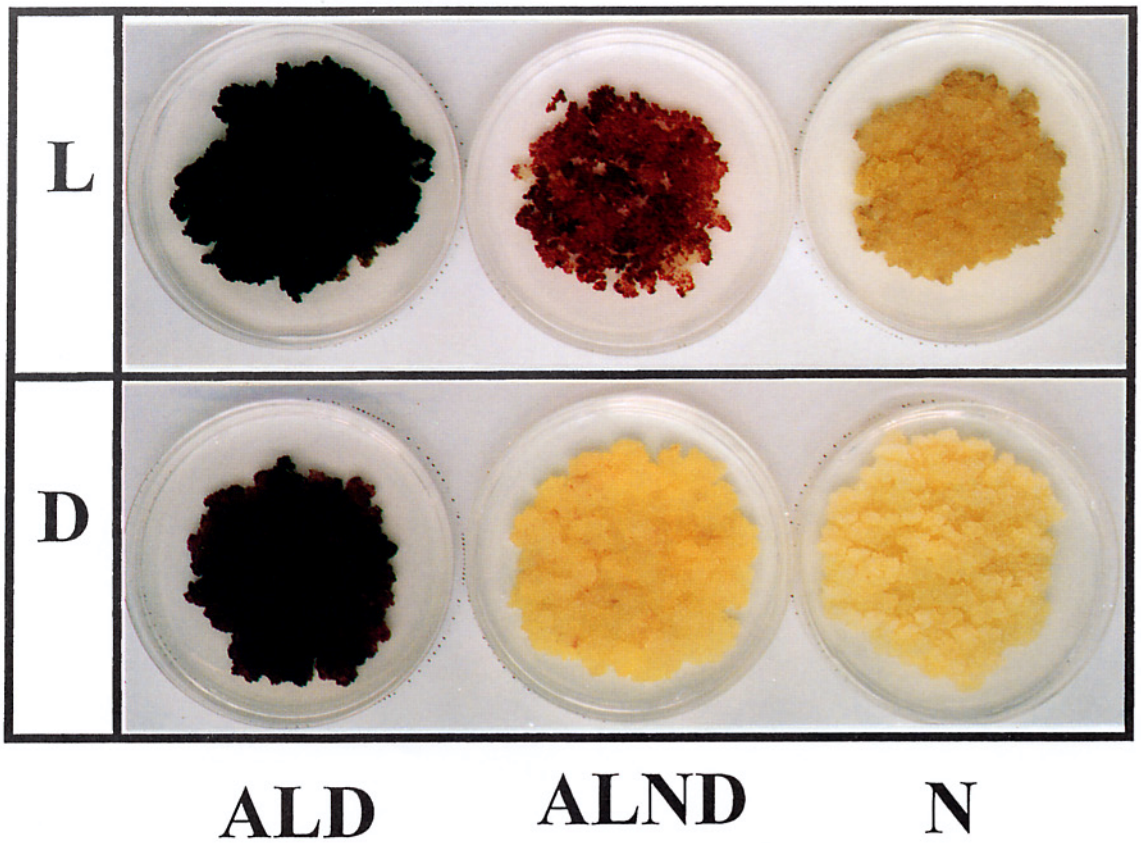

Fig. 1 Anthocyanin synthesis in the three cell lines (ALD, ALND and N) of sweet potato (*Ipomoea batatas*) callus. Each callus was maintained on 0.1 mgl^{-1} 2, 4-D-containing agar medium for 14 days in the darkness, then transferred onto a fresh preparation of the same medium and cultured for 21 days under continuous light (L) or in darkness (D).

PRL-4C medium were used for the immunocytochemical analysis of VP36. Irradiated ALD cells produced the largest amounts of anthocyanin among the three cell lines (**Fig. 1**), and also formed a large number of cyanoplasts (Nozue *et al.*, 1997). Immunocytochemical labeling with protein A-gold particles was performed as described previously (Nishimura *et al.*, 1993; Nozue *et al.*, 1997). Antibodies against the fusion protein described above (dilution 1 : 1,000) were used for the analysis. Thin sections were examined with a transmission electron microscope (1200 EX; JEOL, Tokyo, Japan) operated at 80 kV.

Results

Expression of VP36 and VP24 in three cell lines

Expression of VP24 has been found to be closely correlated with the accumulation of anthocyanin in three different cell lines (ALD, ALND and N) (Nozue *et al.*, 1997). The accumulation of VP36 in these cell lines was examined in this study. Both VP36 and VP24 were extracted from the suspension-cultured cells of each cell line and analyzed by SDS-PAGE with subsequent immunoblotting. **Fig. 2A** shows that large amounts of VP36 were expressed in both ALD and ALND cells cultured in the light, with a small amount detected in N cells. However, no VP36 was detected in ALND or N cells cultured in the dark. The dark-cultured ALD cells expressed VP36 and synthesized anthocyanin (**Fig. 1**). Larger amounts of both VP36 and anthocyanin were produced in the irradiated ALD cells than in the dark-cultured ALD cells. The accumu-

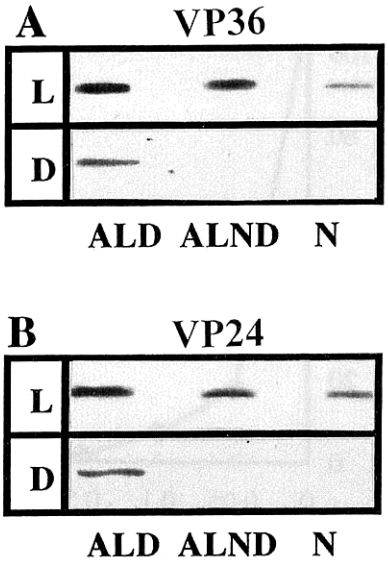

Fig. 2. The expression of VP 36 and VP24 in the three cell lines (ALD, ALND and N) of sweet potato cells in suspension culture. Both VP24 and VP36 were analyzed by SDS-PAGE (15 μg of protein per lane) with subsequent immunoblotting with antibodies against VP36 (A) or VP24 (B), respectively. Each callus was maintained for 14 days on 2,4-D-containing agar medium in the darkness as described in **Fig. 1.** Suspension-cultured cells from each cell line were initiated by transfer of a 14-day-old callus to 2,4-D-free liquid medium, followed by culture for 7 days under continuous light (L) or in darkness (D).

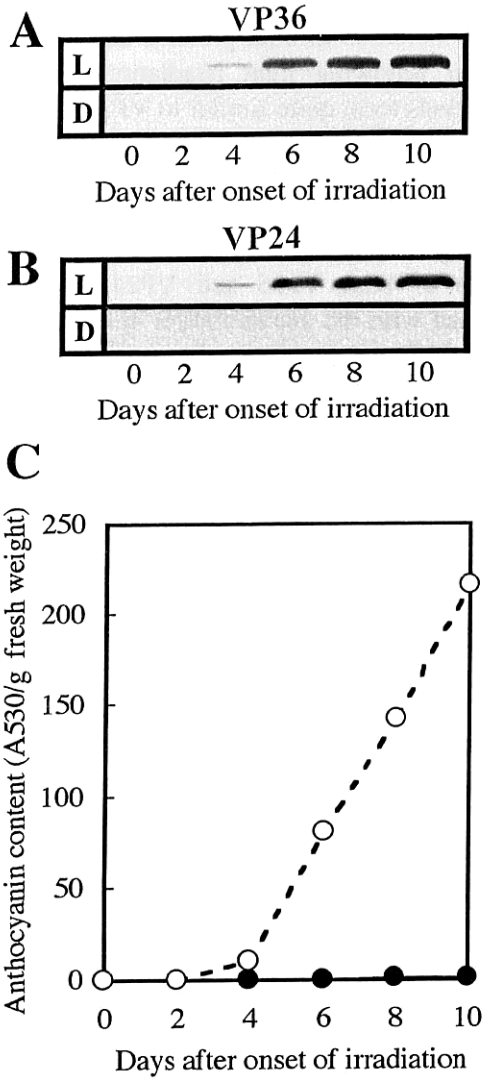

Fig. 3. Light-induced accumulation of VP36, VP24 and anthocyanin synthesis in ALND cells in suspension culture. Suspension-cultured cells were initiated by transfer of a 14-day-old callus to 2,4-D-free liquid medium, then cultured under continuous light (L) or in darkness (D). Cell extracts (15 μg of protein per lane) were separated by SDS-PAGE with subsequent immunoblotting with antibodies against VP36 (A) or VP24 (B). Anthocyanin content (C) was quantitated using the same suspension-cultured cells as the immunoblot analysis under continuous light (○) or in darkness (●).

lation patterns of VP36 in the three cell lines were very similar to those of VP24 (**Fig. 2B**).

Light-induced expression of VP36

When the ALND callus that had been cultured in darkness was transferred to 2,4-D-free liquid medium and then cultured under continuous illumination, changes in relative amounts of VP36 and VP24, and anthocyanin content were examined (**Fig. 3**). Little amounts of both VP36 and VP24 were detected in the dark-cultured cells, but marked increases in both proteins and anthocyanin occurred following light irradiation. Changes in VP36 levels were quite similar to VP24 levels in the irradiated ALND cells (**Fig. 3, A** and **B**). Anthocyanin formation also began between 2 and 4 days after onset of irradiation, but not occur in darkness (**Fig. 3C**). These results indicated the accumulation patterns of both VP36 and VP24 were closely correlated with the accumulation of anthocyanin in the vacuoles.

Effect of 2,4-D on the light-induced VP36 expression

High concentrations of 2,4-D have been found to markedly inhibit anthocyanin formation and VP24 expression in irradiated cells (Nozue *et al.*, 1995, 1997). In this study, the effect of various concentrations of 2,4-D on light-induced VP36 accumulation pattern was examined. The dark-cultured 14-day-old ALND callus was transferred to liquid medium containing 0, 0.05, 0.1, 0.5 or 1.0 mg l^{-1} of 2,4-D. After 7 days of culture, relative amounts of VP36 and VP24 were examined by immunoblot analysis. Little amounts of both VP24 and VP36, and only a small amount of anthocyanin formation were observed in cells cultured in 0.5 and 1.0 mg l^{-1} of 2,4-D-containing medium (**Fig. 4**). Higher concentrations of 2,4-D completely inhibited light-induced VP36 accumulation in a manner very similar to VP24 (**Fig. 4, A** and **B**).

Localization of VP36

VP36 was found to be a 36-kDa vacuolar protein as detected by immunoblot analysis with antibodies against the fusion protein including the C-terminal region expressed in *E. coli* (Xu *et al.*, 2000). The C-terminal region contains seven or eight putative transmembrane domains, and the VP36 peptide may include one or two of these according to the predicted molecular size. However, it is not yet clear whether VP36 is a tonoplast protein or vacuolar matrix protein. In this study, to clarify the localization of the VP36 peptide in anthocyanin-containing vacuoles, we subjected suspension-cultured ALD cells that had been cultured for 14 days under continuous illumination to immunocytochemical electron microscopic observation. The immunocytochemical analysis showed that the VP36 peptide was localized in cyanoplasts in the anthocyanin-containing vacuoles, and not in the tonoplast (**Fig. 5**). In previous study, large numbers of cyanoplasts were found in vacuoles of the suspension-cultured ALD cells, and VP24 was also found to localize in the cyanoplasts (Nozue *et al.*, 1997). Both peptides may be involved in the formation of the cyanoplast via interaction with anthocyanins.

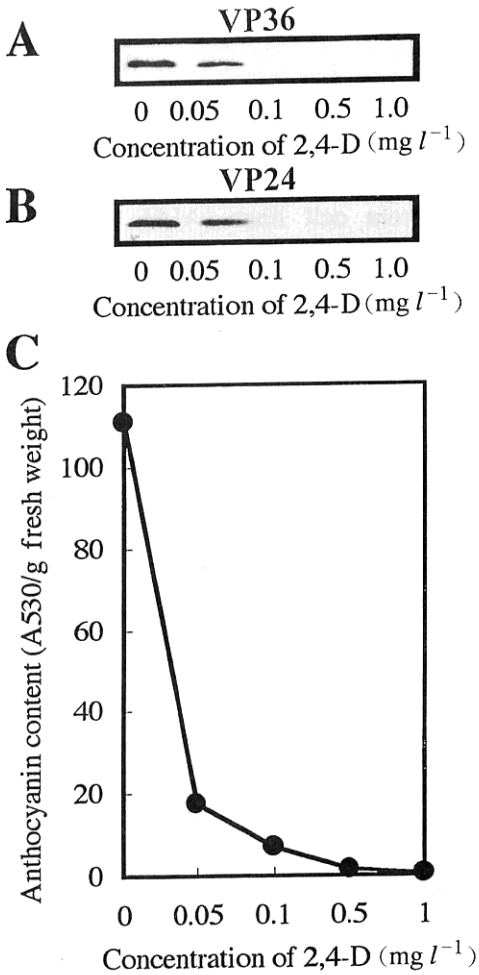

Fig. 4. Effect of 2,4-D on the light-induced accumulation of VP36, VP24, and anthocyanin synthesis in suspension-cultured ALND cells. The 14-day-old ALND callus was transferred to liquid medium supplemented with 2,4-D at various concentrations, then cultured under continuous illumination. Cells were harvested 7 days after the start of irradiation. Cell extracts (15 μg of protein per lane) were separated by SDS-PAGE with subsequent immunoblotting with antibodies against VP36 (A) or VP24 (B). Anthocyanin content (C) was quantitated using the same suspension-cultured cells as the immunoblot analysis (●).

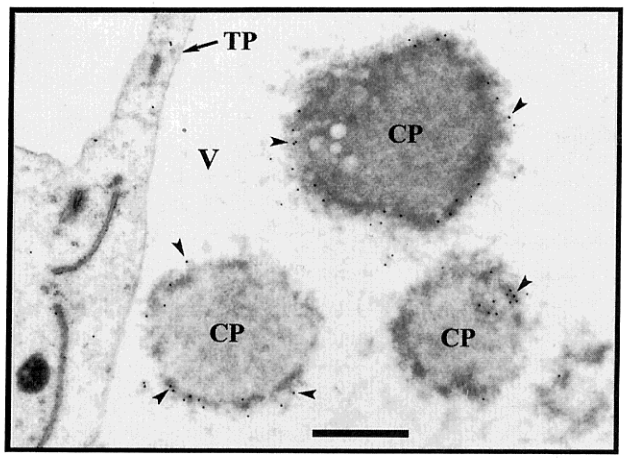

Fig. 5. Immunocytochemical detection of VP36 in suspension-cultured ALD cells that had been cultured for 14 days under continuous illumination with antibodies against VP36. Arrowheads indicate immunogold particles. Bar is 1 μm. CP, Cyanoplast; V, vacuole; TP, tonoplast.

Discussion

Most vacuolar proteins in higher plants are synthesized as proprotein precursors in the rough endoplasmic reticulum (rER), then transported to the vacuoles (Okita and Rogers, 1996). VP24 may also be synthesized first as a 96.3-kDa protein precursor in the rER lumen, then transported to the vacuole and processed into the mature 24-kDa form by proteolytic cleavage. The most salient feature of the VP24 protein precursor is the presence of multiple transmembrane domains in the C-terminal region, whose biological function remains unknown. The deduced amino acid sequence of these domains has no similarities to other known vacuolar membrane proteins (Xu *et al.*, 2001). A 36-kDa peptide (VP36) was also found in the vacuolar fraction prepared from ALND cells by immunoblot analysis using the polyclonal antibodies developed against the fusion protein containing the C-terminal region without transmembrane domains (Xu *et al.*, 2000). Antibodies against the other fusion proteins containing transmembrane domains were not expressed in *E. coli*. All the results obtained by immunoblot analyses in the present experiments showed that the accumulation pattern of VP36 in sweet potato cells in suspension culture is consistent with that of VP24 and also with anthocyanin formation. These results further suggest that both VP24 and VP36 originate from the same protein precursor.

The C-terminal region of the VP24 protein precursor contains five potential *N*-glycosylation sites (Xu *et al.*, 2001), two of which are located in the peptide that was used for preparation of the antigen. Immunoblot analysis and the glycoprotein-detection assay indicated that VP36 was an *N*-glycosylated protein (Xu *et al.*, 2000). Therefore, the molecular size of VP36 polypeptide may be slightly smaller than 36 kDa. The C-terminal region contains seven or eight putative transmembrane domains, and VP36 may include one or two of these according to the predicted molecular size. However, immunocytochemical detection of VP36 using the antibodies described above showed that VP36 was localized in the cyanoplast in anthocyanin-containing vacuoles, but not in the tonoplast. Therefore, VP36 may be a vacuolar protein which is not highly hydrophobic, derived from the C-terminal region of VP24 protein precursor, but is not a tonoplast protein.

Highly purified anthocyanin-containing vacuoles have been prepared from sweet potato cells in suspension culture (Nozue *et al.*, 1995), and the vacuolar proteins analyzed by SDS-PAGE. The N-terminal amino acid sequences of major vacuolar proteins that could be detected by CBB staining were determined (data not shown). However, peptides sharing the amino acid sequence of the VP24 protein precursor have not yet been found. Further investigation is necessary to characterize the function of the C-terminal region of VP24 protein precursor and to determine whether other parts of the transmembrane domains are expressed in the tonoplast of anthocyanin-producing sweet potato.

Anthocyanin pigments are normally found dissolved uniformly in the vacuolar solution, but are sometimes detected in discrete regions of the vacuoles as intensely pigmented-intravacuolar spherical bodies such as in *Solanaceae* (Politis, 1959); red cabbage (Pecket and Small, 1980); radish (Yasuda and Shinoda, 1985); sweet potato (Nozue and Yasuda, 1985); radish, mung bean and cabbage (Nozzolillo and Ishikura, 1988); and *Polygonum cuspidatum* (Kubo *et al.*, 1995). We termed these intravacuolar inclusions cyanoplasts in the present study. The complete biological function of the cyanoplast in anthocyanin-containing vacuoles has not yet been clarified, however, we have proposed that the cyanoplast may play a role in the trapping of large amounts of anthocyanins in vacuoles (Nozue *et al.*, 1997). Recently, Markham *et al.* (2000) reported that the petals of carnations and lisianthus contain intensely coloured intravacuolar bodies (anthocyanic vacuolar inclusions, AVIs), which have a substantial effect on flower colour by trapping specific anthocyanins. Three major proteins were found in the AVI complexes isolated from the petals of the lisianthus flower. It is not yet clear what relation these AVI proteins have to the

cyanoplast-localizing proteins in anthocyanin-containing vacuoles of the sweet potato, however, these vacuolar proteins are probably involved in the formation of anthocyanin-containing intravacuolar inclusions via interaction with anthocyanin.

Acknowledgment

This study was carried out under the NIBB Cooperative Research Program (99-104).

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