Primary Structure and Expression of a 24-kD Vacuolar Protein (VP24) Precursor in Anthocyanin-Producing Cells of Sweet Potato in Suspension Culture

Wenxin Xu, Hidenari Shioiri, Mineo Kojima, and Masayuki Nozue*

Graduate School of Science and Technology (W.X., M.K., M.N.) and Department of Applied Biology, Faculty of Textile Science and Technology (H.S., M.K., M.N.), Shinshu University, 3–15–1 Tokida, Ueda, Nagano 386–8567, Japan

A 24-kD vacuolar protein (VP24) accumulates abundantly in intravacuolar pigmented globules in anthocyanin-containing sweet potato (*Ipomoea batatas*) cells in suspension culture. A cDNA clone encoding VP24 was isolated from a cDNA library constructed from light-irradiated suspension-cultured cells. Sequence analysis revealed that a 2.9-kbp VP24 cDNA encodes a protein of 893 amino acid residues with a molecular mass of 96.3 kD. According to the deduced amino acid sequence of VP24 cDNA, VP24 is probably synthesized as a large precursor protein with an N-terminal extension composed of a signal peptide and a propeptide, plus the polypeptide of the mature VP24 and its C-terminal propeptide, which contains the multiple transmembrane domains. A search in the ProDom database revealed the mature VP24 domain belongs to the zinc metalloprotease family. Northern analysis revealed that the single 2.9-kb VP24 mRNA increases rapidly after light irradiation, whereas VP24 mRNA was undetectable in the dark-cultured cells or in the presence of a high concentration of 2,4-dichlorophenoxyacetic acid. Light-induced VP24 gene expression closely correlated with the accumulation of anthocyanin in the vacuoles. These results suggested that proteins derived from the VP24 precursor protein may be involved in vacuolar transport and/or accumulation of anthocyanin synthesized in the cytosol.

Many hydrophilic water-soluble secondary metabolites accumulate in the central vacuole of plants, though most of them are usually synthesized in the cytoplasm. Biosynthesis of anthocyanins, which are the major water-soluble pigments, occurs in the cytosol, but anthocyanins and their intermediates are never detected there. Only the end product, anthocyanins, not their intermediates, are detected in the vacuoles. Cytosolic localization of many enzymes related to anthocyanin biosynthesis has been reported (Hrazdina et al., 1978; Jonsson et al., 1983; Hrazdina et al., 1987; Hrazdina and Jensen, 1992; Fujiwara et al., 1998), and it is thought that most of them assemble as a membrane-associated multi-enzyme complex or a macromolecular complex having contact with multiple proteins in the cytosol (Stafford, 1981; Hrazdina and Wagner, 1985; Burbulis and Winkel-Shirley, 1999). Anthocyanins that have been synthesized by the enzyme complex may be effectively transported into the vacuole by a specific transporter without any loss of product in the cytosol by diffusion. Anthocyanin synthesized in maize cells lacking *Bronze-2* (*Bz2*) gene was found retained in the cytosol, not transported into the vacuole (Marrs et al., 1995). The mechanism of anthocyanin transport into the vacuole has been shown through the functional analysis of the maize *Bz2* gene (Marrs et al., 1995) and the petunia *Anthocyanin 9 (An9)* gene encoding glutathione *S*-transferase (GST; Alfenito et al., 1998). The results suggested that anthocyanins were glutathionated in the cytosol by GST, then transported into the vacuole through a Mg^{2+}-ATP-dependent glutathione pump (GS-X pump) that is involved in the detoxification of xenobiotics in higher plants (Martinoia et al., 1993; Li et al., 1995; Lu et al., 1997; Rea et al., 1998). These analyses indicated that anthocyanin synthesized in the cytosol was recognized and glutathionated as a xenobiotic compound by GST, then transported into the vacuole by the GS-X pump.

In our previous papers (Nozue and Yasuda, 1985; Nozue et al., 1987, 1993) we demonstrated that cultured cells of sweet potato (*Ipomoea batatas* Lam. cv Kintoki) produced large amounts of anthocyanin, and formed intensely pigmented globules, cyanoplasts (Politis, 1959), or anthocyanoplasts (Pecket and Small, 1980) within the central 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 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). The level of VP24 continued to increase, along with the accumulation of anthocyanin in light-induced cells, but no transient increase of Phe ammonia-lyase or chalcone synthase that are involved in the biosynthesis of anthocyanins was noted (Lawton et al., 1983;

* Corresponding author; e-mail msnozue@giptc.shinshu-u.ac.jp; fax 81–268–21–5352.

Chappell and Hahlbrock, 1984). It is generally believed that the biosynthesis of anthocyanin is a cytosolic event after which it is transported and retained in the vacuoles. VP24 combines easily with anthocyanins and the largest amount of it is recovered from the reddish pellet after ultracentrifugation of the homogenate prepared from anthocyanin-containing cells. Our previous study suggested that VP24 may play a role in the intravacuolar trapping of anthocyanins via the formation of cyanoplasts through the hydrophobic interaction with anthocyanin in the vacuoles. However, whether VP24 has other biological roles in addition to the formation of cyanoplast is not clear. Although the non-producing cell line (N cell line) of sweet potato produces little anthocyanin and no cyanoplasts are formed, a small amount of VP24 accumulates in N cells after light irradiation (Nozue et al., 1997).

Most vacuolar proteins in higher plants are synthesized as proprotein precursors in the rough endoplasmic reticulum, then transported to the vacuoles (Okita and Rogers, 1996). The precursor proteins are processed post-translationally into their mature forms by the vacuolar-processing enzyme (Hara-Nishimura et al., 1993). VP24 may be synthesized in this way as a precursor protein, and converted into the mature form by proteolytic cleavage in the vacuole. To clarify the function of VP24 in the anthocyanin-producing cells it is important to obtain the structural information of the VP24 precursor protein. In the present study we isolated and characterized a cDNA clone for VP24. We discuss the predicted structure and possible function of VP24 protein that is synthesized as a large precursor with the multiple transmembrane hydrophobic domains.

RESULTS

Detection and Determination of Amino-Terminal Amino Acid Sequence of VP24

Vacuoles were prepared from anthocyanin-containing protoplasts of a high anthocyanin-producing cell line (ALND) of sweet potato cells in suspension culture that had been cultured for 7 d under continuous illumination. More than 95% of these ALND cells contained anthocyanin in the vacuoles, and little contamination by other organelles was observed in the vacuole fraction prepared by the methods described previously (Nozue et al., 1995). Both samples prepared from vacuoles and whole cells, which contained almost the same amount of anthocyanin, were subjected to SDS-PAGE and immunoblot analysis (Fig. 1). VP24 was detected as one of the prominently stained proteins in the vacuole fraction. The N-terminal amino acid sequences of VP24 were determined and are shown in Figure 2. Two specific primers (F1 and R1) used as primers for PCR were synthesized based on these deduced nucleotide sequences as described in "Materials and Methods."

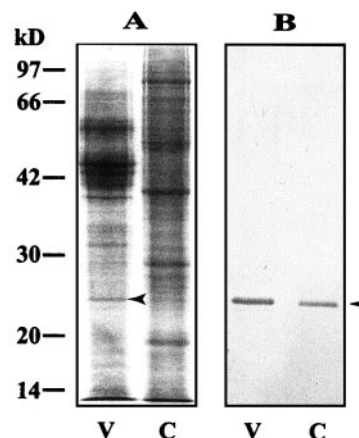

Figure 1. Detection of VP24 in vacuoles prepared from anthocyanin-producing ALND cells of sweet potato by SDS-PAGE (A) and immunoblot analysis (B). Suspensions of cells were initiated from the ALND callus by transfer to 2,4-D-free liquid medium and cultured for 7 d under continuous illumination. Vacuoles were isolated and purified by the methods previously described (Nozue et al., 1995). Both samples prepared from vacuoles (V) and whole cells (C) contained an almost equal amount of anthocyanin. They were subjected to SDS-PAGE (14% [w/v] gel) and stained by Phast Gel Blue (Pharmacia Biotech, Piscataway, NJ) or analyzed by immunoblotting with antibodies against VP24. Arrowhead indicates VP24.

cDNA Cloning and Sequence Analysis

A cDNA library was constructed with a λ ZAP II and poly(A)$^+$ RNA from the ALND cells that had been cultured in 2,4-dichlorophenoxyacetic acid-(2,4-D) free liquid medium for 84 h under continuous illumination. Eight clones were selected after tertiary immunoscreening of 1×10^6 primary plaques with specific polyclonal antibodies against VP24. Of these, three were identified as VP24 cDNA clones by PCR using Uni-ZAP XR vector primers (SK and T3) and the VP24-specific primers (F1 and R1), and these were subcloned into pBluescript SK$^-$. One clone (VP246 cDNA) with the longest cDNA insert (2.7 kbp) was selected and used to determine the complete DNA sequence for both strands. However, the typical nucleotide sequence for a signal peptide necessary for transport to the endoplasmic reticulum lumen was not detected in the nucleotide sequence of VP246 cDNA. To characterize a further sequence upstream of the 5′ end of VP246 cDNA, the missing 5′ end was isolated by 5′ RACE PCR as described in "Materials and Methods." The complete nucleotide sequence of VP24 cDNA (named VP24G) was determined and the deduced amino acid sequence is shown in Figure 2. Nucleotide sequence analysis revealed that VP24G is 2,899 bp in length with an open reading frame of 2,679 bp encoding 893 amino acids. The N-terminal amino acid sequence of VP24 was found in positions 141 to 179 of the deduced amino acids from VP24G. The 3′-untranslated region contained a polyadenylation signal (AATAAA; Joshi, 1987). The M_r calculated from the deduced amino acid

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atcatcacttctctatatatagtctttacagtctttttttgttgctcgaccactcagtcatatatatatactctaacgttatatagtgaattgaaggatta  100
ttataagagggacacaaaaaaaaaaaaaaaaATGGCAGAGAGGTCTGGGTATGAGATATTGGTGTTGTTTGCGTTTGTGATATGTGGGACTTGGGGGGTG  200
                                M  A  E  R  S  G  Y  E  I  L  V  L  F  A  F  V  I  C  G  T  W  G  V
TATCATTATCAGTTTAAGGTTCTCCCTGAGGCCCTCAGCCCGGAGGATGCCGGCGTCACAGGCTTCTCTGAGGAGGCGGCCATGGCTCATGATAAGGCAT  300
Y  H  Y  Q  F  K  V  L  P  E  A  L  S  P  E  D  A  G  V  T  G  F  S  E  E  A  A  M  A  H  D  K  A  L
TGAGTTCACTCGGCCCCCATCCTCTTGGCTCTGCCGTTCTTGACACTGCCCTCCAGTATGTGTTGAAAGCAGCAAAAACTATTGAGGAAGAGGCATACGG  400
  S  S  L  G  P  H  P  L  G  S  A  V  L  D  T  A  L  Q  Y  V  L  K  A  A  K  T  I  E  E  E  A  Y  G
GGATGTTAACGTGGAAGTGCAATGTTTTCATGCAAACACTGGGGTTAATACTCTGTCTGGAGGATCATATTATGGGAAAACCCTGGTTTACTCGGATATG  500
 D  V  N  V  E  V  Q  C  F  H  A  N  T  G  V  N  T  L  S  G  G  S  Y  Y  G  K  T  L  V  Y  S  D  M
AAGCATGTTCTAATCAGAATATCATCAAAATCTGCAGCAACTAAACTAAGATCAGGCGAGGAGGACAATGCTATTCTTGTTTCTGCCCACGTCGATACCG  600
K  H  V  L  I  R  I  S  S  K  S  A  A  T  K  L  R  S  G  E  E  D  N  A  I  L  V  S  A  H  V  D  T  V
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  F  A  A  E  G  A  G  D  D  S  S  N  V  A  V  M  L  E  L  A  R  G  L  S  K  Q  A  S  G  F  K  N  S
TGTCATCTTTCTGTTTAATACTGGGGAGGAAGAAGGCTTGGATGGCTCCCATAGCTTTGTAACTCAGCACCCTTGGATTAATACTGTCAGAGTGGCTGTT  800
 V  I  F  L  F  N  T  G  E  E  E  G  L  D  G  S  H  S  F  V  T  Q  H  P  W  I  N  T  V  R  V  A  V
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N  L  E  A  M  G  I  G  G  K  S  G  I  F  Q  A  G  P  D  P  W  A  I  Q  N  F  A  K  V  A  K  R  P  S
CCGGCCAGATTGTGTCACAGGATTTGTTTGGTTCCGGGGTAATAAAATCTACAACTGATTTTCAAGTGTATAAAGAGATTGCTGGTCTGTCTGGCATGGA  1000
  G  Q  I  V  S  Q  D  L  F  G  S  G  V  I  K  S  T  T  D  F  Q  V  Y  K  E  I  A  G  L  S  G  M  D
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P  F  L  L  H  V  A  T  S  P  D  F  P  T  G  K  N  T  L  S  Q  G  D  S  E  E  E  V  D  T  V  V  Y  F
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  D  I  L  G  R  F  M  V  V  Y  P  Q  S  L  A  D  M  I  N  T  S  V  I  A  L  A  L  F  L  W  S  A  L
ACTAAATCAGGGAGGGCTTTCATCTCTAGTTTCTCTGGCACTTTCAGTTTTGAGTATTGTACTCATGTGGATATGCTCTCTCGGTCTCTCCATCCTGGTT  1400
 L  N  Q  G  G  L  S  S  L  V  S  L  A  L  S  V  L  S  I  V  L  M  W  I  C  S  L  G  L  S  I  L  V
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A  Y  V  L  P  S  I  S  E  S  P  V  P  F  I  A  S  P  W  L  V  I  G  L  F  A  A  P  A  L  L  G  A  F
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  I  G  Q  H  V  V  Y  L  L  L  H  K  F  L  S  Y  T  F  S  E  T  K  G  F  L  P  L  S  L  Q  G  D  E
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 E  D  V  A  V  L  D  S  E  K  W  M  F  K  A  G  L  L  Q  W  L  L  V  L  V  V  G  N  Y  L  N  V  G
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  A  I  G  L  T  V  P  L  V  V  S  S  G  V  F  V  Q  L  V  N  T  L  I  G  N  L  V  R  F  V  S  N  P
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 G  E  Q  A  D  W  I  S  T  A  I  V  A  A  L  I  A  A  I  V  C  L  T  M  V  Y  V  L  P  Y  I  H  N
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TTTCTCAGTATGGGAAGTCAGCTTCACCCTTTACCTTAGCTTACTTGGATACTCTGTATGTTGCAGACCCTTCTCTAACAATGGGCAGTTCAATTTCTGT  2800
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GGCAAGTGAGTAGaactatcaccctatgggttgtAATAAAtttctactacaaataataaaaagaatattgtaatttagccgtaaaaaaaaaaaaaaaaa  2899
 A  S  E  *
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Figure 2. Complete nucleotide sequence of the VP24 cDNA and deduced amino acid sequence. The coding region is in capital letters. The deduced amino acid sequence is indicated below the nucleotide sequence in single-letter code. The dotted line indicates the putative signal peptide, and the underlined sequence denotes the N-terminal amino acid sequence of purified VP24. The putative polyadenylation signal at the 3′ end is indicated in bold letters. The asterisk indicates the termination codon. The wavy lines denote potential sites of *N*-glycosylation.

sequence was 96,318—about 4-fold larger than that of a purified VP24 protein estimated by SDS-PAGE.

These sequence analyses indicate that VP24 is synthesized as large precursor protein and is processed during and/or after transport to the vacuole. The hydropathy plot and predicted primary structure of the VP24 protein precursor are shown in Figure 3, A and B. The deduced precursor sequence of VP24G contains a hydrophobic N terminus with characteristics of a signal sequence about 22 amino acid residues as proposed by von Heijne (1986). After cleavage of the signal peptide, the VP24 precursor must be further cleaved to remove N- and C-terminal propeptides. The N-terminal propeptide sequence extends from the end of the signal sequence to the N-terminal amino acid residues of mature VP24 protein, and the C-terminal propeptide sequence that contains highly hydrophobic regions extends from the end of the mature VP24 protein (Fig. 3B). Seven or eight transmembrane domains have been predicted in the C-terminal propeptide by the Tmpred program (Hofmann and Stoffel, 1993) and by the PSORT program based on Klein's method (Klein et al., 1985), respectively. The C-terminal propeptide domain is com-

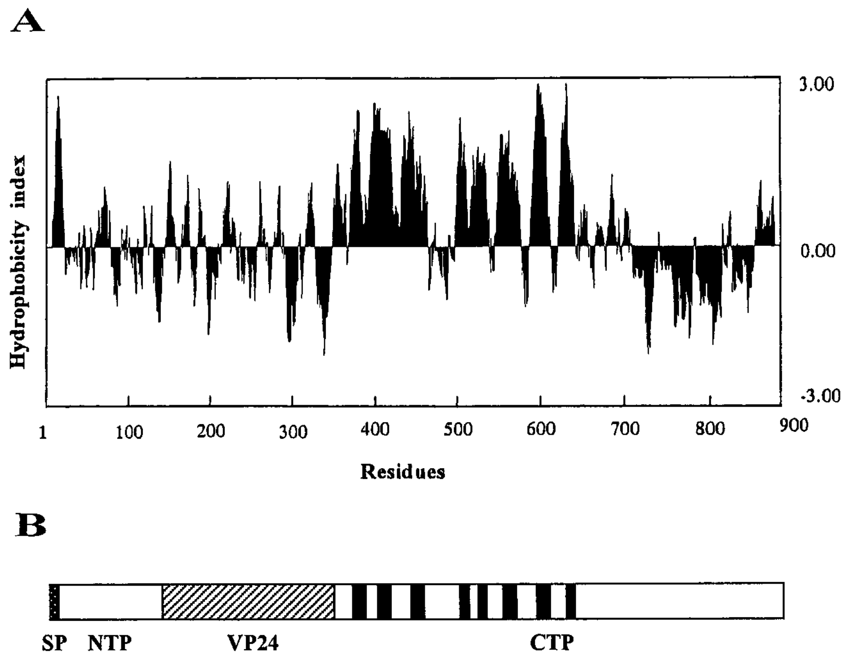

Figure 3. Structural features of VP24 precursor protein deduced from VP24G sequence. Hydropathy plot (A) and schematic diagram showing primary structure (B). Hydropathy was analyzed by the methods of Kyte and Doolittle (1982) for a window size of 12 amino acid residues. In B, SP represents signal peptide; NTP, N-terminal propeptide; VP24, mature VP24 peptide; and CTP, C-terminal propeptide. Putative transmembrane domains predicted by the PSORT program (http://psort.nibb.ac.jp/index.html) are marked with black boxes.

prised of five potential *N*-glycosylation sites (Fig. 2). The deduced amino acid sequence of VP24G was compared with sequences of other proteins in the search of the protein domain database ProDom (http://protein.toulouse.inra.fr/prodom.html). It is interesting that the N-terminal portion of the deduced VP24 precursor is found to be a member of the zinc metalloprotease family. The closely related domain is found in a hypothetical 98.3-kD protein (YP67_CAEEL) of *Caenorhabditis elegans* and a hypothetical 95.7-kD protein (O94479) of *Schizosaccharomyces pombe* with similarity scores of 10^{-19} and 10^{-10}, respectively. These two proteins also contained multiple transmembrane domains in the C-terminal region; however, there is no homology with the sequence of transmembrane domains in the VP24 protein precursor.

Southern-Blot Analysis

Southern-blot analysis showed that digestion of genomic DNA of sweet potato ALND cells with *Mlf* I produced four major bands and one faint band. Digestion with *Hin*dIII produced two main bands and one faint band, and digestion with *Eco*RI produced three major bands (Fig. 4). VP24G does not contain the restriction sites of *Hin*dIII and *Mlf* I. The size of DNA probe used in Southern-blot analysis, which was obtained by PCR using genomic DNA of sweet potato ALND cells as a template, was about 0.4 kbp longer than the probe obtained using VP24 cDNA as template (data not shown). The use of two specific primers resulted in probes of different sizes, indicating that the genomic DNA of VP24 probably contains an intron in the amplified region. The probe did not contain restriction sites of the three enzymes (data not shown). The results of Southern hybridization suggest that there may be more than three closely related VP24G genes in the genome of sweet potato ALND cells.

VP24 Gene Expression

A marked increase in VP24 was previously found in vacuoles isolated from cells that had been cultured in 2,4-D-free medium under continuous illumination (Nozue et al., 1995, 1997). The accumulation of anthocyanin also began and continued to increase after exposure to light. These results indicated that the expression of VP24 was closely accompanied by the accumulation of anthocyanin in sweet potato cells in suspension culture under the condition of light. To

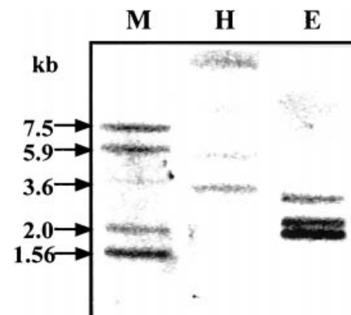

Figure 4. Southern-blot analysis of VP24 genes of sweet potato ALND callus. Genomic DNA (15 μg) was digested with *Mlf* I (M), *Hin*dIII (H), and *Eco*RI (E), then separated on a 0.8% (w/v) agarose gel and transferred to nylon membranes. The probe used was prepared by PCR using the genomic DNA as a template as described in the text.

further investigate the expression pattern of the VP24 gene transcript, northern-blot analysis was performed on total RNA from suspension-cultured cells initiated by transfer of a callus to 2,4-D-free liquid medium. A single RNA transcript of about 2.9 kb was detected in cultured cells 3 d after onset of irradiation, and the amount increased rapidly over the next 3 d (Fig. 5, A and B). This high expression level continued during culture under continuous illumination, however, little VP24 gene transcript was detected in the dark-cultured cells. The levels of anthocyanin and VP24 detected by immunoblot increased 3 d after light irradiation (Fig. 5B) when the same suspension-cultured cells as those examined in northern-blot analysis were used (Fig. 5A). These increases of VP24 and anthocyanin correlated well with the increase of VP24 gene expression.

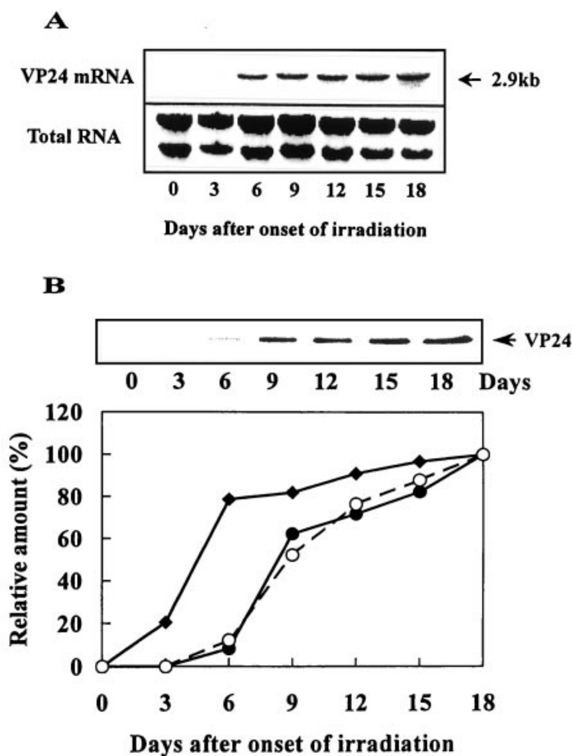

Figure 5. Induction of VP24 mRNA, anthocyanin and VP24 protein in light-irradiated suspension-cultured cells. Suspensions of cells were initiated from the 11-d-old callus that had been cultured in PRL-4C agar medium containing 0.1 mg L^{-1} 2,4-D in the dark by transfer to 2,4-D-free liquid medium, then cultured under continuous illumination. A, Northern-blot analysis of the expression of VP24 gene on various days after light irradiation. Total RNA (10 μg) prepared from the cultured cells was detected with a ^{32}P-labeled VP24 cDNA probe. Ethidium bromide staining of electrophoresed RNA is shown as a loading control for RNA blot in the bottom panel. B, Changes in VP24 mRNA level, VP24 protein, and anthocyanin content. Relative amounts of VP24 mRNA, VP24 protein, and anthocyanin content were measured by densitometric analysis of the corresponding bands shown in the top panels and expressed as a percentage of the value of the cells 18 d after irradiation. Cell extracts (3.6 μL per lane) from cultured cells (0.1 g fresh weight) extracted with 200 μL of SDS-sample buffer were fractionated by SDS-PAGE with subsequent immunoblotting analysis using antibodies against VP24. Anthocyanin was measured as described in the text. ◆, VP24 mRNA; ○, VP24; ●, anthocyanin.

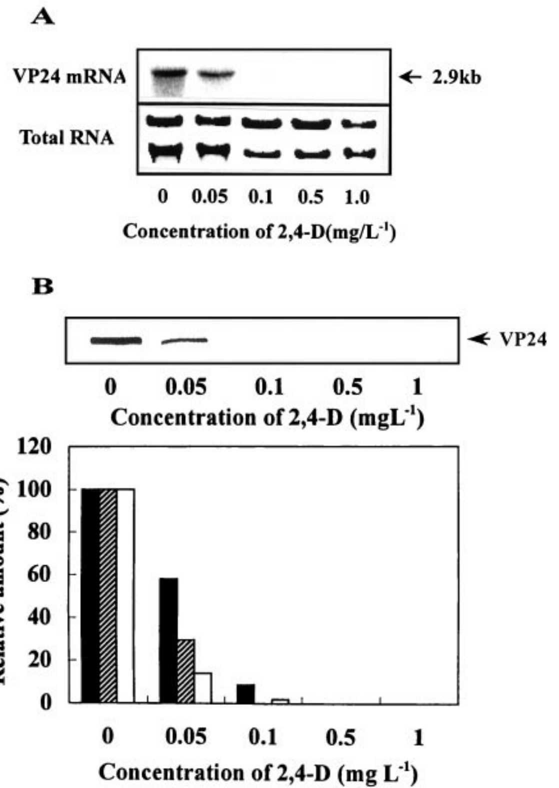

Figure 6. Effect of 2,4-D on the expression of VP24 gene in suspension-cultured cells. Eleven-day-old callus samples cultured in PRL-4C agar medium containing 0.1 mg L^{-1} 2,4-D in the dark were transferred to liquid medium containing 2,4-D at various concentrations and then the cells were cultured under continuous illumination. Cells were harvested 7 d after the start of irradiation. A, Northern-blot analysis of the expression of VP24 gene. Total RNA (10 μg) prepared from the cultured cells was detected with a ^{32}P-labeled VP24 cDNA probe. Ethidium bromide staining of electrophoresed RNA is shown as a loading control for RNA blot in the bottom panel. B, Effect of 2,4-D on VP24 mRNA level, VP24 protein, and anthocyanin content. Relative amounts of VP24 mRNA and VP24 protein were measured by densitometric analysis of the corresponding bands shown in the top panels and expressed as a percentage of the value of the cells cultured without 2,4-D medium under continuous illumination. Cell extracts (3.6 μL per lane) from cultured cells (0.1 g fresh weight) extracted with 200 μL of SDS-sample buffer were fractionated by SDS-PAGE with subsequent immunoblotting analysis using antibodies against VP24. Anthocyanin was measured as described in the text. ■, VP24 mRNA; ▨, VP24; □, anthocyanin.

As reported previously (Nozue and Yasuda, 1985; Nozue et al., 1995, 1997), 2,4-D markedly inhibited the expression of VP24 and the formation of anthocyanin in illuminated sweet potato cells in suspension culture. Figure 6A shows the effect of 2,4-D on the level of VP24 transcript in 7-d-old illuminated ALND cells. The level of VP24 transcript was markedly inhibited by 2,4-D at a concentration of more than 0.1 mg L^{-1}. The levels of anthocyanin and VP24 detected by immunoblot were also inhibited by

higher concentrations of 2,4-D (Fig. 6B) when the same suspension-cultured cells as those examined in northern-blot analysis were used (Fig. 6A).

DISCUSSION

VP24 was originally found to be a 24-kD protein localized in cyanoplasts in the anthocyanin-containing vacuoles in sweet potato cells (Nozue et al., 1997). However, the data presented here indicate that VP24 cDNA encoded a 96.3-kD protein containing a hydrophobic N terminus with characteristics of a signal sequence. Northern-blot analysis also showed a single 2.9-kb VP24 mRNA detected in light-irradiated cultured cells. These results suggest that VP24 may be synthesized first as a large protein precursor in the endoplasmic reticulum lumen, and then transported to the vacuole. According to the search using ProDom database, the mature VP24 domain most probably belongs to the zinc metalloprotease family. 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 the multiple transmembrane domains. However, homology search of the C-terminal hydrophobic domain in the precursor shows no similarities to any other proteins in the database. The hypothetical proteins that have a similar molecular mass and a similar hydropathic character with the VP24 protein precursor are found in fruit fly, *Caenorhabditis*, Arabidopsis, and *Schizosaccharomyces* by Blast 2.0 search using the swisstrembl database. They have a related sequence in N-terminal portion with the VP24 protein precursor, however, there are no homologies in any other regions, including their transmembrane domains. These analyses strongly suggested that VP24 is a new vacuolar protein that is derived from the vacuolar membrane protein precursor via a novel processing scheme.

Soluble vacuolar protein precursors are usually transported to vacuoles by the sorting system involved in the vacuolar sorting determinants (Neuhaus and Rogers, 1998; Marty, 1999), and then processed into their respective mature forms in the vacuoles by proteolytic cleavage (Hara-Nishimura et al., 1995). However, little is known regarding the sorting mechanism that localizes the integral membrane proteins to the vacuoles and their processing after that. The VP24 protein precursor is presumably transported as the integral vacuolar membrane protein, and then the mature VP24 peptide may be proteolytically cleaved from the vacuolar membrane as demonstrated in the carboxypeptidase yscS of *Saccharomyces cerevisiae* (Spormann et al., 1992; Cowles et al., 1997). Processing of the N-terminal propeptide that extends from the end of the putative signal sequence to the N-terminal amino acid residues of mature VP24 protein is thought to occur on the C-terminal side of R-140. Although the processing site has been not identified, even further proteolytic cleavage of the C-terminal propeptide is necessary to reach the mature form of VP24 to release into the lumen of vacuole.

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) as the antigen. Although the exact position of the C-terminal propeptide in the VP24 precursor protein has not yet been identified, a 36-kD protein has been found, which accumulates in the anthocyanin-containing vacuoles of sweet potato cultured cells, by immunoblot analysis using antibodies against the fusion protein containing the hydrophilic region of C-terminal propeptide (see Fig. 3B; data not shown). A preliminary examination shows the possibility that another peptide derived from VP24 precursor protein may be expressed in addition to the mature VP24 protein in the vacuole.

The light-induced expression of VP24 gene closely correlated with the accumulation of anthocyanin in the vacuole of ALND cells. The ALND cell line used in the present study produced large amounts of anthocyanin under continuous illumination, but little anthocyanin in the dark (Nozue et al., 1997). Light irradiation is also indispensable for the expression of the VP24 gene in ALND cells. Higher concentrations of 2,4-D markedly inhibited the formation of anthocyanin in light-irradiated ALND cells. The occurrence of cyanoplasts (Nozue and Yasuda, 1985) and the expression of VP24 gene and mature VP24 protein localized in cyanoplasts of the anthocyanin-containing vacuoles was completely inhibited in cells cultured in 2,4-D-containing medium. These results indicate that the light-induced expression of VP24 and possibly other peptides derived from the VP24 protein precursor may be regulated at the transcriptional level.

It was recently demonstrated that the enzymes involved in anthocyanin synthesis most likely formed a macromolecular complex with contacts between multiple proteins in the cytosol (Burbulis and Winkel-Shirley, 1999). After synthesis, anthocyanins are never found in the cytosol in normal plant cells, but are expeditiously transported to the vacuole by a specific transporter. Marrs et al. (1995) demonstrated that cyanidin-3-glucoside conjugated with glutathione by GST was recognized as a substrate for transport into vacuoles by GS-X pump in maize. Four genes encoding GS-X pumps have been cloned from Arabidopsis and characterized (Rea et al., 1998), but VP24 cDNA was distinct from these genes. It is possible that the putative transmembrane domain we found in the primary structure of the VP24 protein precursor might be a new unique vacuolar membrane protein involved in transport during anthocyanin formation.

VP24 may be involved in the formation of cyanoplasts (Nozue et al., 1997); however, the mature VP24 protein may have another as yet unknown biological role in the vacuoles of anthocyanin-producing cells. Sequence analysis indicated that matureVP24 peptide is a member of the metalloprotease family. Why should the metalloprotease be concentrated in the cyanoplasts? Although the protease activity of VP24 has not been yet confirmed, one possibility is that VP24 is involved in the degradation of glutathione *S* conjugates in the vacuoles. It was proposed that carboxypeptidase degraded glutathione moieties of the glutathione *S* conjugates in the plant vacuoles (Wolf et al., 1996). Further studies are necessary to elucidate the function of the domains derived from VP24 protein precursor in anthocyanin-producing sweet potato cells.

MATERIALS AND METHODS

Cell Cultures

ALND cells of sweet potato (*Ipomoea batatas* Lam. cv Kintoki) in suspension culture was used in the present experiments. The ALND cells were established by visual clonal selections from callus culture that had been initiated from root tissue (Nozue et al., 1987). The ALND cell line produced large amounts of anthocyanin under continuous illumination, but little anthocyanin in the dark. The ALND callus was maintained in 30 mL of PRL-4C (Gamborg, 1966) agar medium that contained 3% (w/v) Suc and 0.1 mg L^{-1} 2,4-D in a 100-mL Erlenmeyer flask in the dark at 25°C with subculture at 3-week intervals. To induce anthocyanin synthesis, 7-d-old callus (1 g wet weight) was transferred to 20 mL of liquid PRL-4C medium without 2,4-D and exposed to continuous illumination as described previously (Nozue et al., 1993).

Determination of the Amino-Terminal Sequence of VP24

VP24 was purified by SDS-PAGE on 12.5% (w/v) gel as described by Laemmli (1970) from the vacuole fraction prepared from 7- to 10-d-old anthocyanin-producing cells in suspension culture. Vacuoles were isolated and purified by the method described in the previous paper (Nozue et al., 1995). VP24 was transferred from the gel after SDS-PAGE onto a polyvinylidene difluoride membrane that was stained with Coomassie Brilliant Blue R-250. After excision of the bands, the amino-terminal amino acid sequence was determined with a gas-phase protein sequencer (Shimadzu PPSQ-23, Kyoto).

Construction and Screening of the cDNA Library

Total RNA was extracted from the ALND cells that had been cultured for 84 h in 2,4-D-free PRL-4C liquid medium under continuous illumination by the SDS-phenol method (Logemann et al., 1987; Verwoerd et al., 1989; Shirzadegan et al., 1991). Poly $(A)^+$ RNA isolated by Oligotex-dT30 super (Takara Biomedicals, Kyoto) was used to construct a λ ZAP II cDNA library that was screened using a VP24-specific polyclonal antibody (Nozue et al., 1997). Further confirmation of positive clones was carried out by PCR using F1 (5′-GGIGARGARGAYAAYGCIAT-3′) and R1 (5′-TCCATCATIACIGCIACRTT-3′) primers designed from the N-terminal amino acid sequence of the purified VP24. The positive clones were excised and maintained in pBluescript SK^- according to the manufacturer's instructions (Stratagene, La Jolla, CA).

PCR Amplification of the 5′ End of cDNA

The missing 5′-end portion of the gene was obtained by using the 5′-RACE system from Gibco-BRL (Cleveland). First-strand cDNA was synthesized from total RNA, which was isolated from 7-d-old ALND cells cultured as described above under continuous illumination, using VP24-1 primer (5′-ACTATCATCTCCAGCT-3′) designed from the internal cDNA fragment. A poly C tail was added to the 3′ end of the purified first strand cDNA using dCTP with terminal deoxynucleotidyl transferase. After inactivation of terminal deoxynucleotidyl transferase by heating for 10 min at 65°C, the dC-tailed cDNA was amplified using a nested primer VP24-2 primer (5′-CGGTATCGACGTGGG CAGAAAC-3′) and a 5′-RACE Abridged Anchor Primer (Gibco-BRL). The reaction was performed for 35 cycles of denaturation at 94°C for 45 s, annealing at 55°C for 45 s, and elongation at 72°C for 60 s and at 72°C for 10 min. A second PCR amplification was performed as described above using a VP24-2 primer and an Abridged Universal Amplification Primer (Gibco-BRL) with the PCR amplified dC-tailed cDNA as the template. A third PCR amplification was performed to obtain the uracil DNA glycosylase-treated PCR product for subcloning using a VP24-3 primer (5′-CAUCAUCAUCAUCCGTATGCCTCTTCCTCAA-3′) and a Universal Amplification Primer (Gibco-BRL) with the second PCR product as the template. The resultant 5′-RACE product was cloned into pAM1 vector (Gibco-BRL).

DNA Sequence Analysis

DNA sequencing was performed on VP24 positive clones obtained from the cDNA library and the 5′-RACE product using a 373A DNA sequencer (PE-Applied Biosystems, Tokyo) and the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction kit (PE-Applied Biosystems). T3 and T7 primers and newly designed primers from the obtained sequence data were used for DNA sequencing. Nucleotide sequences, molecular mass, and hydrophilic/hydrophobic profiles by the methods of Kyte and Doolittle (1982) were analyzed with the GENETYX-MAC software version 10.1.

Northern-Blot Analysis

Total RNA was isolated from ALND cells in suspension culture as described above. Total RNA (10 μg) was then separated on formaldehyde-containing 1% (w/v) agarose

gel and blotted onto a Hybond-N$^+$ nylon membrane (Amersham, Tokyo). The blotted membrane was hybridized with a [^{32}P]-labeled cDNA probe, which was prepared by PCR amplification using primers F2 (5′-GAAGGAGCTGG AGATGATA-3′) and R6 (5′-GAAGAAGGAGATAAACC AC-3′) with VP24 cDNA as the template. The membrane was washed under the following conditions: three 5- to 10-min washes in 2× SSC containing 0.5% (w/v) SDS at room temperature, a 30-min wash in 2× SSC containing 0.1% (w/v) SDS at 37°C, and three 5-min washes in 0.1× SSC at room temperature.

Genomic Southern-Blot Analysis

Genomic DNA was purified from ALND cells that had been cultured for 11 d in the dark by a modified cetyltrimethyl-ammonium bromide method (Murray and Thompson, 1980; Rogers and Bendich, 1994). Fifteen micrograms of DNA that had been digested with *Mlf* I, *Hin*dIII, and *Eco*RI, respectively, was subjected to electrophoresis on 0.8% (w/v) agarose gel and blotted onto a Hybond-N$^+$ nylon membrane (Amersham). The membrane was hybridized with a [^{32}P]-labeled cDNA probe (1.38 kb), which was prepared by PCR amplification using primers F3 (5′-ATTGCTGGTCTGTCTGGC-3′) and R5 (5′-TATCCAATCTG CCTGCTCT-3′) with genomic DNA of ALND cells as the template. Hybridization was carried out as described in northern-blot analysis.

Extraction and Estimation of VP24 and Anthocyanin Content

Suspension-cultured cells (0.1 g fresh weight) that had been harvested at various intervals as described previously (Nozue et al., 1997) were homogenized with a mortar and pestle in 200 μL of SDS-PAGE sample buffer (20 mM Tris-HCl, pH 6.8, 2% [w/v] SDS, 2% [v/v] 2-mercaptoethanol, and 40% [v/v] glycerol). The homogenate was transferred to microtubes and then boiled for 3 min. After centrifugation at 15,000*g* for 5 min the supernatant was analyzed by SDS-PAGE with subsequent immunoblotting with antibodies against VP24 as described previously (Nozue et al., 1997). The relative amounts of VP24 were measured by densitometric analysis of the corresponding bands on the immunoblots.

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

ACKNOWLEDGMENTS

We thank Prof. Mikio Nishimura from the National Institute for Basic Biology (Okazaki, Japan) for his generous help and advice and Drs. Hiroyoshi Kubo (Shinshu University, Matsumoto, Japan) and Kenji Yamada (Kyoto University) for helpful suggestions.

Received April 10, 2000; modified July 5, 2000; accepted September 8, 2000.

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