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Rapid Paper

The Involvement of Tonoplast Proton Pumps and $Na^+(K^+)/H^+$ Exchangers in the Change of Petal Color During Flower Opening of Morning Glory, *Ipomoea tricolor* cv. Heavenly Blue

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The petal color of morning glory, *Ipomoea tricolor* cv. Heavenly Blue, changes from purplish red to blue during flower opening. This color change is caused by an unusual increase in vacuolar pH from 6.6 to 7.7 in the colored adaxial and abaxial cells. To clarify the mechanism underlying the alkalization of epidermal vacuoles in the open petals, we focused on vacuolar H^+-ATPase (V-ATPase), H^+-pyrophosphatase (V-PPase) and an isoform of Na^+/H^+ exchanger (NHX1). We isolated red and blue protoplasts from the petals in bud and fully open flower, respectively, and purified vacuolar membranes. The membranes contained V-ATPase, V-PPase and NHX1, which were immunochemically detected, with relatively high transport activity. NHX1 could be detected only in the vacuolar membranes prepared from flower petals and its protein level was the highest in the colored petal epidermis of the open flower. These results suggest that the increase of vacuolar pH in the petals during flower opening is due to active transport of Na^+ and/or K^+ from the cytosol into vacuoles through a sodium- or potassium-driven $Na^+(K^+)/H^+$ exchanger NXH1 and that V-PPase and V-ATPase may prevent the over-alkalization. This systematic ion transport maintains the weakly alkaline vacuolar pH, producing the sky-blue petals.

Keywords: Anthocyanin — Flower color — *Ipomoea tricolor* cv. Heavenly Blue — Na^+/H^+ exchanger — Proton pumps — Vacuolar pH.

Abbreviations: DTT, dithiothreitol; HBA, heavenly blue anthocyanin; NHX1, Na^+/H^+ exchanger isoform 1; PM-ATPase, plasma membrane H^+-ATPase; TBS, Tris-buffered saline; V-ATPase, vacuolar H^+-ATPase; V-PPase, vacuolar H^+-translocating inorganic pyrophosphatase.

Introduction

Beautiful flower colors are mostly due to anthocyanins, and the mechanism of color development, especially of the blue color, has been attracting much interest (Goto 1987, Goto and Kondo 1991, Brouillard and Dangles 1994). Since anthocyanins give a blue color in an alkaline solution and a red color in an acidic solution in vitro, Willstätter proposed the pH theory (Willstätter and Everest 1913). However, his pH theory was opposed by Shibata et al. (1919) who argued that the cell sap of petals is generally weakly acidic. This controversial issue remained unresolved until our structural elucidation of a blue metalloanthocyanin, commelinin, from blue dayflower, *Commelina communis* (Kondo et al. 1992).

The petal color of blue morning glory, *Ipomoea tricolor* cv. Heavenly Blue, changes from purplish red in the bud to blue in the fully open flower (Fig. 1A, B). The same pigment, heavenly blue anthocyanin (HBA), is responsible for both colors (Kondo et al. 1987) (Fig. 1C). By direct measurement of vacuolar pH using a pH-sensitive microelectrode, we demonstrated that this change of petal color was accompanied by an increase of pH from 6.6 to 7.7 (Yoshida et al. 1995). This was the first evidence supporting the pH theory in petal color and the interesting physiological phenomenon that vacuolar pH is alkaline. Although simple anthocyanins are easily decolorized in such an alkaline condition, polyacylated anthocyanin, HBA, is stabilized by an intramolecular stacking of three caffeoyl residues to the anthocyanidin chromophore (Kondo et al. 1987, Goto and Kondo 1991, Yoshida et al. 1992, Yoshida et al. 2003a).

A similar change in petal color has been observed in Japanese morning glory, *Ipomoea nil*, and Fukada-Tanaka et al. reported the involvement of the gene for an Na^+/H^+ exchanger (*InNHX1*) (Fukada-Tanaka et al. 2000, Fukada-Tanaka et al. 2001, Yamaguchi et al. 2001). They found an insertion of a transposable element into the *InNXH1* gene in a purple mutant that did not give blue but purple colored open petals. They also reported that the *InNHX1* gene was similar to that in *I. tricolor* (DDBJ/GenBank/EBI accession number, AB054979). However, the localization of NHX1 and its function remain obscure,

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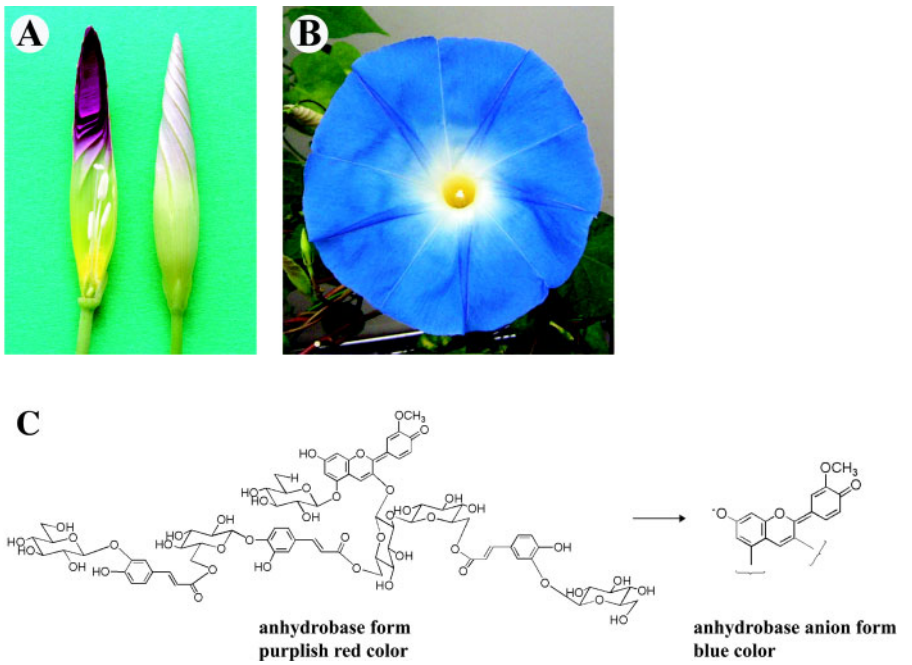

Fig. 1 Flower petal color of *Ipomoea tricolor* cv. Heavenly Blue and their anthocyanin component. (A) Purplish red bud –24 h. (B) Fully open blue flower 0 h. (C) Structure of petal pigment of morning glory, heavenly blue anthocyanin (HBA, 1). The anhydrobase form of HBA (left) shows a purplish red color at pH 6.6 and the anhydrobase anion form (right) exhibits a blue color at pH 7.7 the same as the fully open petals.

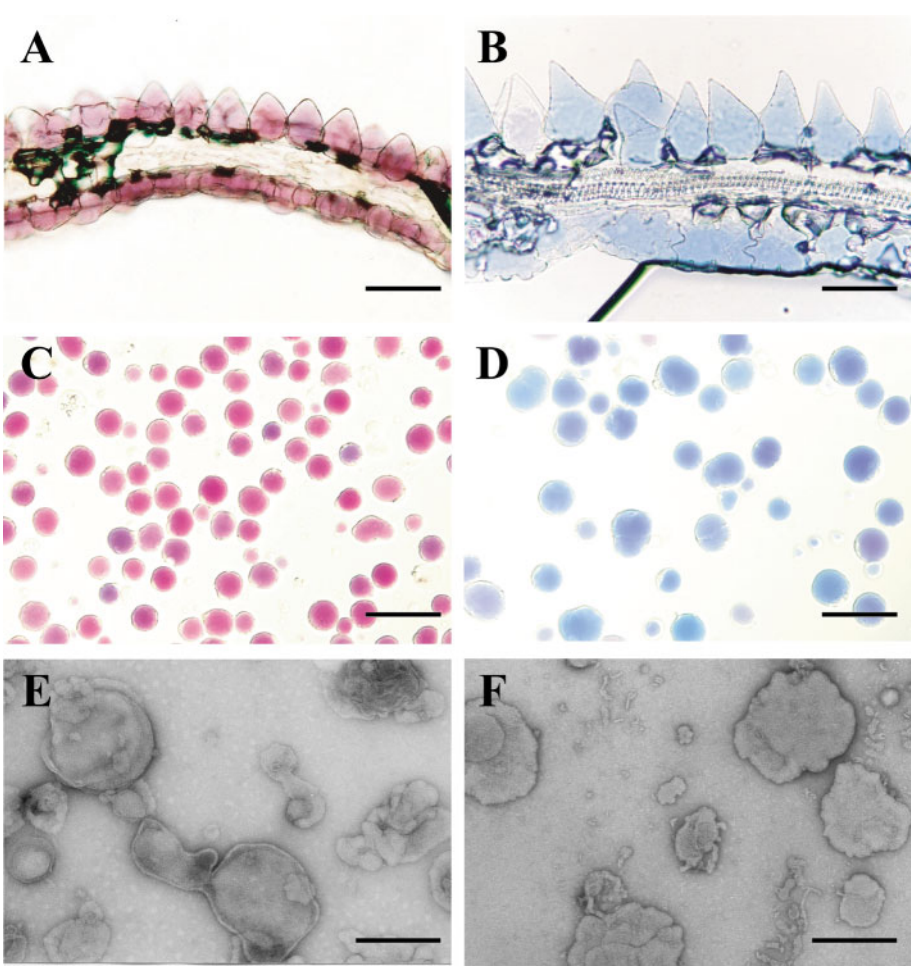

Fig. 2 Transverse section of morning glory petals at different flower opening stages, colored protoplasts prepared from each petal, and vacuolar membrane vesicles purified from each colored protoplast. (A) A transverse section of a petal in a red bud (–24 h) observed by light microscopy. (B) A transverse section of a petal in a fully open blue flower (0 h). (C) Red protoplasts obtained from the red rims of the petals (–24 h) after incubation with cellulase and pectinase for 100 min with gentle shaking. (D) Blue protoplasts obtained from the blue rims of the petals (0 h). (E) The purified vacuolar membrane vesicles from red protoplasts. (F) The purified vacuolar membrane vesicles from blue protoplasts. Bar = 50 μm in A–D. Bar = 200 nm in E, F.

and the detailed mechanism underlying the change of petal color in morning glory is still unknown.

To clarify the mechanism of vacuolar pH increase in morning glory, we isolated the vacuolar membranes of colored epidermal cells and measured the ion transport activities in the membrane. In general, the vacuolar H^+-ATPase (V-ATPase) and H^+-pyrophosphatase (V-PPase) maintain the acidic pH of vacuoles (Maeshima 2001). Also, the plasma membrane H^+-ATPase (PM-ATPase) is a key regulator of the cytosol pH (Sze et al. 1999). Therefore, we determined the protein levels and activities of V-ATPase, V-PPase and PM-ATPase in addition to NHX1 in flower petals. Here we demonstrate that the change in the petal color from red to blue during the flower opening process is accompanied by an increase in the vacuolar pH which is produced by a simultaneous increase in protein levels and activities of V-PPase, V-ATPase, NHX1 and PM-ATPase.

Results

Purification of vacuolar membrane from colored cells

The transverse section of the morning glory petal indicated that both the adaxial and abaxial epidermal layers are colored and the inner parenchyma is colorless (Fig. 2A, B). The adaxial cells were conical and the abaxial cells were flat. HBA was dissolved in the central vacuoles of both epidermal cells. From the microelectrode experiments, only the vacuolar pH of colored cells increased from 6.6 to 7.7 during flower opening and the vacuolar pH of the inner colorless cells was determined to be 5.8 in buds and 6.0 in fully open flowers (Yoshida et al. 1995). These results indicate that only the epidermal colored cells have a special system to increase the vacuolar pH. Therefore, we purified vacuolar membranes from colored cells to examine the mechanism of vacuolar alkalization.

In this study, we developed a method of isolating anthocyanin-containing cells. When the rim of fully open flowers was treated with maceration medium including Sumizyme C, Macerozyme R-200 and 600 mM mannitol in the buffer at pH 7.0, colored protoplasts were selectively released from the tissues as shown in Fig. 2D. Blue protoplasts were obtained from the rim part without any change in color (Fig. 2D). Using the same procedure with slight modification (650 mM mannitol, pH 6.5), red protoplasts were obtained from the buds with a high purity (Fig. 2C). The photographs of a transverse section and the protoplasts indicate that the cell volume of blue petals is almost twice as large as that of red petals, suggesting that cell enlargement by influx of water into the vacuole may occur during flower opening coincident with the increase in pH.

Next, we purified vacuolar membranes from the blue and red protoplasts. The sizes of both membrane vesicles were determined to be about 0.15 μm by transmission electron microscopy (Fig. 2E, F). There was no difference in size between the membrane vesicles prepared from bud (red) and open flowers (blue). The membrane vesicles obtained were used for the following experiments.

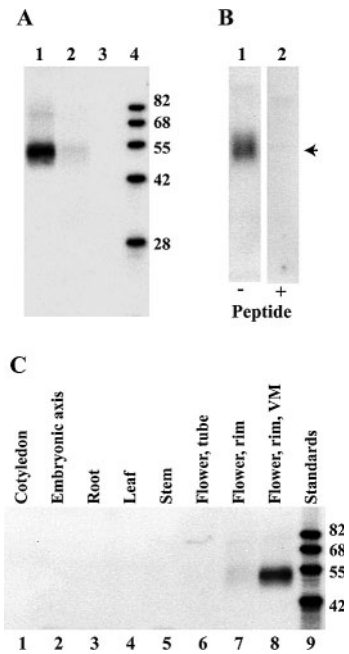

Fig. 3 Specificity and reactivity of anti-NHX1 antibody. (A) Immunoblot of various membrane fractions with the antibody. Lane 1, vacuolar membranes (10 μg) prepared from colored protoplasts from open petals (0 h); lane 2, crude membranes (30 μg) from the flower rim in blue (0 h); lane 3, crude membranes (30 μg) from leaves; lane 4, molecular size markers. (B) Competition of immunological reaction with the antigen peptide. Vacuolar membranes (15 μg) from blue protoplasts were subjected to immunoblot with anti-NHX1 antibody in the presence (+) or absence (–) of the synthetic antigen peptide at a final concentration of 15 μg ml^{-1}. An arrow indicates the position of NHX1. (C) Tissue-specific accumulation of NHX1 protein in morning glory. Crude membrane fractions (40 μg) were prepared from cotyledon (lane 1), embryonic axis (lane 2), roots (lane 3), leaves (lane 4), stems (lane 5), the tube part (lane 6) and rim part of petals (0 h) (lane 7), vacuolar membranes (4 μg) purified from blue protoplasts from petal rims (0 h) (lane 8) and molecular size markers (lane 9).

Immunochemical detection of NHX1 protein in pigmented cells of flower petals

As a result of genetic and biochemical studies, the gene for a Na^+/H^+ exchanger *InNHX1* has been proposed to be involved in the change of petal color in the Japanese morning glory *I. nil* (Yamaguchi et al. 2001). Furthermore organ- and stage-specific accumulation of mRNA of *InNHX1* has been reported (Yamaguchi et al. 2001), but the subcellular localization of its translation product, InNHX1, has not been examined. In this study, we examined whether NHX1 protein specifically exists in fully open flowers in *I. tricolor*. We prepared crude membrane fractions of various parts of the morning glory plant and the petals at different stages of flower opening to analyze the accumulation of NHX1.

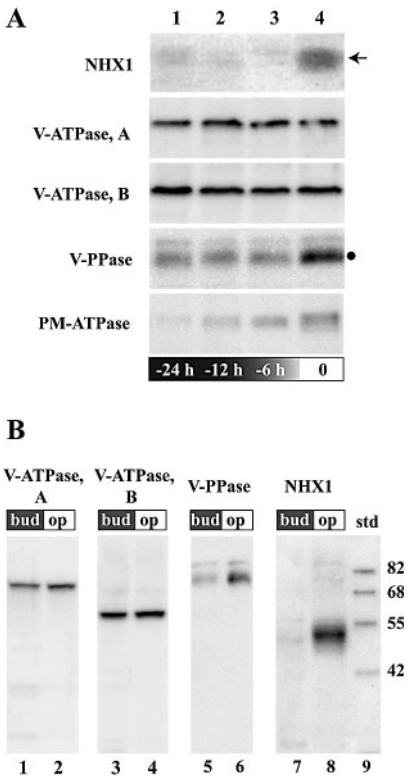

Fig. 4 Stage-specific accumulation of NHX1, V-ATPase, V-PPase and PM-ATPase proteins in crude membrane fractions of colored petal rim and purified vacuolar membranes from colored protoplasts prepared from blue and red petals. (A) Immunoblot analysis of crude membrane fractions (30 μg per lane, for the immunoblot with anti-NHX1 antibody: 40 μg) prepared from flower petals at –24 h (red) before opening, –12 h, –6 h and 0 h (fully open, blue) with antibodies to NHX1, subunit A of V-ATPase, subunit B of V-ATPase, V-PPase and PM-ATPase. The arrow shows the position of NHX1. The black circle shows V-PPase. (B) Immunoblot analysis of vacuolar membranes (10 μg) purified from colored protoplasts of flower buds (bud, –24 h) and open petals (op, 0 h). The right-hand lane shows molecular sizes (kDa) of the standard proteins.

The antibody to the C-terminal sequence of NHX1 shows good reactivity with the NHX1 of *I. tricolor*. As shown in Fig. 3A, the peptide-specific polyclonal antibodies reacted with a 50 kDa protein in the vacuolar membranes prepared from epidermal cells of fully open petals, of which the vacuolar pH increased to pH 7.7. The immunostained band at 50 kDa was also detected in the crude membrane fraction from the pigmented portion of petals, but not in the membrane from leaves. The 50 kDa band was not detected with the antibodies pretreated with a corresponding antigen peptide (Fig. 3B), indicating the specificity of the antibody to NHX1. The 50 kDa protein was detected only in the blue rim part of flower petals and not in the tube part of petals, which does not contain anthocyanin (Fig. 3C). The other organs, such as stem, leaf and root, did not give any immunostained band (Fig. 3C). These results indicate the cell-specific existence of the NHX1 protein in the pigmented cells in flower petals. The distribution of NHX1 in *I. tricolor* was coincident with the tissue-specific expression of *InNHX1* in Japanese morning glory (Yamaguchi et al. 2001).

Stage-specific accumulation of NHX1, V-PPase and PM-ATPase protein in flower petals

Using specific antibodies, we determined the expression level of several membrane proteins including NHX1, V-ATPase, V-PPase and PM-ATPase in flower petals and examined changes in their protein levels during flower opening. Immunoblot analysis of the crude membrane fraction with the anti-NHX1 antibody revealed the stage-specific accumulation of the protein (Fig. 4A). We could not detect NHX1 in crude membranes obtained at –24 h to –6 h. The band of NHX1 was observed in crude membranes obtained at the fully open stage (0 h). The weakly immunostained band may be due to both the low content of the protein compared with proton pumps and low immunoreactivity of the peptide-specific antibodies. In contrast to NHX1, the subunits A and B of V-ATPase remained at a constant level during the flower opening process (Fig. 4A). It should be noted that the protein level of V-PPase was steady until – 6 h, then markedly increased at the fully open stage (0 h).

The protein level of PM-ATPase gradually increased as the flower opened (Fig. 4A). Therefore, we assayed the vanadate-sensitive, molybdate-insensitive, nitrate-insensitive and azide-insensitive plasma membrane H^+-ATPase activity of crude membrane fractions prepared from petals of different stage (–24 and 0 h). The activity increased from 84 ± 1 nmol min^{-1} (mg of protein)$^{-1}$ in the buds to 163 ± 1 (0 h) nmol min^{-1} (mg of protein)$^{-1}$ in the fully open petals. The 2-fold increment in the activity was consistent with the change in the protein level of the enzyme.

The difference in the protein level was more clearly observed in immunoblots of the vacuolar membranes purified from colored protoplasts (Fig. 4B). The level of NHX1 was the highest in the vacuolar membranes from open flowers. The crude membrane fraction of opening flower and vacuolar membrane of colored protoplasts from buds had only a trace amount of NHX1. These results indicate that NHX1 was expressed and accumulated stage-specifically. It should be noted that the petal color at –6 h was purplish red and the color change started at around –5 to –4 h. Therefore, in *I. tricolor*, the change of petal color to blue seemed to be synchronized with flower opening.

Vacuolar membrane localization of NHX1

At present, the vacuolar membrane localization of proteins cannot be identified from their primary sequences. Although the results of immunoblot analysis of the crude membrane fraction of petals and purified vacuolar membranes indicated vacuolar membrane localization of NHX1, it should be clarified by more direct evidence. Thus, we carried out subcellular fractionation and subsequent immunoblotting using antibodies to several membrane markers (Fig. 5). The fractions of NHX1 (nos.

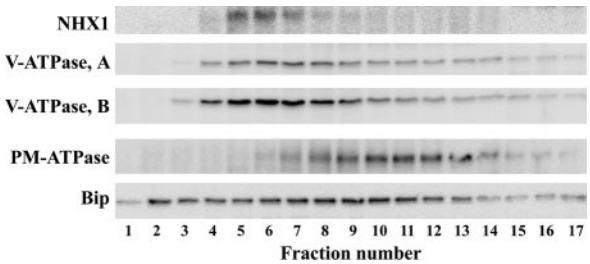

Fig. 5 Subcellular fractionation of NHX1 protein in flower petals. Crude membrane fraction of petals (0 h) was separated by sucrose density gradient centrifugation. The gradient was separated into 0.7 ml fractions from the top (no. 1) and then subjected to immunoblot with antibodies to NHX1, subunit A of V-ATPase, subunit B of V-ATPase, PM-ATPase and Bip.

5–7) were coincident with those of subunits A and B of V-ATPase, but not with that of PM-ATPase (nos 10–12) or an endoplasmic reticulum marker (Bip, nos 8–10). These results indicate that NHX1 is localized in the vacuolar membrane.

To confirm the localization of NHX1 further, we examined the flower petals by electron microscopic immunochemistry. Thin sections of petals from open flowers were treated with an antibody to NHX1 prior to treatment with colloidal gold linked to protein A. As shown in Fig. 6A, gold labeling for NHX1 was detected on the vacuolar membranes, which contained the subunit A of V-ATPase (Fig. 6B) and V-PPase (Fig. 6C), in the epidermal cells, where no gold particles were observed in other organelles such as the plasma membranes. Therefore, NHX1 protein was confirmed to localize at the vacuolar membrane. We could not observe the inner parenchyma of the petal which was easily injured and difficult to fix because of its structural weakness.

Activities of proton pumps and Na^+/H^+ exchanger in the vacuolar membranes

The acidic vacuolar pH value in general plants is maintained mainly by activities of proton pumps on the tonoplast. Other pumps, channels and transporters on the vacuolar and plasma membranes also work together to keep the ionic homeostasis of the cell. Thus, we first measured proton pump activities of vacuolar membrane vesicles prepared from colored protoplasts. The ATP and PP_i hydrolyzing activities were low in flower buds (–24 h) and increased in the fully open flowers (0 h) (Fig. 7A, B). The proton pump activity was measured as the rate of fluorescence quenching of the membrane-permeant amine dye, acridine orange. The ATP- and PP_i-dependent proton pump activities increased in the open flowers (Fig. 7C, D). The pH gradient generated by V-ATPase and V-PPase collapsed after adding membrane-permeable NH_4Cl, indicating the electrogenic proton pump activity. Theoretically, the rate of the fluorescence quenching depends on the internal volume of membrane vesicles even with the same activity of proton pumps. The sizes of the vacuolar membrane vesicles from buds

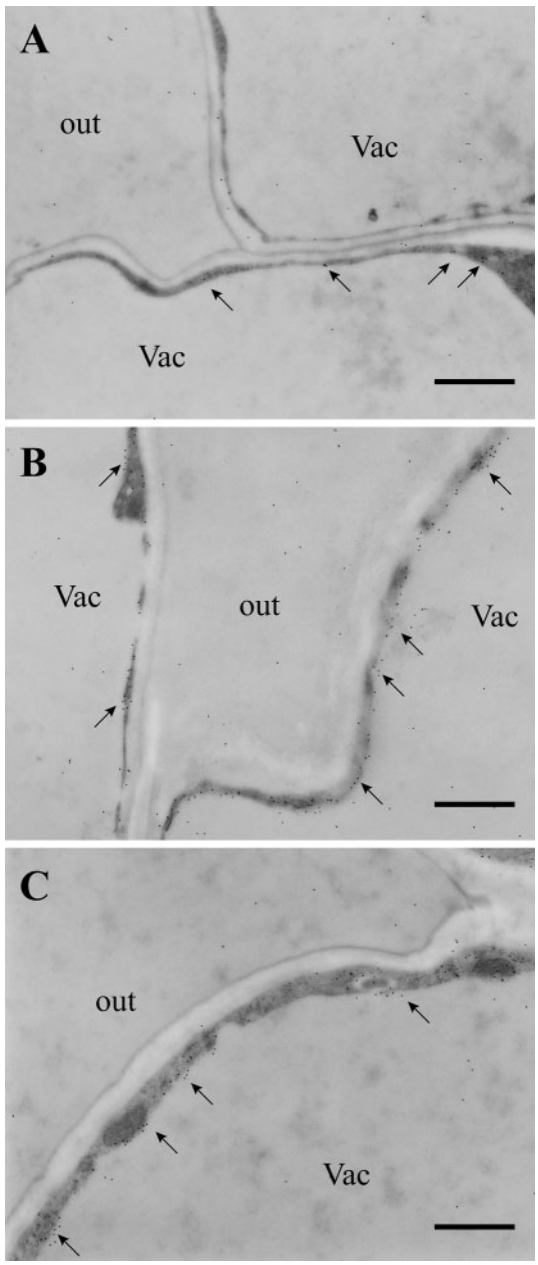

Fig. 6 Immunogold labeling of vacuolar NHX1, V-ATPase and V-PPase in flower petals. Thin sections of a petal from an open flower (0 h) were treated with each antibody and bound antibodies were visualized with protein A coupled to 15 nm of colloidal gold. Arrows indicate the corresponding antigen. Vac, vacuole; out, out of petal tissue. Bar = 1 μm. (A) NHX1. (B) V-ATPase subunit A. (C) V-PPase.

and open petals were measured by electron microscopy (Fig. 2E, F). There was no marked difference between the two preparations (mean diameter, 0.15 μm), indicating that the difference in the fluorescence quenching rates reflected the difference in the activity of V-ATPase and V-PPase.

We examined the Na^+/H^+ exchange activity of the vacuolar membrane vesicles from the colored protoplasts from buds

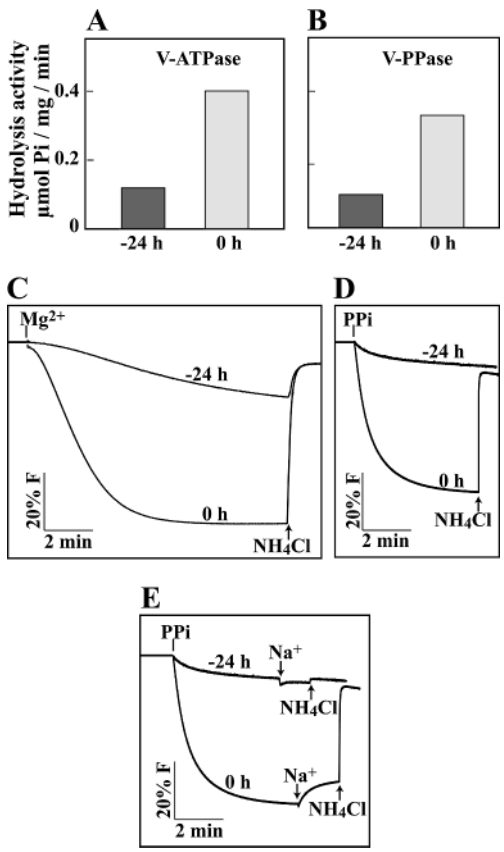

Fig. 7 Activities of proton pumps and Na^+/H^+ exchange in vacuolar membranes vesicles prepared from the epidermal colored cells of buds (–24 h) and open flowers (0 h). A sample (30 μg) was used for the proton pump assay. The pH gradient generated by proton pumps collapsed after adding NH_4Cl at a final concentration of 1.5 mM. (A) Substrate hydrolysis activities of V-ATPase. (B) Substrate hydrolysis activities of V-PPase. (C) The ATP-dependent proton pump activities. (D) The PPi-dependent proton pump activities. (E) The Na^+/H^+ exchange activity of bud (–24 h) and fully open petal (0 h) driven by V-PPase monitored by following the recovery of fluorescence of acridine orange after adding Na_2SO_4 at a final concentration of 50 mM.

(–24 h) and open flowers (0 h). By addition of 50 mM Na_2SO_4 to the suspension of membrane vesicles from open petals (0 h) pre-incubated with PP_i to generate a pH gradient, a marked fluorescent recovery was detected (Fig. 7E). This result indicates that NHX1 functions as a Na^+/H^+ exchanger in the vacuolar membrane of flower petals. On the other hand, the Na^+/H^+ exchange activity of tonoplast membrane vesicles of buds (–24 h) was hardly detected, corresponding to a lack of NHX1 protein. Sodium ions did not inhibit V-PPase even at 100 mM (data not shown). Therefore, the results shown in Fig. 7E certainly indicate the existence of a H^+ gradient-driven Na^+ antiport activity in the vacuolar membrane of the epidermis of fully open petals. The assay medium contained K^+ at 50 mM to activate V-PPase. Therefore, we could not demonstrate the activity of K^+/H^+ exchange, although both sodium and potassium ions are expected to be present in the colored cells in petals.

Discussion

In this study, we identified the NHX1 protein in the vacuolar membranes of anthocyanin-accumulating epidermal cells of morning glory *I. tricolor* and provided direct evidence that NHX1 was involved in the increase in the vacuolar pH in those cells. Yamaguchi et al. (2001) performed molecular genetic studies showing the contribution of the *InNHX1* gene to the change of petal color in *I. nil*. The change of petal color from red to blue in *I. tricolor* is due to the increase in the vacuolar pH from weakly acidic pH (6.6) to weakly alkaline pH (7.7) (Yoshida et al. 1995). The present study demonstrated a close relationship between the slight alkalization of vacuoles and the Na^+/H^+ antiport system.

Immunochemical studies using isolated vacuolar membranes from protoplasts, which contain a large amount of anthocyanin, provided clear evidence that NHX1 specifically exists only in the colored cells (Fig. 3). Furthermore, accompanied by the flower color change from red to blue during flower opening, the protein level of NHX1 increased markedly (Fig. 4). The findings on the vacuolar localization of NHX1 are: (i) vacuolar membranes prepared from isolated colored vacuoles contained NHX1 (Fig. 3); (ii) NHX1 showed the same profile as the subunits of V-ATPase in the subcellular fractionation (Fig. 5); and (iii) NHX1 was detected on the vacuolar membrane of petal epidermal cells by immunoelectron microscopy (Fig. 6). Thus, we concluded that NHX1 is accumulated in the vacuolar membrane of epidermal cells in flower petals at the late stage of flower opening. This cell- and stage-specific accumulation of NHX1 was closely related to the increase in the vacuolar pH followed by the color change of epidermal cells during flower opening.

The Na^+/H^+ exchange activity was detected in the vacuolar membranes from colored petal cells. Cation/H^+ exchangers have been found in various organisms. In plants, Na^+/H^+ exchangers have been investigated mostly in relation to salt tolerance in rice (Fukuda et al. 1998, Fukuda et al. 2004) and *Arabidopsis thaliana* (Zhang and Blumwald 2001, Zhang et al. 2001, Venema et al. 2002, Apse et al. 2003). However, the NHX1 homolog might play a specific role in flower petals. Detailed biochemical analysis of *A. thaliana* vacuolar Na^+/H^+ exchanger revealed that the exchanger transports both potassium and sodium ions, with the K_m for Na^+ and K^+ being 30 and 14 mM, respectively, and the V_{max} value being high for K^+ (Yamaguchi et al. 2003). Thus, NHX1 should be considered as an $Na^+(K^+)/H^+$ exchanger and we should take into account the role of both Na^+ and K^+ in petal cells. Through experiments using K^+-selective microelectrodes, Walker et al. (1996) studied the K^+ concentration of barley roots cells and found that the vacuolar K^+ concentration varied proportionally with tissue K^+ concentration, whereas cytosolic K^+ concentration remained constant over a wide range of tissue K^+ concentration. At a tissue K^+ concentration <100 mM, the cytosolic K^+ concentration was higher than the vacuolar K^+ concentration. If the K^+ con-

centration in the cytosol is higher than that in the vacuole, a K^+ gradient is formed across the vacuolar membranes. Under such conditions, the Na^+/H^+ exchanger can work as a potassium-driven K^+/H^+ exchanger. Therefore, NHX1 might function as a K^+-driven K^+/H^+ exchanger or Na^+-driven Na^+/H^+ exchanger in petal cells. As a result, protons in the vacuole must be released into the cytosolic space, resulting in a slight alkalization of vacuoles. Protons accumulated in the cytosol may be exported to the cell wall space by PM-ATPase, whose level of protein (Fig. 4A) and activity increased 2-fold during flower opening. Therefore, the vacuolar proton pumps, which were also increased, may modulate the vacuolar pH to a weakly alkaline condition. This is one working hypothesis to explain the present results. There is another possibility that vacuolar proton pumps are the main factors for alkalization to drive $Na^+(K^+)/H^+$ exchanger and incorporate Na^+ or K^+ actively. For example, K^+/H^+ exchangers coupled with V-ATPase function to increase the larval lepidopteran midgut to pH 11 (Wieczorek et al. 1991, Azuma et al. 1995, Harvey and Wieczorek 1997). To prove these hypotheses, we have to determine the concentrations of Na^+ and K^+ in both the cytosol and vacuoles in the colored petal cells.

The present study also revealed that V-ATPase was activated in the flower petals during flower opening. V-ATPase activity in cells and tissues is strictly controlled to regulate vacuolar pH and membrane potential values. Some possible mechanisms have been proposed to be involved in regulation of V-ATPase activity in vivo. Reversible disulfide bond formation between cysteine residues at the catalytic sites on subunit A causes reversible inhibition of V-ATPase activity (Feng and Forgac 1994, Oluwatosin and Kane 1997). Alteration of the V-ATPase activity by association of the glycolytic enzyme aldolase has been reported for yeast (Lu et al. 2004). Very recently, molecular interaction of V-ATPase and aldolase has been demonstrated in rice (Konishi et al. 2005). Further investigations are required to resolve the problem of which mechanism is involved in the activation of V-ATPase in flower petal cells.

In conclusion, we observed the tissue- and stage-specific accumulation of NHX1 in blue morning glory petals. We also clarified that the protein level and the activities of NHX1, V-ATPase, V-PPase and PM-ATPase increased toward the final flower opening stage. These proteins may function cooperatively to decrease the water potential of the vacuole, then the cell enlargement during flower opening process occur. Studies to determine the substrates for NHX1, K^+ and Na^+, in the flower and the integrative system for flower opening are in progress.

Materials and Methods

Plant materials

Seeds of *I. tricolor* cv. Heavenly Blue were purchased from Sakata Seed Co. Ltd. (Yokohama, Japan). Plants were cultivated in a phytotron, in a plastic greenhouse and on the University Farm. Plant materials for preparation of the vacuolar membrane were grown on soil. Plants for preparation of the crude membrane fraction were grown on vermiculite. Flowers and other organs at several stages were picked and immediately used for experiments.

Microphotometric observation of transverse section

Transverse section of fresh petals were prepared using a plant microtome (MT-3, Nihon Ikakikai, Tokyo, Japan) and observed with a microscope (BX50WI, Olympus, Tokyo, Japan) (Yoshida et al. 2003b, Yoshida et al. 2003c).

Electron microscopy of vacuolar membrane vesicles

Vacuolar membrane vesicles were prepared according to the general procedure for negative staining. A drop of sample solution was put on a carbon film grid. After partially drying, 2% uranyl acetate was applied. The solvent was removed with filter paper and the sample was observed by transmission electron microscopy (JEM-2000EX, JEOL, Akishima, Japan) at 100 kV.

Protoplast preparation from colored epidermal cells and vacuolar membrane isolation

The upper colored rim part (120 g) was cut from the petals of open flowers. The fresh tissue (20 g) was chopped with a razor blade and incubated in 200 ml of maceration medium containing 50 mM Tris–HCl, pH 7.0, 1% (w/v) Sumizyme C (Shin Nihon Chemical, Anjyo, Japan), 0.1% (w/v) Macerozyme R-200 (Yakult Honsha, Osaka, Japan) and 600 mM mannitol at 25°C for 100 min with gentle shaking. The tissue homogenate was filtered through a layer of nylon cloth (108 μm mesh) and then through a layer of Miracloth (EMD Biosciences, Darmstadt, Germany). After centrifugation of the filtrate at 400×*g* for 10 min, the precipitate portion containing protoplasts was gently collected, suspended in 50 mM Tris–HCl, pH 7.0 and 600 mM mannitol, and then centrifuged at 400×*g* for 10 min. The protoplasts were washed a further three times.

The protoplast suspension in a disruption medium was subjected twice to sonication with a Tomy sonicator (model UR-200, Tokyo, Japan) for 30 s and then centrifuged at 3,600×*g* for 10 min. The supernatant was centrifuged at 120,000×*g* for 40 min. The obtained precipitate was suspended in 20 mM Tris-acctatc, pH 7.5, 600 mM sucrose, 1 mM EGTA, 2 mM $MgCl_2$ and 2 mM dithiothreitol (DTT) and then poured into centrifugation tubes. Vacuolar membranes were isolated by the floating centrifugation method (Maeshima and Yoshida 1989). The obtained membranes were suspended in 20 mM Tris-acetate, pH 7.5, 20% (w/v) glycerol, 1 mM EGTA, 1 mM $MgCl_2$ and 2 mM DTT.

For preparation of protoplast from buds, the incubation reaction was carried out in 50 mM Tris–HCl, pH 6.5 and 650 mM mannitol solution.

Crude membrane preparation

All experimental procedures were carried out at 0–4°C. Flower buds expected to open 24, 12 and 6 h later were cut from plants. Flower petals that had just opened were also picked. In some experiments, flower petals were divided into the upper (blue rim) and lower parts (white tube). Cotyledons, hypocotyls and roots from 11-day-old seedlings, and leaves and stems from 33-day-old seedlings were used for membrane preparation. Tissues (5–17 g) were chilled, chopped with a razor blade in a homogenizing medium, and then homogenized in a mortar. The homogenizing medium comprised 50 mM Tris-acetate, pH 7.5, 250 mM sorbitol, 1 mM EGTA, 20 μM APMSF (*p*-amidinophenyl methanesulfonyl fluoride hydrochloride), 1% (w/v) polyvinylpyrrolidone and 2 mM DTT. The tissue homogenate was filtered through a layer of nylon cloth (108 μm mesh) and then through a layer of Miracloth (EMD Biosciences). The filtrate was centrifuged at 3,600×*g* for 10 min and the resulting supernatant was centrifuged at

120,000×*g* for 40 min. The precipitate was suspended in 20 mM Tris-acetate, pH 7.5, 20% (w/v) glycerol, 1 mM EGTA, 1 mM $MgCl_2$ and 2 mM DTT, and used as the crude membrane fraction.

Sucrose density gradient centrifugation

Crude membrane fraction was prepared from the blue rim part (30 g) of open flowers as described above, and then suspended in 20 mM Tris-acetate, pH 7.5, 5% (w/v) sucrose, 1 mM EGTA, 2 mM $MgCl_2$ and 2 mM DTT. The suspension (0.5 ml) was overlaid on 10.5 ml of a 15–40% (w/w) sucrose gradient in 30 mM Tricine-KOH, pH 7.5 and 1 mM EDTA, and centrifuged in an SW41-Ti rotor (Beckman, Palo Alto, CA, U.S.A.) at 100,000×*g* for 20 h. The gradient was separated into 0.7 ml fractions and then immunoblot analysis was performed with antibodies to marker proteins and NHX1.

Enzyme assay

Protein content was determined by the method of Bradford (1976) with a Bio-Rad protein assay kit. The PP_i hydrolysis activity of V-PPase (Maeshima and Yoshida 1989) and the ATP hydrolysis activity of V-ATPase (Matsuura-Endo et al. 1990) were measured as described previously. The basic assay medium (125 μl) for PP_i hydrolysis activity comprised 1 mM sodium PP_i, 10 mM $MgSO_4$, 50 mM KCl, 1 mM Na_2MoO_4, 30 mM Tris-MES, pH 7.2 and vacuolar membranes (4 μg of protein). The assay medium (125 μl) for ATP hydrolysis activity comprised 30 mM Tris-acetate, pH 7.2, 3 mM Na_2ATP, 3 mM $MgSO_4$, 50 mM KCl, 0.04% (w/v) Triton X-100 and 1 mM Na_2MoO_4. After the enzyme reaction, released Pi was measured. The activity of PM-ATPase (Ballesteros et al. 1998) was determined in 30 mM MES-Tris, pH 6.5, 3 mM Na_2ATP, 3 mM $MgSO_4$, 50 mM KCl, 1 mM Na_2MoO_4, 0.04% (w/v) Triton X-100, 100 mM KNO_3, 5 mM NaN_3 and with/without 200 μM Na_3VO_4. The vanadate-sensitive activity was calculated as that of plasma membrane H^+-ATPase.

The assay medium (2.0 ml) for proton pump activity of V-PPase comprised 5 mM Tris-MES, pH 7.2, 3 mM $MgSO_4$, 250 mM sorbitol, 0.5 mM DTT, 50 mM KCl, 0.50 μM acridine orange and 30 μg of vacuolar membranes (Maeshima and Yoshida 1989). After the addition of potassium PP_i at a final concentration of 1 mM, fluorescence quenching of acridine orange (excitation, 493 nm; emission, 540 nm) was measured at 20°C with a Jasco FP-6600 fluorescence spectrophotometer (Tokyo, Japan). The assay medium (2.0 ml) for proton pump activity of V-ATPase comprised 5 mM Tris-MES, pH 7.2, 3 mM Tris-ATP, 250 mM sorbitol, 200 mM KCl, 0.5 μM acridine orange and 30 μg of vacuolar membranes. The reaction was started by adding $MgSO_4$ at a final concentration of 3 mM. For recording NHX activity, 50 mM Na_2SO_4 was added to the assay medium which was pre-treated with potassium PP_i to generate a H^+ gradient, and fluorescence recovery (excitation, 493 nm; emission, 540 nm) was measured at 20°C.

Preparation and affinity purification of antibodies

For preparation of antibodies, a peptide corresponding to the C-terminal region of NHX1 (AB055062, Cys-VPFVAGSPVEQSPR) of morning glory (*I. nil*), which was previously cloned (Yamaguchi et al. 2001), was synthesized, linked with keyhole limpet hemocyanin, and then injected into rabbits. The obtained antiserum was subjected to affinity column chromatography to purify the NHX1-specific antibody. The antigen peptide was linked to agarose resin (Affi-Gel 102; Bio-Rad, Hercules, CA, U.S.A.) by using *trans*-4-(*N*-maleimidylmethyl)-cyclohexane1-caboxylate-*N*-hydroxysuccinimide ester. Antiserum was applied to a column of this resin (3 ml) pre-equilibrated with Tris-buffered saline (TBS; 150 mM NaCl, 20 mM Tris–HCl, pH 7.5) and then washed with 40 ml of TBS and 10 ml of 150 mM NaCl. The specific antibodies were eluted by 100 mM glycine-HCl, pH 2.5. The eluted fraction was immediately neutralized by addition of 1 M Tris and was used as anti-NHX1 antibody.

The peptide-specific antibodies to the subunit A and B of mung bean V-ATPase (Matsuura-Endo et al. 1992), V-PPase (Takasu et al. 1997) and PM-ATPase (Kobae et al. 2004) were prepared previously.

Immunoblotting

Proteins were separated by SDS–PAGE on 12.5% gels and transferred to a polyvinylidene difluoride membrane (Millipore, Billerica, U.S.A.) using a semi-dry blotting apparatus (Bio-Rad, Hercules, CA, U.S.A.). Immunoblotting was carried out using the obtained polyclonal antibodies, together with horseradish peroxidase-linked protein A and enhanced chemiluminescence (ECL) Western blotting detection reagents (Amersham Biosciences, Buckinghamshire, U.K.).

Immunoelectron microscopy

Open flower petals were vacuum infiltrated for 1 h in fixation mixture consisting of 4% paraformaldehyde, 1% glutaraldehyde and 60 mM sucrose in 50 mM cacodylate buffer, pH 7.4. The petals were then cut into slices <1 mm thick and treated for another 2 h with the freshly prepared fixation mixture. Samples washed with 50 mM cacodylate buffer, pH 7.4, were dehydrated in a graded dimethyl formamide series at –20°C and embedded in LR White acrylic resin (London Resin). Blocks were polymerized under a UV lamp at –20°C for 24 h. Ultrathin sections were prepared on a Reichert ultramicrotome and mounted on uncoated nickel grids. Immunocytochemical labeling with protein A–gold was performed as described previously (Nishimura et al. 1993). Antibodies to NHX1, V-PPase, the subunit A of V-ATPase and protein A–gold (Amersham) were used for analysis. Thin sections were examined with a JEOL 1200EX transmission electron microscope at 80 kV.

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