Synchrony between flower opening and petal-color change from red to blue in morning glory, \textit{Ipomoea tricolor} cv. Heavenly Blue

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Abstract: Petal color change in morning glory \textit{Ipomoea tricolor} cv. Heavenly Blue, from red to blue, during the flower-opening period is due to an unusual increase in vacuolar pH (pHv) from 6.6 to 7.7 in colored epidermal cells. We clarified that this pHv increase is involved in tonoplast-localized Na\textsuperscript{+}/H\textsuperscript{+} exchanger (NHX). However, the mechanism of pHv increase and the physiological role of NHX1 in petal cells have remained obscure. In this study, synchrony of petal-color change from red to blue, pHv increase, K\textsuperscript{+} accumulation, and cell expansion growth during flower-opening period were examined with special reference to ItNHX1. We concluded that ItNHX1 exchanges K\textsuperscript{+}, but not Na\textsuperscript{+}, with H\textsuperscript{+} to accumulate an ionic osmoticum in the vacuole, which is then followed by cell expansion growth. This function may lead to full opening of petals with a characteristic blue color.

Keywords: ItNHX1, \textit{Ipomoea tricolor} cv. Heavenly Blue, vacuolar pH increase, K\textsuperscript{+}/H\textsuperscript{+} exchange, cell expansion growth, flower opening

Introduction

Beautiful flower colors are mostly due to anthocyanins which change with the pH, like litmus, becoming blue under alkaline conditions and red when acidic \textit{in vitro}.\textsuperscript{1–4} Thus, the first proposed idea for flower color difference was pH theory by Willstätter that blue petal color was due to an alkaline cell sap.

...and the red was caused by an acidic cell condition.\textsuperscript{5} However, anthocyanins are dissolved in the central vacuoles of petal cells and vacuolar pH (pHv) is generally kept as weakly acidic, in order to maintain various secondary transport activities in the tonoplast\textsuperscript{6} and vacuolar functions.\textsuperscript{7} Therefore, with respect to blue flower color development, there has long been a controversy between pH theory\textsuperscript{5} and metal complex theory.\textsuperscript{8} Until now the metal complex theory was proven by X-ray crystallographic analysis of blue pigments from \textit{Commelina communis}\textsuperscript{9–11} and \textit{Centaurea cyanus}.\textsuperscript{10,12}

The petals of blue morning glory, \textit{Ipomoea tricolor} cv. Heavenly Blue is the first example that proved the flower color change by pH change. The petals exhibit sky-blue when full-opened, but in the bud stage the petal color is red (Fig. 1A), although the responsible pigment is the same heavenly blue anthocyanin, HBA (Fig. 1C),\textsuperscript{13} during all the flowering stages.\textsuperscript{14} The petal color change was in fact found to be due to an unusual increase of pHv from 6.6 to 7.7 in colored cells.\textsuperscript{14} Followed by this observation, we also found that this pHv increase was linked with up-regulation of the tonoplast-localized Na\textsuperscript{+}/H\textsuperscript{+} exchanger (ItNHX1).\textsuperscript{15}

In plants, the NHXs identified so far are almost...
all linked with salt tolerance.16–18) But in morning glory petals NHXs seemed to be express without relation to salts. Furthermore, a similar pHv increase, from 5.8 to 6.8, was observed during the flower-opening period in Japanese morning glory I. nil cv. Danjuro.19) Experiments using purple-mutant line of I. nil lacking InNHX1 suggested a close correlation between NHX protein expression and blue-petal color.20) These findings indicate that the pHv increase may be common in morning glory petals, although the physiological role of NHX in flower petals has not been clarified. Thus, we studied to determine function of pHv increase by ItNHX1 in morning glory petals. We analyzed petal color change from red to blue, pHv increase, change in ion contents, and cell expansion growth during flower-opening with a special reference to NHX1 expression. In late autumn we occasionally observed strange petals in which red parts existed in blue petals even at fully-opened stage. We studied cell features and expression of ItNHX1 protein of the red cells. We here report these results and discuss the role of ItNHX1 during the last period of flower-opening in morning glory petals.

Materials and methods

Plant materials and growth conditions. Seeds of Ipomoea tricolor cv. Heavenly Blue were purchased from Sakata Seed Co. Ltd. (Yokohama,
Plants were cultivated in an incubator, in a plastic greenhouse and at the Nagoya University Farm, as previously reported. For gene cloning and quantitative real time PCR, the colored rim part of petals was collected and immediately frozen with liquid nitrogen, and stored at −80°C until use.

**Transverse sectioning and calculation of cell volume.** Transverse sections of fresh petals were prepared as described previously. The cell volumes of colored adaxial cells were calculated assuming a cone shape.

**Cryo-SEM observation.** Cryo-scanning electron microscopy (cryo-SEM) was carried out with a HITACHI S-2300 apparatus (Hitachi, Hitachi, Japan). Petal tissue was set on a cryo stage, frozen with liquid N₂, and then fractured. The sample was observed by SEM at 3.0 kV.

**HPLC analysis of petal anthocyanin.** Red and blue rim portions of chimera-colored petals were collected and extracted with 50% acetonitrile (CH₃CN) containing 5% trifluoroacetic acid (TFA), at rt for 3 h. The extracts were analyzed by HPLC equipped with an ODS-column (Develosil ODS-HG-5 2.0 mm φ × 250 mm, Nomura Chemical, Seto, Japan).

**pHv measurement in chimera-colored petal cells.** pHv of red and blue epidermal cells in chimera-colored petals was measured using proton-selective microelectrodes in accordance with our previous report.

**Preparation of protoplasts from petals.** Colored protoplasts were prepared as previously reported with several modifications. Colored rim parts (ca. 2 g) were incubated in 30 ml of maceration medium at 27°C for 40 min. Maceration medium was composed of 20 mM MES-Tris, pH 6.5, 2% (w/v) Sumizyme C (Shin Nihon Chemical, Anjyo, Japan), 0.1% (w/v) Sumizyme MC (Shin Nihon Chemical, Anjyo, Japan), and 500 mM mannitol. The reaction mixture was then filtered through a layer of Miracloth (EMD Biosciences, Darmstadt, Germany) and centrifuged (320 × g for 5 min). The precipitated protoplasts were suspended in q. 500 mM mannitol, purified by centrifugation (320 × g for 5 min), and washed three times. The cell number in the suspension was counted using a Thoma cell counter plate (ERMA Inc., Tokyo, Japan). The average cell volume of the protoplasts was calculated from photographs by using ImageJ 1.36b software (NIH, Bethesda, USA).

**Preparation of protoplasts from chimera-colored petals and crude membrane fractions.** Using the above procedure with a 100 min incubation period, red and blue protoplasts were separately prepared from the rim part of chimera-colored petals. Crude membranes were isolated by the floating centrifugation method as previously described and suspended in 20 mM Tris-acetate, pH 7.5, 20% (w/v) glycerol, 1 mM EGTA, 1 mM MgCl₂, and 2 mM DTT.

**Microspectrophotometry of protoplasts.** The absorption spectra of colored protoplasts were recorded with a micro-spectrophotometer (MCPD-7000, Otsuka Electronics, Osaka, Japan). Ion analysis of colored cell sap by capillary electrophoresis. Precipitated protoplasts were suspended in water, mixed vigorously, and centrifuged (1,000 × g for 1 min). The supernatant was then analyzed with a capillary electrophoresis apparatus (CAPI-3200, Otsuka Electronics, Osaka, Japan), using a polyimide-coated fused-silica capillary (length, 80 cm; effective length, 67.5 cm; inner diameter, 75 µm; GL Science, Tokyo, Japan). Sample solutions were introduced under gravity (25 mm, 30 sec for cations; 25 mm, 60 sec for anions), and the temperature in the chamber was maintained at 25°C. UV absorption detection at 215 nm for cations and 230 nm for anions was utilized to obtain electrophorograms. For cation analysis, α-CFI-101O buffer (Otsuka Electronics, Osaka, Japan) was used as the mobile phase, and the applied voltage was 20 kV. For anion analysis, α-AFQ-105 buffer (Otsuka Electronics, Osaka, Japan) was used with an applied voltage of −20 kV. Under each condition, standard ion mixtures were analyzed, and the contents were determined from calibration curves. All samples were analyzed in triplicate.

**Salt treatment of protoplasts.** Protoplasts prepared from the red rim parts of bud petals (at −7.5 h) or the red part of chimera-colored petals (at 0 h) were suspended in solution (20 mM MES-Tris, 500 mM mannitol, pH 6.5) with the indicated salts. When incubated without salt (control), the concentration of mannitol was adjusted to 600 mM. The suspension was incubated at rt for 3 h. The cells were washed with 500 mM mannitol, three times before ion analysis.

**cDNA cloning of ItNHX1.** Gene cloning was generally performed using total RNA from morning glory bud petals (−12 h). cDNA containing
the complete ItNHX1 open reading frame was obtained by reverse transcription-PCR amplification using specific primers for the 5' and 3' regions of ItNHX1, a BamHI-site-containing sense primer, 5'-AAGGGATCCATGGCGTTCCAGTCATCTAGGGCTCTGCTGTCAG-3', and a PstI-site-containing sense primer, 5'-ATCTCTGCAAGTCATCTAGGGCTCTGCTGTCAG-3'.

Analysis of ItNHX1 gene expression. Quantitative real time PCR analysis was performed using an ABI PRISM 7300 and a SYBR Premix Ex Taq (Takara, Otsu, Japan). Total RNA (500 ng) from blue or red parts of chimera-colored petals were quantified using the following primers: ItNHX1-F (5'-CTATTATATCATTTGGTGCGGTC-3') and ItNHX1-R (5'-CCGTTGCAGCAATATTGCAATCAGC-3'); γ-subunit of mitochondrial F1F0 ATP synthase (γ-sub),23 γ-sub-F (5'-GGATGTCTGCCATCGACAGCAGTC-3') and γ-sub-R (5'-TCAGCCCTCAGATGTCTGCTC-3'). Thermal cycling conditions were 95°C for 10 s, followed by 40 cycles of 95°C for 5 s and 60°C for 31 s.

Immunoblot analysis. Antibodies specific to ItNHX1,15 V-PPase,24 PM-ATPase25 and to the α-subunit of mung bean V-ATPase26 were used. Immunoblotting of crude membranes (30 μg of protein) from colored protoplasts, prepared from either the blue or red part of chimera-colored petals was carried out using horseradish peroxidase-linked protein A and enhanced chemiluminescence (ECL) Western blotting detection reagent (GE Healthcare, Piscataway, USA), as previously described.15 Antigen levels on immunoblots were quantified from duplicate blotting experiments using an image analyzer (Bio-Rad, Hercules, USA).

Statistics. A statistical analysis between two groups was performed by use of Student’s t-test. Significant differences were accepted at P < 0.05.

Results

Changes in petal color, pHv and cell volume during flowering-period. The time-course of morning glory petal-color change during the flowering-period is shown in Fig. 1A. At −24 h, petal color is red. Blue color development starts around −6 h, and reaches completion after −3 h. Both adaxial and abaxial epidermal vacuoles are colored (Fig. 1B) due to the presence of a single anthocyanin, HBA,13 in solution (Fig. 1C). Adaxial cells have a typical conical shape, while abaxial cells are flat and it was clearly shown that cell expansion growth occurred during blooming (Fig. 1B, 1D).

We calculated cell volume on the adaxial side using photographs of transverse sections. In normal growing petals, the average cell volume at −24 h was 3.7 ± 0.9 pl, and the volume increased gradually in two separate phases, reaching 11.0 ± 3.2 pl at 0 h (Fig. 1E). In the first stage (−24 h to −6 h), a slow increase in cell volume was observed without cell color change, whereas during the second stage (−6 h to 0 h) the cell volume increase was more rapid and coupled with bluing. Because the cell color change from red to blue is solely dependent upon the change in pHv from acidic to alkaline conditions,14,15 the pHv of colored cells at each flowering stage can be estimated by the petal color. Prior to −12 h, the pHv of colored epidermal cells was weakly acidic, around 6.6; after −6 h, pHv increased rapidly to become weakly alkaline.

Ion content in colored epidermal cells. To clarify the changes in ionic state during flowering we analyzed ion species and content in colored epidermal cells. Colored protoplasts were prepared from petals at each flowering stage, and the content of cations and anions in the cell sap were quantified by capillary electrophoresis (Fig. 2). The most abundant cation in colored epidermal cells at each flowering stage was K⁺, and the K⁺ content gradually increased in two distinct phases while approaching the full-opening stage (Fig. 2A). In the first phase (−24 h to −6 h), the K⁺ content increased slowly from 146 to 218 fmol cell⁻¹. In the second stage (−6 h to 0 h), K⁺ content increased rapidly from 218 to 411 fmol cell⁻¹. The beginning of K⁺ uptake and cell color bluing were coincident. In contrast to K⁺ content, Mg²⁺ (< 11 fmol cell⁻¹) and Ca²⁺ (ca. 3 fmol cell⁻¹) content were maintained at low levels throughout the time period examined. At all stages, the Na⁺ content was below the detection limit (< 5 fmol cell⁻¹). Anions detected at −24 h were Cl⁻, PO₄³⁻, and malate, at levels of 32, 35, and 2 fmol cell⁻¹, respectively, and changes in these levels were very small over the entire period (Fig. 2B).

The profile of K⁺ concentration change was shown in Fig. 2C. From −24 h to −12 h, the K⁺ concentration decreased from 47 mM to 27 mM; at −3 h, it increased to 37 mM; and then it decreased once again. The K⁺ content increase from −6 h to
0 h may be correlated with expression of ItNHX1 and this may be coupled with cell-volume increase.

**Cellular features of red cells in chimera-colored petals.** In late autumn, *I. tricolor* cv. Heavenly Blue sometimes displays at 0 h, peculiar, chimera-colored petals with red sectors, parts, or spots in the blue-colored petal (Fig. 3A). The shape, size, and location of red parts in these petals vary with each particular flower and the appearance may be due to a response against cold stress. These red-colored parts always seemed to be shrunken, and the chimera-colored petals sometimes showed a deficiency in full flower-opening. Interestingly, at 0 h, transverse sections of chimera-colored petals neighboring the red-blue border exhibited a gradual cell color-change from red through purple to blue (Fig. 3C).
3B). Thus we analyzed the cellular features of those red cells.

The average volume of red cells was significantly smaller \((4.1 \pm 1.4 \text{ pl}; P < 0.001)\), even at 0 h, when compared to that of blue cells \((10.5 \pm 2.5 \text{ pl})\) (Fig. 3C). Thus, the cell expansion of red cells in chimera-colored petals appeared to be arrested at the bud stage. The average pHv of red cells, \(6.9 \pm 0.20\), was significantly lower, than that of blue cells, \(7.7 \pm 0.26\) \((P < 0.001)\) (Fig. 3D). But anthocyanin components in both blue and red parts analyzed by HPLC was the same (Fig. 3E). These results indicate a strong relationship between pHv increase (equal to cell bluing) and cell expansion growth in the chimera phenotype. The ion content of red and blue cells in chimera-colored petals at 0 h were analyzed. (Fig. 3F). The K\(^+\) content in red colored cells \((257 \pm 41 \text{ fmol cell}^{-1})\) was approximately 60% that of blue colored cells \((434 \pm 86 \text{ fmol cell}^{-1})\). Na\(^+\) was not detected in any cells examined. The content of other ions, Mg\(^{2+}\), Cl\(^-\), PO\(_4^{3-}\) and malate anion, were low and did not show significant variation with color. Thus, only K\(^+\) levels were found to be lower in red colored cells than in blue colored cells.

Salt treatment of colored epidermal cells in vitro. To monitor the cation/H\(^+\) exchange activity of ItNHX1 in living cells, red-colored protoplasts prepared from bud petals at \(-7.5\) h were treated with various mono- and divalent metal salts (Fig. 4). When the protoplast suspension was incubated in the absence of salt for 3 h, the cells showed no color change (Fig. 4A, Control). Addition of 50 mM KCl or NaCl to the medium caused an obvious bluing of the cells, similar color to that of protoplasts prepared from fully-opened petals at 0 h (Fig. 4A, 0 h petal cell). In contrast, cells treated with 50 mM LiCl, MgCl\(_2\) or CaCl\(_2\) remained red (Fig. 4A). Other metal ions, Al\(^{3+}\), Fe\(^{3+}\), Co\(^{2+}\) and Zn\(^{2+}\), also failed to affect cell-color bluing.

The ion content of protoplasts treated with alkali salts was quantified (Fig. 4B, 4C). In control protoplasts, the K\(^+\) content was \(133 \pm 14 \text{ fmol cell}^{-1}\), which increased to \(260 \pm 53 \text{ fmol cell}^{-1}\) after KCl-treatment for 3 h (Fig. 4B). In cells treated with NaCl for 3 h, the Na\(^+\) content dramatically increased to \(132 \pm 48 \text{ fmol cell}^{-1}\), while little change in K\(^+\) content \((158 \pm 32 \text{ fmol cell}^{-1})\) was observed (Fig. 4B). In contrast, the addition of LiCl did not induce cell color bluing, although the Li\(^+\) content increased to \(66 \pm 13 \text{ fmol cell}^{-1}\) (Fig. 4B). Regarding anions, a small increase in Cl\(^-\) (ca. 15 fmol cell\(^{-1}\)) was observed after treatment with any salt, but the content of PO\(_4^{3-}\) and malate anion was not appreciably changed (Fig. 4C).
Next, red protoplasts prepared from the red parts of chimera-colored petals at 0 h were incubated with KCl (Fig. 5). Only slight bluing occurred and corresponding increase in cellular K$^{+}$ content was only 30%, from 214 ± 39 to 279 ± 52 fmol cell$^{-1}$ (Fig. 5B). These results suggest that the red cells in chimera-colored petals are dysfunctional in K$^{+}$ uptake.

**Expression of ItNHX1 mRNA and gene product in red cells of chimera-colored petals.** To confirm the relationship between pHv increase by K$^{+}$ uptake and ItNHX1, ItNHX1 transcript levels in red-colored cells of chimera-colored petals were quantified. Sequencing of ItNHX1 cDNA cloned from *I. tricolor* petals revealed an open reading frame for a predicted protein of 542 amino acid residues and molecular size of 55 kD. The deduced ItNHX1 amino acid sequence is 98% identical to that of InNHX1. The relative *ItNHX1* mRNA level in normally colored petals was shown in Fig. 6A. *ItNHX1* mRNA expression was observed at −24 h, increased from −24 to 0 h, and then decreased. This expression profile is consistent with previously reported *ItNHX1* protein expression levels. On the other hand, relative *ItNHX1* mRNA expression levels in red- and blue-colored cells of chimera-colored flowers showed no significant difference (Fig. 6B).

Expression of the *ItNHX1* gene product in chimera-colored petals was determined immunchemically using crude membrane fractions prepared from red- or blue-colored cells of chimera-colored petals separately. The level of *ItNHX1* in red-colored cells was very low; only 14% that of blue-colored cells (Fig. 6C). Also, the level of PM-ATPase protein in red cells was approximately 56% that of blue cells. The levels of V-ATPase, V-PPase and Bip did not vary appreciably with cell color; relative levels in red cells to those in blue cells were 94%, 81% and 99%, respectively.

**Discussion**

**Substrate of *ItNHX1* in morning glory petal.** NHX1 has been thought to exchange Na$^{+}$ for H$^{+}$ and excrete toxic Na$^{+}$ from cytosol to apoplasts or to vacuoles to protect against salt stress. However, Na$^{+}$ was not detectable
in colored cells during any flower-opening stage as shown in Fig. 2A. Furthermore, K⁺ uptake (Fig 2A) was synchronized with the ItNHX1 expression level in normal blue petals. The strong correlation between K⁺ content in the cells and expression level of ItNHX1 was also confirmed in red cells of chimera petals (Fig. 3F, 6C). Thus, we conclude that ItNHX1 exchanges K⁺ for H⁺ in epidermal colored cells in morning glory petals.

However, some biochemical studies showed that both Na⁺ and K⁺ could be transported by NHX. This was confirmed by the results of salt-treatment experiments of red-bud cells at −7.5 h (Fig. 4). Treatment with KCl or NaCl produced blue cells (evidence for pHc increase) with an increase in cellular Na⁺ or K⁺ content (>120 fmol cell⁻¹), although anion content was not significantly increased (Cl⁻, 15 fmol cell⁻¹). These data suggest that Na⁺ and K⁺ may be incorporated into vacuoles by a cation/H⁺ exchange activity of ItNHX1, and not by functions of channels and/or co-transport systems. The reason why K⁺ is the substrate of ItNHX1 in vivo may be due to a very low tissue Na⁺ content. In pressed juice from petals, Na⁺ concentration was very low, below 2 mM, in contrast, K⁺ concentration was approximately 60–80 mM (unpublished).

**Role of NHX1 in morning glory petals as K⁺ influx system.** For flower-opening, deviation growth with cell expansion growth occurs, and accumulation of osmoticum into vacuoles is essential. In several flowers organic solutes plays the role. In petals of *I. tricolor* cv. Heavenly Blue, the colored epidermal cells grew to more than three times their original size during the last 24 h of flower-opening (Fig. 1B, 1E). This expansive cell growth may be caused by two different mechanisms. Cell enlargement during the first stage (−24 h to −12 h) without a change in cell color may be coordinated with the uptake of neutral organic compounds, since the K⁺ concentration was shown to decrease during this period. However, in the second stage (−6 h to 0 h) K⁺ may contribute as a major osmoticum for cell expansion growth. This was also suggested by the analysis of small red cells from chimera-colored petals with low K⁺ content (257 fmol cell⁻¹). Furthermore, when cell enlargement was inhibited, interference with petal-opening frequently occurred (Fig 3A). These phenomena suggest the importance of cell expansion growth in flower-opening.

In normal cells the following phenomena occurred synchronously with the expression of ItNHX1: cell-color bluing (Fig. 1B), pHc increase, K⁺ accumulation (Fig. 2A), and cell expansion growth (Fig. 1E). At the final flower stage (−6 h to 0 h), up-regulation of V-ATPase, and V-PPase was observed, presumably activating H⁺-pumps and thereby facilitating ItNHX1 K⁺/H⁺ exchanger activity. The simultaneous increase in PM-ATPase activity upon flower-opening contributes to H⁺ efflux out of cells and may drive K⁺ influx through hyperpolarization-activated voltage-gated K⁺ channels. Furthermore, this up-regulated PM-ATPase may also act in apoplast acidification, a process which may induce cell wall-loosening and the coupled uptake of solutes with water to facilitate cell expansion growth, similar to that of auxin-induced rapid growth.

**Mechanism of vacuolar alkalization of colored cells by ItNHX.** In the tonoplast, a number of channels, transporters and pumps work to regulate ion homeostasis. Therefore, pHc is determined by the ion balance in a dynamic equilibrium condition. We calculated whether such a high pHc level (7.7 at 0 h) could be maintained through the proton motive force (pmf)-driven ItNHX1 exchange of K⁺ with H⁺. Pmf (Δp) is represented by Eq. 1, where \( \mu_H \) is the electrochemical potential of H⁺, \( F \) is the Faraday constant, \( \Delta \Psi \) is electric potential difference across the tonoplast membrane, \( R \) is the gas constant, \( T \) is absolute temperature, and \( \Delta \dbar \text{H} \) is the difference of pH across the tonoplast membrane (ΔpH = pHv − pHc; pHc, cytosolic pH). At 300 K, Eq. 1 is written in Eq. 2, and ItNHX1 can function until the Δp in Eq. 2 becomes 0. At that time, the colored cell reaches dynamic equilibrium and the electric potential difference across the tonoplast is represented as follows (Eq. 3).

\[
\text{pmf} (\Delta p) = \Delta \mu_H / F = \Delta \Psi - 2.303RT \Delta \dbar \text{H} / F \tag{1}
\]
\[
\text{pmf}(\Delta p) = \Delta \Psi - 59 \Delta \dbar \text{H} \text{ (mV)} \tag{2}
\]
\[
\Delta \Psi = 59 \Delta \dbar \text{H} \text{ (mV)} \tag{3}
\]
\[
\Delta \Psi = 59 (7.7 - 7.2) = 30 \text{ mV} \tag{4}
\]

If the pHc value is around 7.2, a value common in plants, the vacuolar membrane potential \( (E_v) \) is +30 mV at equilibrium, where pHv = 7.7 (Eq. 4). Although it is very difficult to measure the \( E_v \) of morning glory cells, \( E_v \) values of other plants indi-
cated that +30 mV should be possible value; *Arabidopsis* root hair vacuoles: +22 mV, 41) *blue-colored* petal cells of Meconopsis grandis: +25 mV (unpublished). Thus, the pmf value for morning glory (+30 mV), as determined in Eq. 4, seems adequate to maintain pHv at 7.7 in petal cells, if tonoplasts were energized with H⁺ pumping by V-ATPase and V-PPase. In fact, V-ATPase and V-PPase were upregulated at the fully opened-flower stage. 15) Therefore, it is reasonable that ItNHX1 can exchange K⁺ in cytosol, which was incorporated from apoplast through some channels, with H⁺ in vacuoles under such high pHv conditions (Fig. 7). At 0 h HBA, a polyphenol pigment with a pKa about 7, 1) plays as a counter anion and dissociates in response to K⁺ accumulation to give an anhydrobase anion-form resulting in blue-colored flowers (Fig. 7).

In conclusion, tonoplast-localized ItNHX1 may act as a K⁺/H⁺ exchanger in morning glory petals cultivated under normal, salt-free conditions. The role of ItNHX1 appears, therefore, to involve the accumulation of ionic osmoticum during the final flower-opening stage (after −6 h) to achieve cell expansion growth with vacuolar alcalization, causing synchronized full-opening and the beautiful blue color which attracts pollinators. This integrated flower opening system does not appear to be particular to *I. tricolor* cv. Heavenly Blue, but rather is observed in general in morning glories. Recent genetic studies also indicated a potential contribution of the NHX family gene products to cell size. 42) The fact that NHX1 is involved not only in salt tolerance but also petal opening with color change is of clear interest in the context of plant evolution.

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The sequences reported in this paper have been deposited in the DNA Data Bank of Japan (accession nos. AB292774 and AB292775).

**References**

1) Goto, T. and Kondo, T. (1991) Structure and molecular stacking of anthocyanins—Flower color variation. Angew. Chem. Int. Ed. Engl. 30, 17–33.
2) Brouillard, R. and Dangles, O. (1994) Flavonoids and flower color. In The Flavonoids Advances in Research Since 1986 (ed. Harborne, J.B.). Chapman & Hall, London, pp. 565–588.

3) Andersen, O.M. and Monica, J. (2006) The anthocyanins. In Flavonoids, Chemistry, Biochemistry and Applications (eds. Anderson, O.M. and Markham, K.R.). CRC Press, Florida, pp. 471–511.

4) Yoshida, K., Mori, M. and Kondo, T. (2009) Blue Flower Color Development by Anthocyanins: from chemical structure to cell physiology. Nat. Prod. Rep. DOI: 10.1039/b800165k.

5) Willstätter, R. and Everest, A.E. (1913) Untersuchungen über die anthocyane; I. Über den farbstoff der kornblume. J. Liebig. Ann. Chem. 401, 189–232.

6) Martinoia, E., Maeshima, M. and Neuhaus, H.E. (2001) Tonoplast transmitters and their essential role in plant metabolism. J. Exp. Bot. 52, 83–102.

7) Maeshima, M. (2001) Structure determination of heavenly blue corn/C13ower pigment. Nature 415, 515–518.

8) Shibata, K., Shibata, Y. and Kashiwagi, I. (1919) Studies on anthocyanins: Color variation in anthocyanins. J. Am. Chem. Soc. 41, 208–220.

9) Kondo, T., Yoshida, K., Nakagawa, A., Kawai, T., Tamura, H. and Goto, T. (1992) Structural basis of blue-colour development in flower petals: Structure determination of commelinin from Commelina communis. Nature 358, 515–518.

10) Takeda, K. (2006) Blue metal complex pigments involved in blue flower color. Proc. Jpn. Acad., Ser. B 82, 142–154.

11) Shiono, M., Matsugaki, N. and Takeda, K. (2005) Structure of commelinin, a blue pigment from the blue flowers of Commelina communis. Proc. Jpn. Acad., Ser. B 84, 452–456.

12) Shiono, M., Matsugaki, N. and Takeda, K. (2005) Structure of the blue cornflower pigment. Nature 436, 791.

13) Kondo, T., Kawai, T., Tamura, H. and Goto, T. (1987) Structure determination of heavenly blue anthocyanin, a complex monomeric anthocyanin from the morning glory Ipomoea tricolor by means of the negative NOE method. Tetrahedron Lett. 28, 2273–2276.

14) Yoshida, K., Kondo, T., Okazaki, Y. and Katou, K. (1995) Cause of blue petal colour. Nature 373, 291.

15) Yoshida, K., Kawachi, M., Mori, M., Maeshima, M., Kondo, M., Nishimura, M. et al. (2005) The involvement of tonoplast proton pumps and Na⁺/K⁺ exchangers in the change of petal color during flower opening of morning glory, Ipomoea tricolor cv. Heavenly Blue. Plant Cell Physiol. 46, 407–415.

16) Apsé, M.P., Aharon, G.S., Suedden, W.A. and Blumwald, E. (1999) Salt tolerance conferred by overexpression of a vacuolar Na⁺/H⁺ antiporter in Arabidopsis. Science 285, 1250–1258.

17) Zhu, J-K. (2002) Salt and drought stress signal transduction in plants. Annu. Rev. Plant Biol. 53, 247–273.

18) Yamaguchi, T. and Blumwald, E. (2005) Developing salt-tolerant crop plants: challenges and opportunities. Trends Plant Sci. 10, 615–620.

19) Yoshida, K., Osanai, M. and Kondo, T. (2003) Mechanism of dusky reddish-brown “kaki” color development of Japanese morning glory, Ipomoca nil cv. Danjuro. Phytochemistry 63, 721–726.

20) Fukada-Tanaka, S., Inagaki, Y., Yamaguchi, T., Saito, N. and Iida, S. (2000) Colour-enhancing protein in blue petals. Nature 407, 581.

21) Yoshida, K., Kitahara, S., Ito, D. and Kondo, T. (2006) Ferric ions involved in the flower color development of the Himalayan blue poppy, Meconopsis grandis. Phytochemistry 67, 992–998.

22) Yamaguchi, T., Fukada-Tanaka, S., Inagaki, Y., Saito, N., Yonekura-Sakakibara, K., Tanaka, Y. et al. (2001) Genes encoding the vacuolar Na⁺/H⁺ exchanger and flower coloration. Plant Cell Physiol. 42, 451–461.

23) Ohnishi, M., Fukada-Tanaka, S., Hoshino, A., Takada, J., Inagaki, Y. and Iida, S. (2005) Characterization of a novel Na⁺/H⁺ antiporter gene InNHX2 and comparison of InNHX2 with InNHX1, which is responsible for blue flower coloration by increasing the vacuolar pH in the Japanese morning glory. Plant Cell Physiol. 46, 259–267.

24) Takasu, A., Nakamishi, Y., Yamauchi, T. and Maeshima, M. (1997) Analysis of the substrate binding site and carboxyl terminal region of vacuolar H⁺-pyrophosphatase of mung bean with peptide antibodies. J. Biochem. 122, 883–889.

25) Kobae, Y., Uemura, T., Sato, M.H., Ohnishi, M., Minura, T., Nakagawa, T. et al. (2004) Zinc transporter of Arabidopsis thaliana AtMTP1 is localized to vacuolar membranes and implicated in zinc homeostasis. Plant Cell Physiol. 45, 1749–1758.

26) Matsuura-Endo, C., Maeshima, M. and Yoshida, S. (1992) Mechanism of the decline in vacuolar H⁺-ATPase activity in mung bean hypocotyls during chilling. Plant Physiol. 100, 718–722.

27) Hasegawa, P.M., Bressan, R.A., Zhu, J-K. and Bohne, H.J. (2000) Plant cellular and molecular responses to high salinity. Annu. Rev. Plant Physiol. Plant Mol. Biol. 51, 463–499.

28) Zhang, H-X. and Blumwald, E. (2001) Transgenic salt-tolerant tomato plants accumulate salt in foliage but not in fruit. Nature Biotechnol. 19, 765–768.

29) Venema, K., Quintero, F.J., Pardo, J.M. and Donaire, J.P. (2002) The Arabidopsis Na⁺/H⁺ exchanger AtNHX1 catalyzes low affinity Na⁺ and K⁺ transport in reconstituted liposomes. J. Biol. Chem. 277, 2413–2418.

30) Yamaguchi, T., Ape, M.P., Shi, H. and Blumwald, E. (2003) Topological analysis of a plant vacuolar Na⁺/H⁺ antiporter reveals a luminal C terminus that regulates antiporter cation selectivity. Proc. Nat. Acad. Sci. 100, 12510–12515.

31) Collier, D.E. (1997) Changes in respiration, protein and carbohydrates of tulip tepals and Alstroemeria during development. J. Plant Physiol. 150, 446–451.

32) Bieleski, R., Elgar, J. and Heyes, J. (2000) Mechani-
cal aspects of rapid flower opening in Asiatic Lily. Annal. Botany 86, 1175–1183.
33) Vergauwen, R., Van den Ende, W. and Van Laere, A. (2000) The role of fructan in flowering of Campanula rapunculoides. J. Exp. Bot. 51, 1261–1266.
34) Okamoto, H., Ichino, K. and Katou, K. (1978) Radial electrogenic activity in the stem of Vigna sesquipedalis: involvement of spatially separate pumps. Plant Cell Environ. 1, 279–284.
35) Okamoto, H., Katou, K. and Ichino, K. (1979) Distribution of electric potential and ion transport in the hypocotyl of Vigna sesquipedalis VI. The dual structure of radial electrogenic activity. Plant Cell Physiol. 20, 103–114.
36) Bellando, M., Marre, M.T., Sacco, S., Talarico, A., Venegoni, A. and Marre, E. (1995) Transmembrane potential-mediated coupling between H+ pump operation and K+ fluxes in Elodea densa leaves hyperpolarized by fusicoccin, light or acid load. Plant Cell Environ. 18, 963–976.
37) Very, A-A. and Sentenac, H. (2003) Molecular mechanism and regulation of K+ transport in higher plants. Annu. Rev. Plant Biol. 54, 575–603.
38) Katou, K. and Furumoto, M. (1986) A mechanism of respiration-dependent water uptake enhanced by auxin. Protoplasma 133, 174–185.
39) Pardo, J.M., Cubero, B., Leidi, E.O. and Quintero, F.J. (2006) Alkali cation exchangers: roles in cellular homeostasis and stress tolerance. J. Exp. Bot. 57, 1181–1199.
40) Nicholls, D.G., and Ferguson, S.J. (2002) Quantitative bioenergetics: the measurement of driving forces. In Bioenergetics 3. Academic Press, London, pp. 31–54.
41) Lew, R.R. (2004) Osmotic effect on the electrical properties of Arabidopsis root hair vacuoles in situ. Plant Physiol. 134, 352–360.
42) Mäser, P., Thomine, S., Schroeder, J.I., Ward, J.M., Hirshi, K., Sze, H. et al. (2001) Phylogenetic relationships within cation transporter families of Arabidopsis. Plant Physiol. 126, 1646–1667.

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