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Rapid Paper

Genes Encoding the Vacuolar Na^+/H^+ Exchanger and Flower Coloration

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Vacuolar pH plays an important role in flower coloration: an increase in the vacuolar pH causes blueing of flower color. In the Japanese morning glory (*Ipomoea nil* or *Pharbitis nil*), a shift from reddish-purple buds to blue open flowers correlates with an increase in the vacuolar pH. We describe details of the characterization of a mutant that carries a recessive mutation in the *Purple* (*Pr*) gene encoding a vacuolar Na^+/H^+ exchanger termed InNHX1. The genome of *I. nil* carries one copy of the *Pr* (or *InNHX1*) gene and its pseudogene, and it showed functional complementation to the yeast *nhx1* mutation. The mutant of *I. nil*, called *purple* (*pr*), showed a partial increase in the vacuolar pH during flower-opening and its reddish-purple buds change into purple open flowers. The vacuolar pH in the purple open flowers of the mutant was significantly lower than that in the blue open flowers. The *InNHX1* gene is most abundantly expressed in the petals at around 12 h before flower-opening, accompanying the increase in the vacuolar pH for the blue flower coloration. No such massive expression was observed in the petunia flowers. Since the *NHX1* genes that promote the transport of Na^+ into the vacuoles have been regarded to be involved in salt tolerance by accumulating Na^+ in the vacuoles, we can add a new biological role for blue flower coloration in the Japanese morning glory by the vacuolar alkalization.

Key words: Blue flower coloration — Japanese morning glory (*Ipomoea nil*) — *NHX1* gene — Vacuolar Na^+/H^+ exchanger — Vacuolar pH.

Abbreviations: ANS, Anthocyanidin synthase; CHI, chalcone isomerase; CHS, chalcone synthase; DFR, dihydroflavonol 4-reductase; F3H, flavanone 3-hydroxylase; HBA, heavenly blue anthocyanin; *Pr*, *Purple*; *pr-m*, *purple-mutable*; *Pr-r*, *Purple-revertant*; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; RT, reverse transcription; UF3GT, UDP-glucose:flavonoid 3-*O*-glucosyl-transferase.

The nucleotide sequences reported in this paper have been submitted to DDBJ under accession numbers AB055062 (*InNHX1* from *Pr-r* plant), AB055063 (*InNHX1* pseudogene from *Pr-r* plant), AB054978 (*InACT4*), AB054979 (*ItNHX1*), AB051817 (*PhNHX1*), AB051819 (*ThNHX1*) and AB051818 (*NcNHX1*).

Introduction

The vacuolar pH plays an important role in the flower coloration (Davies and Schwinn 1997, Mol et al. 1998, Tanaka et al. 1998). In petunia (*Petunia hybrida*), seven loci affecting the vacuolar pH have been identified, and recessive mutations in these *Ph* loci display blueing of flower colors due to increased pH in the vacuole (de Vlaming et al. 1983, Chuck et al. 1993, van Houwelingen et al. 1998). In the morning glory, *Ipomoea tricolor*, reddish-purple buds change to blue open flowers, and the vacuolar pH in the epidermal cells of flower limbs increases from about 6.6 to 7.7 (Asen et al. 1977, Yoshida et al. 1995). A similar change in the flower color of the Japanese morning glory (*Ipomoea nil* or *Pharbitis nil*) also occurs, and the blue flowers of *I. tricolor* and *I. nil* contain the same anthocyanin named heavenly blue anthocyanin (HBA) as a major pigment (Goto and Kondo 1991, Lu et al. 1992).

Thanks to an extensive history of genetic studies in *I. nil*, a number of mutable loci conferring variegated flowers have been documented (Imai 1934, Iida et al. 1999). One of them named *purple-mutable* (*pr-m*) confers purple flowers with blue sectors (Fukada-Tanaka et al. 2000). The variegation is due to recurrent somatic mutation from the recessive *purple* to the blue revertant allele, *Purple-revertant* (*Pr-r*). To characterize the *Purple* (*Pr*) gene, we obtained germinal revertants producing blue flowers. Since such revertants are usually heterozygotes (*Pr-r/pr-m*), we selfed and chose pairs of siblings carrying either the *pr-m* or *Pr-r* allele homozygously for further characterization (Fig. 1). Subsequently, we were able to identify that the mutation *pr-m* is caused by integration of an *En/Spm*-related transposable element, *Tpn4*, into the *Pr* gene encoding a vacuolar Na^+/H^+ exchanger (Fukada-Tanaka et al. 2000, Fukada-Tanaka et al. 2001). The *Pr* gene comprises 15 exons, and *Tpn4* is integrated into the first untranslated exon. Thus, the *pr-m* and *Pr-r* lines are isogenic, and the difference between these lines must be the sequences within exon 1 where the *pr-m* and *Pr-r* plants carry the 12-kb *Tpn4* insertion and a footprint sequence (3 bp CAG insertion) generated by the excision of *Tpn4*, respectively.

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In this paper we describe details of the characterization of the *Pr* gene and discuss our observations with regard to the blue flower coloration. No alterations were detected in the anthocyanin pigment compositions between the *pr-m* and *Pr-r* lines and the vacuolar pH in *pr-m* was significantly lower than that in *Pr-r*. The isolated *Pr* gene was able to show functional complementation to a deletion mutation in the *NHX1* gene encoding a vacuolar Na^+/H^+ exchanger in yeast (*Saccharomyces cerevisiae*), indicating that the Pr gene product bears NHX1 activity. The *Pr* gene was most extensively expressed in the petals at around 12 h before flower-opening of *I. nil*, whereas no such massive expression was observed in the flowers of *P. hybrida*. Since reddish-purple buds change purple and blue open flowers in *pr-m* and *Pr-r* plants, respectively, the Pr gene products must be involved in a process for conversion from the purple to blue coloration. The NHX1 proteins have been shown to be important for salt tolerance and intracellular protein trafficking in yeast and plants (Nass et al. 1997, Apse et al. 1999, Fukuda et al. 1999, Gaxiola et al. 1999, Bowers et al. 2000, Darley et al. 2000, Quintero et al. 2000). We have thus added a new biological role of the NHX1 protein in blue flower coloration in the Japanese morning glory.

Materials and Methods

Plant material

The *pr-m* and *Pr-r* lines of *I. nil* were previously described (Fukada-Tanaka et al. 2000, Fukada-Tanaka et al. 2001). The *c-1* mutant 78WW*c-1* of *I. nil* displaying white flowers with red stems is thought to be deficient in a transcriptional regulator because the accumulation of mRNAs in the structural genes encoding the enzymes for the anthocyanin biosynthesis, *CHS-D*, *CHI*, *F3H*, *DFR-B*, *ANS* and *UF3GT*, is reduced in the flower buds of the *c-1* mutant (Abe et al. 1997, Fukada-Tanaka et al. 1997, Hoshino et al. 1997, Hoshino et al. 2001). The cultivar Heavenly Blue of *I. tricolor* displaying blue flowers and the *P. hybrida* varieties, cultivar Surfinia Purple Mini and its derivative Surfinia Violet Mini bearing reddish-purple and violet flowers, respectively, were previously described (Yoshida et al. 1995, Fukui et al. 1998). The other petunia varieties used were: the *ph* mutants MO59 (*ph1*), V26 (*ph1*), Pr57 (*ph2*) and Rw14 (*ph2*), cultivars Titan Red and Surfinia Light Blue Mini. *Torenia hybrida* cv. Summerwave Blue and *Nierembergia caerulea* cv. Fairy Bells were also used for cDNA isolation.

pH measurement

We employed the following three methods to estimate the vacuolar pH in the epidermal cells of flowers. (1) About 50 µl sap was prepared from the whole flower limbs in either pigmented buds or open flowers by centrifugation and measured with a Horiba B-212 twin pH meter (Kyoto, Japan). (2) The epidermal tissues of adaxial pigmented flower limbs were peeled off mechanically from three open flowers, and the pH of about 50 µl sap prepared by centrifugation was also measured with the same pH meter. We noticed that the color of the sap became gradually reddish when we peeled off the epidermis, and we assumed that the pH value obtained might be lower than the actual pH in the vacuole. We were unable to peel off the epidermal tissues from the pigmented flower buds. (3) The sap (30 µl) was diluted in 1 ml of 100 mM potassium phosphate buffer at various pH values and subjected to spectrophotometric analysis. The vacuolar pH was estimated by comparing absorption spectra of the sap in various pH solutions with reflective spectra of the intact limbs of open flowers which was performed as described by Yokoi and Saito (1973).

Southern and Northern blot hybridization

Preparation of DNAs and RNAs and their characterization by Southern and Northern blot hybridization were carried out as previously described (Hoshino et al. 1997, Fujiwara et al. 1998, Takahashi et al. 1999, Fukada-Tanaka et al. 2001). For poly$(A)^+$ RNAs preparation, total RNAs were first isolated with TRIZOL Reagent for total RNA isolation (Gibco BRL, Rockville, MD, U.S.A.) and poly$(A)^+$ RNAs were subsequently prepared with Dynabeads mRNA purification kit (DYNAL, Oslo, Norway). The cDNAs of the *DFR-B*, *ANS* and *Pr* genes used for probes were previously described (Inagaki et al. 1999, Fukada-Tanaka et al. 2000, Fukada-Tanaka et al. 2001, Hoshino et al. 2001), and the *Actin 4* cDNA of *I. nil* (*InACT4*) was provided by Y. Morita (accession number AB054978). The probe for the 5′ region of the *Pr* cDNA containing exons 1 and 2 (probe A) was prepared by PCR amplification with the primers PrEX1F1 (5′-AGAATGTAGGCTACAGA-3′) and PrEX2R2 (5′-AATTATAAGGGCAGTAATGGATTC-3′), and the probe for its 3′ region corresponding to exons 9 to 14 (probe B) was with the primers PrEX9F1 (5′-GCACTCAACCGATCGTGAGG-3′) and PrEX14R1 (5′-AACTGTGCTGAACAGAACAACCG-3′). For the positions of the primers and probes, see Fig. 5. The cycle of reactions were: initial denaturation at 95°C for 5 min, 30 cycles consisting of denaturation (95°C for 30 s), annealing (55°C for 30 s) and extension (72°C for 1 min), and then final extension at 72°C for 10 min.

Isolation of the Pr gene and its pseudogene from the Pr-r plant

Using the *Pr* cDNA as a probe, 40 positive λZAP Express (Stratagene, La Jolla, CA, U.S.A.) clones were isolated from about 1,000,000 recombinant plaques in an *Xba*I-digested genomic library from the *Pr-r* revertant plant as previously described (Habu et al. 1998, Fukada-Tanaka et al. 2000, Fukada-Tanaka et al. 2001). From these λ clones, four clones hybridized with both probes A and B, represented by λZExp:Pr-r1, were characterized further, and they carried approximately 7.5-kb *Xba*I fragment containing the *Pr* gene (see Fig. 5). We also characterized two clones hybridized with probe B but not with probe A, represented by λZExp:psPr-1, and they carried about 6.9-kb *Xba*I fragment containing the *Pr* pseudogene.

Isolation of the Pr gene homologs from floricultural plants

Using the *Pr* (*InNHX1*) cDNA as a probe, 14 *PhNHX1*, 7 *ThNHX1* and 12 *NcNHX1* clones were isolated from approximately 200,000 λZAPII (Stratagene) recombinant plaques in cDNA libraries prepared from petals of pigmented buds with emerging corolla of *Petunia hybrida* cv. Surfinia Purple Mini, *Torenia hybrida* cv. Summerwave Blue and *Nierembergia caerulea* cv. Fairy Bells, respectively.

To determine the entire *ItNHX1* cDNA sequence of *Ipomoea tricolor* cv. Heavenly Blue, we obtained two overlapping segments of about 1.3 kb containing 5′ and 3′ regions of the *ItNHX1* cDNA from the pigmented flower buds by RT-PCR amplification with appropriate primers. The 5′ *ItNHX1* cDNA segment was prepared by a 5′ RACE technique using a kit, 5′ RACE System for Rapid Amplification of cDNA Ends, version 2.0 (Gibco BRL) with the PrEX11R1 primer (5′-TCTGCGACAAATGACAGAGTTG-3′) (see Fig. 9). The 3′ *ItNHX1* cDNA segment was prepared by a 3′ RACE technique. The RT for synthesizing the first-strand cDNA was carried out with the oligo-dT adaptor primer PrdTadpr (5′-GCGGCTGAAGACGGCCTATGTGGCC$(T)_{17}$-3′) in 20 µl reaction mixture containing 2 µg total RNAs by using Omniscript RT Kit (Quiagen, Hilden, Germany). The subse-

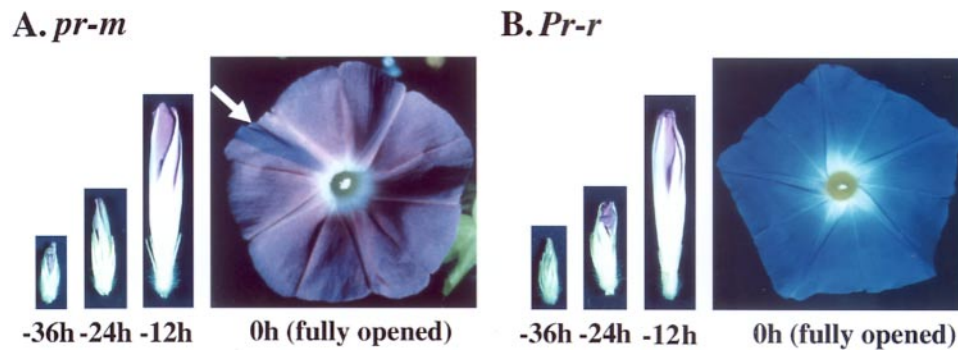


Fig. 1 Coloration of the flowers in the *pr-m* (A) and *Pr-r* (B) plants. The numerals below are the hours before flower-opening. The white arrow points to a blue sector owing to recurrent somatic mutation from the recessive *pr-m* to the blue *Pr-r* allele.

quent PCR amplification was performed with the primers PrdTadpr and PrEX5F1 (5′-TCAAGCACTTAGACATTGACTTTCTG-3′) in 40 μl reaction mixture containing 1 μl of the synthesized first-strand cDNA for initial denaturation at 94°C for 1 min, 32 cycles consisting of denaturation (94°C for 15 s), annealing (55°C for 30 s) and extension (72°C for 1 min). The resulting PCR-amplified fragments were cloned by pGEM-T Easy Vector (Promega, Madison, WI, U.S.A.). At least three different clones in each segment were independently sequenced, and the entire *ItNHX1* cDNA sequence was reconstructed by combining these two sequences.

Detection of scarcely expressed transcripts

To detect the scarcely expressed transcripts from the *Pr* gene in various tissues, we employed RT-PCR amplification with appropriate primers (see Fig. 9). The entire region of the *Pr* cDNAs was amplified with the primers PrEX1F2 (5′-CCATTTGTCTGAAGCTCTTCATC-3′) and PrEX15R2 (5′-CATAGAGCCAAATTGATAATTCAGC-3′) after RT reaction with Omniscript RT Kit (Quiagen). The PCR amplification was performed for 30 cycles in the same way as described above. Subsequently, nested PCR amplification of the *Pr* coding region was performed with the primers PrEX2F1 (5′-GTATGTTTTCG-GGAGGGATTGGAATGG-3′) and PrEX15R1 (5′-CTGCGGCCGCT-CATCTAGGGCTCTGCTCAACTGGT-3′), which contains the *Not*I adapter sequence (underlined) fused with the *Pr* stop codon, in the same way as the PCR amplification.

To examine whether transcripts were produced from the *Pr* pseudogene, we also used RT-PCR amplification followed by nested PCR amplification with appropriate primers. Since the region covering exon 6 to exon 8 of the *Pr* gene was deleted in the *Pr* pseudogene (see Fig. 5), 582- and 432-bp fragments are expected to be seen from the *Pr* gene and its pseudogene by RT-PCR amplification using the primers PrEX4F1 (5′-ATTCATGACAATTATGTTGTTTGGAGC-3′) and PrEX10R1 (5′-CTAGTAGTGACCCTTGAGCTC-3′), respectively. By nested RT-PCR amplification using the primers PrEX4F2 (5′-CTATT-GGCACACTTATTAGCTGTTC-3′) and PrEX9R1 (5′-AAGTAAGA-CATGAGCATCATAAG-3′), 293- and 143-bp fragments are produced from the 582- and 432-bp fragments, respectively.

Functional complementation in yeast

The *Pr* cDNA was fused with the *GAL1* promoter in pINA147 that is a derivative of the multi-copy plasmid vector pYES2 (Invitrogen, San Diego, CA, U.S.A.) carrying the *LEU2* gene of pJJ250 (Jones and Prakash 1990). The resultant plasmid pINA151 was introduced into the yeast strains K601 (*NHX1 leu2-3*) and R100 (*Δnhx1 leu2-3*) obtained from Dr R. Rao (Nass et al. 1997). To test the NaCl tolerance, the yeast cells were grown on APG medium (Nass et al. 1997, Gaxiola et al. 1999) adjusted to pH 4.0 by adding phosphoric acid. Although the APG medium used contains no galactose, the *GAL1* promoter in the high-copy vector appears to activate the *Pr* gene.

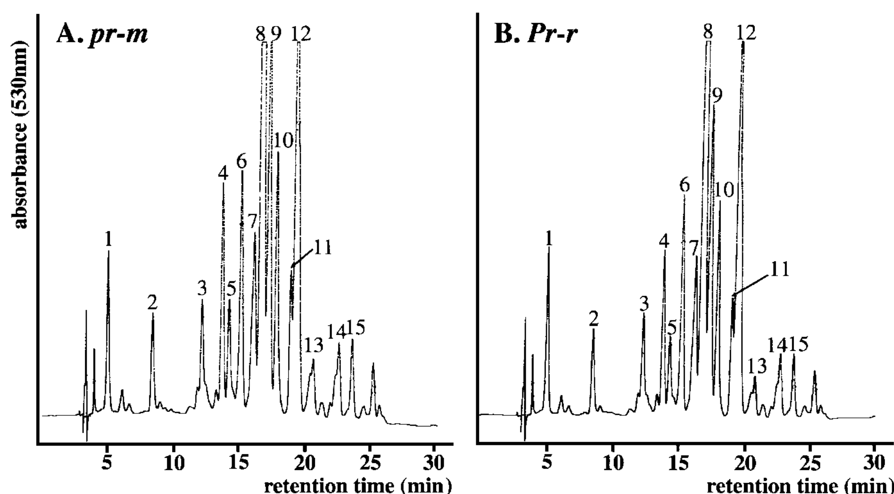
Results and Discussion

Flower coloration and anthocyanin pigment composition in the pr-m and Pr-r lines

In the *Pr-r* revertant plant, reddish-purple buds change to blue open flowers (Fig. 1), and the same change also occurs in the wild-type plant blooming blue open flowers (data not shown). The blue color in the open flowers is not very stable and turns gradually to purple and finally to reddish-purple in the wilted flowers. Although the color in the reddish-purple buds of the *pr-m* plant is indistinguishable from that of the *Pr-r* plant, the open flowers of the *pr-m* plant remain purple. HPLC analysis revealed that the open flowers of the *pr-m* and *Pr-r* plants contained several anthocyanin pigments previously identified in violet or blue flowers of *I. nil* (Lu et al. 1992) and that HBA, the most abundant pigment, accounts for about 28% of the total flower pigments in both lines (Fig. 2). No significant alterations were detected in the pigment compositions between the *pr-m* and *Pr-r* lines, indicating that the *pr* mutation affects the vacuolar pH associated with flower-opening.

Estimation of pH in the flowers of the pr-m and Pr-r lines

In *I. nil*, the pigmentation occurs mostly in the epidermal cells although the subepidermal cells are slightly pigmented, and a large portion of anthocyanin pigments are accumulated in the vacuoles of the epidermal cells (Imai 1934, Kihara 1934, Inagaki et al. 1996). Since the vacuolar pH in the extensively pigmented epidermal cells is known to be higher than that in the relatively colorless parenchyma cells of flowers in *I. tricolor* (Yoshida et al. 1995), we first attempted to obtain the sap from the pigmented adaxial epidermal cells and compared the pH from epidermis with the pH from whole flower limbs (Table 1). Although the pH values obtained from the epidermal



Composition of the pigments in the *pr-m* and *Pr-r* flowers (%)

pigments	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	total
pr-m	2.0	1.7	2.9	4.3	2.7	5.5	5.1	28.2	9.3	5.7	2.9	14.7	2.5	2.6	2.2	92.3
Pr-r	2.2	1.5	3.3	4.5	2.2	5.8	5.7	26.7	8.4	5.3	2.0	16.4	1.9	2.8	2.0	90.7

Fig. 2 HPLC analysis of the pigments in the *pr-m* (A) and *Pr-r* (B) flowers. The analysis was performed as previously described (Lu et al. 1992). The pigment 8 is HBA, and the other pigments identified are: pigment 1, peonidin 3-sophoroside-5-glucoside; pigment 2, peonidin 3-[2-(glucosyl)-6-(caffeoyl)-glucoside]-5-[glucoside]; pigment 3, peonidin 3-[2-(glucosyl)-6-(4-(glucosyl)-caffeoyl)-glucoside]-5-[glucoside]; pigment 6, peonidin 3-glucosylcaffeoylglucoside-5-glucoside; and pigment 7, peonidin 3-[2-(glucosylcaffeoylglucosyl)-6-(caffeoyl)-glucoside]-5-glucoside.

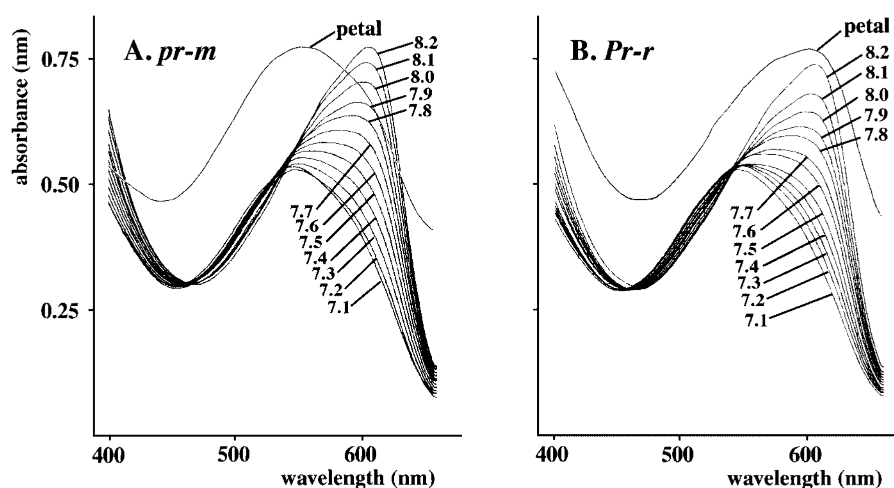


Fig. 3 Spectrophotometric estimation of the vacuolar pH in the *pr-m* (A) and *Pr-r* (B) flowers. The numerals indicate the pH values of the solutions for absorption spectra of the pigments in the sap. Petal indicates the reflective spectra of the intact limbs of open flowers.

cells fluctuated from experiment to experiment, they were always higher than the pH values in the sap prepared from the whole flower limbs. The pH value of the sap from the *Pr-r* flower epidermis was higher than that in *pr-m*, supporting our assumption that the *pr* mutant fails to increase the vacuolar pH. The saps prepared from the pigmented *pr-m* and *Pr-r* buds gave the same pH value, suggesting that the vacuolar pH values in the reddish-purple buds of these lines are identical.

We also tried to estimate the vacuolar pH in the open flowers of the *pr-m* and *Pr-r* plants by a spectrophotometric approach. The reflective spectra of the intact limbs were compared with the absorption spectra of the sap at various pH val-

Table 1 Summary of pH measurement

Plants	Flower stage	Sap from whole petal	Sap from epidermal tissue	Spectra
pr-m	Reddish-purple bud (–12 h)	5.6±0.1 (7)	–	–
	Purple open flower	5.9±0.1 (13)	6.4±0.1 (7)	7.5
Pr-r	Reddish-purple bud (–12 h)	5.6±0.1 (9)	–	–
	Blue open flower	6.2±0.1 (11)	7.0±0.1 (8)	8.0
HB	Reddish-purple bud (–12 h)	6.5±0.1 (10)	–	–
	Blue open flower	7.4±0.1 (4)	–	7.5

pH of sap measured with a pH meter is shown as mean ± standard deviation, and numerals in parentheses are the number of experiments. For the cultivar Heavenly Blue (HB) of *I. tricolor*, the vacuolar pH in the epidermal cells of reddish-purple buds and blue open flowers measured directly by inserting a double-barreled pH microelectrode has been reported to be 6.6±0.4 (22) and 7.7± 0.2 (26), respectively (Yoshida et al. 1995).

ues (Fig. 3). The shape of the spectra of the intact open flower matched closely the spectra of the sap at around pH 7.5 and pH 8.0 in the *pr-m* and *Pr-r* plants, respectively (Table 1).

The pH value obtained by measuring the sap in the flower epidermis appeared to be lower than the actual vacuolar pH probably because of the contamination of other components derived from cytosol and parenchyma. The pH value estimated from the spectra was higher than the observed pH value of the sap from the flower epidermis. To assess these different methods, we employed the cultivar Heavenly Blue of *I. tricolor*, because its vacuolar pH values in the epidermal cells of reddish-purple buds and blue open flowers were measured directly by inserting a double-barreled pH microelectrode (Yoshida et al. 1995). Interestingly, the difference in the pH values between the sap from whole petals and spectra in Heavenly Blue is much narrower than those in the *pr-m* and *Pr-r* plants, and these pH values in Heavenly Blue are close to the vacuolar pH in the epidermal cells measured by inserting a double-barreled pH microelectrode. The cause of wider differences in the pH values in *I. nil* obtained by the three methods than those in *I. tricolor* is currently unknown. In this respect, it would be interesting to measure the vacuolar pH values in the epidermal cells of *I. nil* with the pH microelectrode, because the method is regarded to be more reliable even though it is much more laborious than the procedures we used here.

The results in Table 1 indicate that the increase in the vacuolar pH for blue pigmentation in *I. nil* consists of at least two components, (1) from reddish-purple in buds to purple open flowers and (2) from the purple to blue flowers, and that the *Pr* gene is involved in the latter component.

The genome of I. nil contains the Pr gene and its pseudogene

Cloning of the *Pr* gene in the 7.5-kb *Xba*I fragment of the wild-type plant revealed that it comprises 15 exons, and *Tpn4* is integrated into its first untranslated exon (Fukada-Tanaka et al. 2000, Fukada-Tanaka et al. 2001). Close examination of the Southern hybridization patterns suggested that the genome of *I. nil* contains not only the *Pr* gene in the 7.5-kb *Xba*I fragment but also a second copy in a 6.9-kb *Xba*I band that is hybridizable to the 3′ region (probe B) but not to the 5′ region (probe A) containing the exon 1 and 2 sequences (Fig. 4). This notion was supported by the hybridization patterns in *Eco*RI and *Bst*XI digests (data not shown). We thus cloned both 7.5- and 6.9-kb *Xba*I fragments from the *Pr-r* plant and found that the 7.5-kb segment indeed carries the *Pr* gene of the *Pr-r* plant (accession number AB055062). Sequence comparison of the *Pr* genes between the *Pr-r* plant and the wild-type line previously sequenced (Fukada-Tanaka et al. 2000, accession number AB033990) revealed that 13 polymorphisms occurred in these *Pr* genes and that no alteration was detected in their coding regions (Fig. 5).

The *Pr* sequence in the other 6.9-kb *Xba*I fragment lacks exons 1, 2, 6, 7 and 8, and the 3′ part of the exon 11 sequence. The structural feature indicates that the second copy of the *Pr* gene is a non-functional pseudogene (accession number AB055063). To examine whether transcripts from the *Pr* pseudogene could be produced, we employed RT-PCR amplification followed by nested PCR amplification with appropriate primers to detect the amplified fragments covering exon 4 to exon 10 of the *Pr* gene where the *Pr* pseudogene has the region from exon 6 to exon 8 deleted (see Fig. 5). Although the patterns of the transcripts from the *Pr* gene in various tissues conformed exactly to those in Fig. 6, no mRNA transcribed from

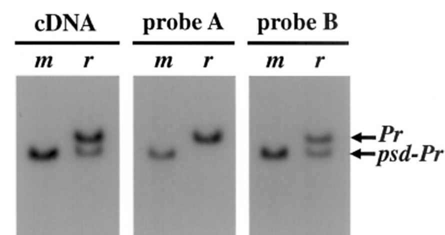


Fig. 4 Southern blot analysis of the *Pr* sequences. Genomic DNAs (10 μg) digested with *Xba*I were hybridized with the probes indicated (see Fig. 5). Symbols *m* and *r* indicate the plants *pr-m* and *Pr-r*, respectively. The arrows with *Pr* and *psd-Pr* point to the 7.5- and 6.9-kb *Xba*I fragments containing the *Pr* gene and its pseudogene, respectively.

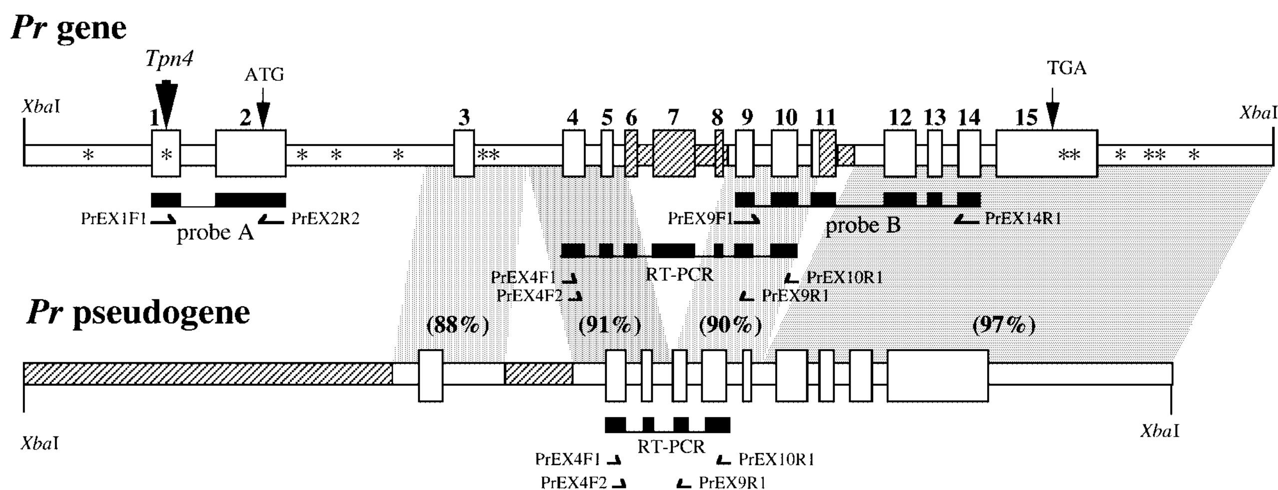


Fig. 5 Comparison of the genomic structures of the *Pr* gene and the pseudogene. The open boxes with the numerals indicate exons (e.g. 1 indicates exon 1). The open areas represent homologous regions between the two segments whereas the hatched parts indicate non-homologous regions. The small vertical arrows with ATG and TGA indicate the positions of the initiation and termination codons of the *Pr* gene, respectively. The large vertical arrow with *Tpn4* indicates the *Tpn4* insertion site, and the asterisks represent the positions where polymorphisms were found between the wild-type and *Pr-r* lines. Of 13 polymorphisms, 8 are single nucleotide polymorphisms, 4 are a few nucleotides polymorphisms including a footprint generated by the *Tpn4* excision, and the remaining one is a length difference in T-stretches. The small horizontal arrows below the map indicate the positions of the primers used. The nucleotide identity (%) between the *Pr* gene and the pseudogene is indicated in parentheses. The locations of probes A and B carrying the exon sequences for Southern blot analysis (Fig. 4) are indicated by the thick bars, and the positions of the primers for PCR amplification of the probes by the small horizontal arrows under the probes. The thick bars with RT-PCR represent the locations of the expected fragments to be amplified by RT-PCR amplification using appropriate primers indicated, in order to detect the production of the transcripts from the *Pr* gene or its pseudogene. The primers for subsequent nested PCR amplification are also shown below the RT-PCR bars.

the pseudogene could be detected in the tissues examined including the flower limbs (data not shown). We can thus conclude that all the *Pr* transcripts are derived from the single *Pr* gene previously characterized (Fukada-Tanaka et al. 2000, Fukada-Tanaka et al. 2001).

Spatial and temporal expression of the Pr gene

The 2.3-kb *Pr* transcripts were previously detected in pigmented flower buds of both wild-type and *Pr-r* plants (Fukada-Tanaka et al. 2000, Fukada-Tanaka et al. 2001). The expression of the *Pr* gene in various tissues of the *Pr-r* plant was examined by Northern blot hybridization (Fig. 6A). For the flower tissues, we used pigmented flower buds at 12 h before flower-opening when the maximum accumulation of the *Pr* transcripts in the flower limbs was observed (see Fig. 7). The *Pr* gene is most abundantly expressed in the flower limbs, moderately in the tubes and stamens, and weakly in the sepals and pistils (Fig. 6A). Both the 2.3-kb *Pr* mRNAs and the short transcripts of about 1.7 kb can be seen in these floral tissues. Although the structure of the short transcripts remains to be elucidated, we noticed that they could be detected with not only probe A but also probe B (data not shown).

No transcripts were detectable in leaves, stems and roots in Northern blot analysis. To examine whether the *Pr* gene is expressed at a low level in these tissues, we employed the RT-PCR amplification technique (Fig. 6B). Although no apparent amplified bands could be detected, clear bands can be seen in the subsequent nested PCR-amplification. We thus concluded that the *Pr* gene is expressed very scarcely, but significantly, in leaf, stem and root. This notion was supported by the observation that the 2.3-kb *Pr* transcripts were detectable by Northern blot hybridization in the poly$(A)^+$ RNA samples prepared from 2-week-old *Pr-r* seedlings with the first true leaf (Fig. 6C). No apparent increase in *Pr* mRNA level was observed after exposure of the *Pr-r* seedlings to 400 mM NaCl for 6 h.

We have also examined the temporal expression of the *Pr* gene in the flower limbs and found that the highest expression of the 2.3-kb *Pr* mRNA is at around 12 h before flower-opening (Fig. 7). This massive production of the *Pr* transcripts must be important for the blue flower coloration during blooming as shown in Fig. 1. The longer transcripts in the *pr-m* flowers, probably due to insertion of *Tpn4* into the *Pr* gene, were also seen at the same stage. Similar hybrid transcripts were previously characterized in the *DFR-B* gene having transposon *Tpn1* inserted (Takahashi et al. 1999). Fig. 7 also shows that the *Pr* mRNAs accumulate at 12 h before flower-opening whereas the transcripts of the structural genes for anthocyanin biosynthesis, represented by *DFR-B* and *ANS*, accumulate before the expression of the *Pr* gene. Similar patterns of the mRNA accumulation were also observed in the other structural genes,

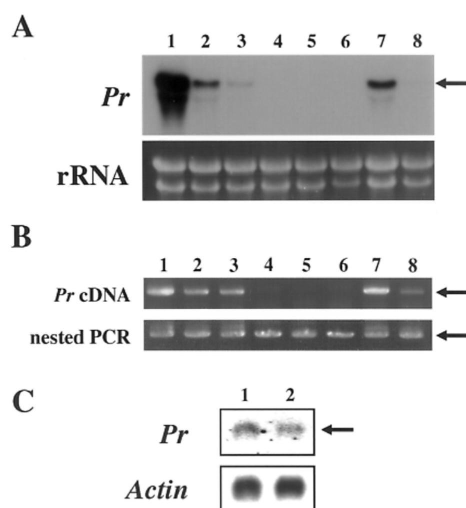


Fig. 6 Expression of the *Pr* gene in various tissues. (A) Northern blot analysis. Total RNAs (10 μg) prepared from flower limbs (1), flower tubes (2), sepals (3), leaves (4), stems (5), roots (6), stamens (7) and pistils (8) of the *Pr-r* line were subjected to Northern blot analysis using the *Pr* cDNA as a probe. The RNAs from floral tissues were obtained at 12 h before flower-opening. The arrow points to the 2.3-kb *Pr* transcripts. The ethidium bromide-stained rRNA bands are shown as a loading control. (B) RT-PCR analysis. RT-PCR amplification was performed with the primers PrEX1F2 and PrEX15R2, and subsequent nested PCR amplification was with the primers PrEX2F1 and PrEX15F1 (see Fig. 9). The lanes are as in (A). The arrows in RT-PCR amplification and nested PCR amplification point to the 2.2- and 1.7-kb amplified fragments, respectively. (C) Northern blot analysis. Poly(A)$^+$ RNAs (1 μg) obtained from 2-week-old seedlings of the *Pr-r* line (1) and the seedlings treated with 400 mM NaCl for 6 h (2). The bands of the *Actin 4* mRNA of *I. nil* are shown as a loading control. The arrow points to the 2.3-kb *Pr* transcripts.

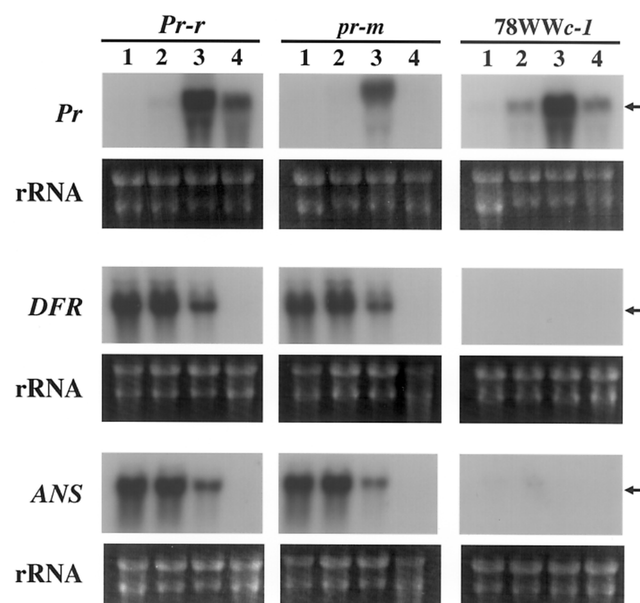


Fig. 7 Temporal expression of the *Pr* gene and its regulation. Total RNAs (10 μg) were prepared at 36 (1), 24 (2) and 12 h (3) before flower-opening, and at 0 h or fully opened (4) from the flower limbs of the *I. nil* lines indicated, and were subjected to Northern blot analysis using the probes indicated. The plant 78WW*c-1* is a *c-1* mutant displaying white flowers. The arrows point to the mRNAs examined. The ethidium bromide-stained rRNA bands are shown as a loading control.

CHS-D, *CHI*, *F3H* and *UF3GT* (data not shown). The results indicated that the *Pr* gene is controlled differently from the structural genes for anthocyanin biosynthesis.

The notion finds the support in the *c-1* mutant bearing white flowers with red stems, which is believed to be deficient in a transcriptional regulator necessary for the expression of the structural genes (Hoshino et al. 2001). In the *c-1* mutant, the *Pr* gene is expressed normally while the expression of the *DFR-B* and *ANS* genes was drastically reduced (Fig. 7). It is clear that the *Pr* gene is not controlled by the *C-1* regulator gene that regulates the structural genes for anthocyanin biosynthesis. It should be noted here that mutations in the *An1*, *An2* and *An11* genes for transcriptional regulators necessary for the expression of the structural genes for anthocyanin biosynthesis in petunia flowers affect the vacuolar pH of the flowers (Mol et al. 1998). The regulation of the *Pr* gene expression of *I. nil* remains to be elucidated.

Functional analysis of the Pr gene product

The 2.3-kb *Pr* cDNA cloned previously carries a 1,626-bp open reading frame for the Pr gene product (Fukada-Tanaka et al. 2000, Fukada-Tanaka et al. 2001), and its deduced amino acid sequence shows around 30 and 75% identities with ScNHX1 and AtNHX1, respectively (Nass et al. 1997, Apse et al. 1999, Gaxiola et al. 1999). Since the AtNHX1 protein is shown to suppress some defects in the yeast *nhx1* mutant (Gaxiola et al. 1999), we have tested whether the *Pr* gene is able to show functional complementation to the yeast *nhx1* mutation (Nass et al. 1997). Introduction of the plasmid with the *Pr* cDNA into the yeast *nhx1* mutant led to growth of the *nhx1* mutant in the presence of 400 mM NaCl whereas the plasmid without the *Pr* sequence failed to confer the NaCl tolerance (Fig. 8). Since the results strongly support the notion that the *Pr* gene encodes a vacuolar Na^+/H^+ exchanger, we can use the *InNHX1* gene for the *I. nil* NHX1 protein as a synonym for the *Pr* gene.

The Pr gene homologs in other floricultural plants

Like *I. tricolor* (Yoshida et al. 1995), we showed that raising the vacuolar pH in the flowers of *I. nil* is responsible for blue coloration and that the recessive *pr* mutation in the *InNHX1* gene fails to increase the vacuolar pH. Apart from the morning glories, petunia is the best studied plant concerning the flower coloration and the pH in the vacuole (Davies and Schwinn 1997, Mol et al. 1998). Phenotypes of the *ph* mutants affecting the vacuolar pH in the flowers of *P. hybrida* are in clear contrast to the phenotype of the *pr* mutant in *I. nil.* A

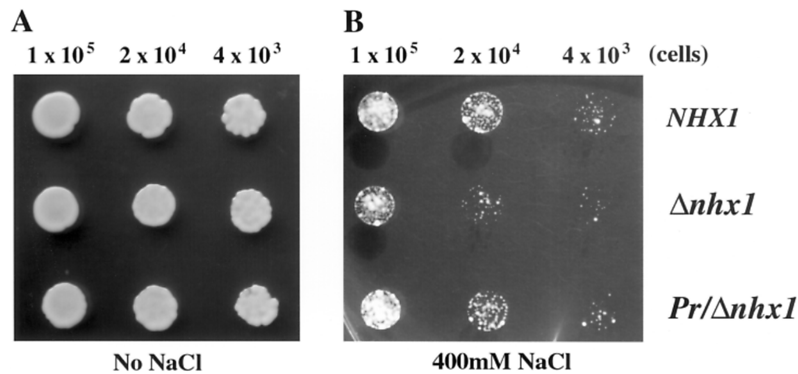


Fig. 8 Complementation of NaCl tolerance by the *Pr* gene in the *nhx1* yeast mutant. Serial dilutions (starting at 10^5 cells) of the yeast strains indicated were grown at 30°C for 3 days on APG medium without NaCl (A) or for 4 days on APG supplemented with 400 mM NaCl (B). *NHX1*, *Δnhx1* and *Pr/Δnhx1* indicate K601 carrying pINA147, R100 containing pINA147, and R100 harboring pINA151, respectively.

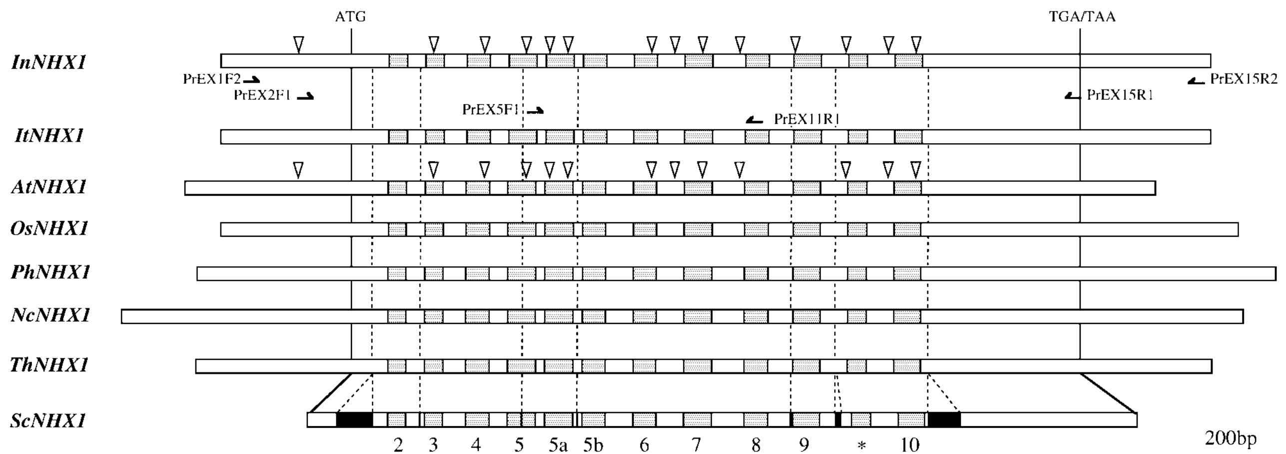


Fig. 9 Comparison of the cDNA structures of the NHX1 genes. The open boxes represent cDNA sequences available: *InNHX1* (Fukada-Tanaka et al. 2000), *ItNHX1* (this study, accession number AB054979), *AtNHX1* (Gaxiola et al. 1999, Darley et al. 2000), *OsNHX1* (Fukuda et al. 1999), *PhNHX1* (this study, accession number AB051817), *NcNHX1* (this study, accession number AB051818), *ThNHX1* (this study, accession number AB051819) and *ScNHX1* (Nass et al. 1997). The thin vertical lines with ATG and TGA/TAA indicate the positions of the initiation and termination codons, respectively. The black boxes and solid vertical bars within the *ScNHX1* cDNA box represent insertions, and the broken vertical lines indicate the corresponding positions in the other cDNAs. The shaded boxes with the numerals or the asterisk under the *ScNHX1* map indicate the regions encoding hydrophobic putative transmembrane domains previously described in *ScNHX1* (Nass et al. 1997, Wells and Rao 2001) and their corresponding regions in the plant *NHX1* sequences. The open arrowheads above the *InNHX1* and *AtNHX1* maps indicate the intron positions (Darley et al. 2000, Fukada-Tanaka et al. 2000). The small horizontal arrows at both ends under the *InNHX1* map indicate the positions of the primers for RT-PCR amplification and subsequent nested PCR amplification to detect production of the *Pr* transcripts in various tissues (Fig. 6B). The positions of the primers used for isolation of the segments of the *ItNHX1* cDNA are also indicated in the central region under the *InNHX1* map.

recessive mutation in one of the seven *Ph* loci fails to decrease pH in the vacuole, resulting in the blueing of flower colors (de Vlaming et al. 1983, Chuck et al. 1993, van Houwelingen et al. 1998). To examine whether the gene encoding a vacuolar Na^+/H^+ exchanger in *P. hybrida* is also expressed extensively in the flower limbs, we first cloned a petunia cDNA homologous to the *InNHX1* cDNA. In addition, the *InNHX1* cDNA homologs from *Torenia hybrida* and *Nierembergia caerulea* were isolated and their sequences were compared with the known *NHX1* cDNAs (Fig. 9). We have included the reconstructed cDNA sequence of *I. tricolor* in the comparison. Like *InNHX1* and *AtNHX1*, cDNAs of *I. tricolor*, *P. hybrida*, *T. hybrida* and *N. caerulea*, which we tentatively named *ItNHX1*, *PhNHX1*, *ThNHX1* and *NcNHX1* cDNAs, respectively, contain long 5′-untranslated regions. Both *InNHX1* and *AtNHX1* genes carry untranslated exon 1 sequences, and the positions of other introns are identical except for exons 11 and 12 of *InNHX1* are fused into one exon in *AtNHX1* (Darley et al. 2000, Fukada-Tanaka et al. 2000). The coding region of the yeast *ScNHX1* cDNA is longer than those of the plant cDNAs due to seven

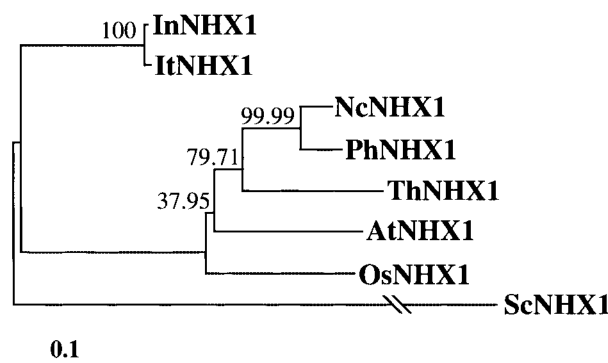


Fig. 10 A phylogenetic tree of the *NHX1* sequences. The tree was constructed using the neighbor-joining method (Saitou and Nei 1987) in GENETYX-MAC (SOFTWARE DEVELOPMENT, Tokyo, Japan). The numerals in the tree are bootstrap percentages. The relative branch of ScNHX1 is shortened by half.

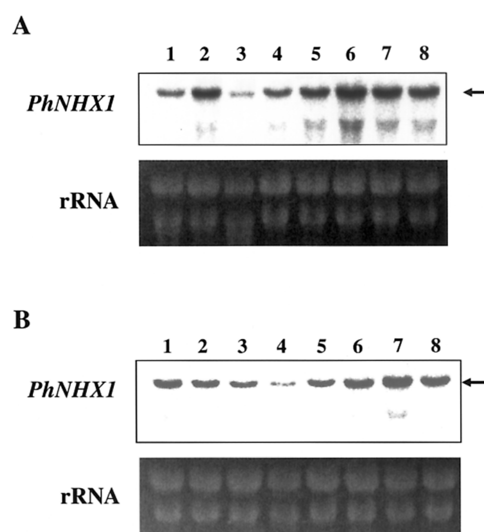


Fig. 11 Expression of the petunia *PhNHX1* gene. (A) Expression of the *PhNHX1* gene in various tissues. Total RNAs (10 µg) from *P. hybrida* cv. Surfinia Purple Mini were analyzed by Northern blot hybridization using the *PhNHX1* cDNA as a probe. RNAs used were: leaves (1), stems (2), sepals (3), petals of closed buds with first signed of pigmentation (4), petals of pigmented closed buds (5), petals of pigmented buds with emerging corolla (6), opened flowers with pre-dehiscent anthers (7) and opened flowers with all anthers dehisced (8). The arrow points to the 2.5-kb *PhNHX1* transcripts. The ethidium bromide-stained rRNA bands are shown as a loading control. (B) Expression of the *PhNHX1* gene in various petunia lines. Total RNAs (10 µg) from petals of pigmented buds with emerging corolla were subjected to Northern blot analysis using the *PhNHX1* cDNA as a probe. Plants used were: MO59 (*ph1*) (1), V26 (*ph1*) (2), Pr57 (*ph2*) (3), Rw14 (*ph2*) (4), Titan Red (5), Surfinia Purple Mini (6), Surfinia Violet Mini (7) and Surfinia Light Blue Mini (8). The arrow points to the 2.5-kb *PhNHX1* transcripts. The ethidium bromide-stained rRNA bands are shown as a loading control.

discrete insertions, and the longer 5′ terminal coding region of *ScNHX1* is reported to encode a signal peptide for membrane targeting (Darley et al. 2000). The amino acid sequences in the regions encoding hydrophobic putative transmembrane domains in all the predicted NHX1 proteins are relatively well conserved.

The deduced amino acid sequences of ItNHX1, AtNHX1, PhNHX1, NcNHX1, ThNHX1, OsNHX1 and ScNHX1 showed 92.7, 74.5, 76.4, 76.6, 73.6, 74.9 and 30.4% identity with InNHX1, respectively. A phylogenetic tree showed that the ScNHX1 gene product is distantly related to the plant proteins (Fig. 10). Nonetheless, both *AtNHX1* and *InNHX1* genes can suppress the salt-sensitive phenotypes of the yeast mutant having the *ScNHX1* gene deleted (Fig. 8, Gaxiola et al. 1999, Darley et al. 2000, Quintero et al. 2000).

Northern blot hybridization revealed that the *PhNHX1* gene is expressed in leaves, stems and flowers and that no drastic increase of the mRNA accumulation was observed during flower development (Fig. 11A). It is clear that the mode of expression of the *PhNHX1* gene is different from that of the *InNHX1* gene. Preliminary Southern blot analysis suggested that the genome of the petunia variety cv. Surfinia Purple Mini probably contains one or two copies of the *PhNHX1* gene (data not shown).

The accumulation of the *PhNHX1* transcripts at the beginning of the flower-opening stage was similar in several petunia cultivars examined including the *ph1* and *ph2* mutants (Fig. 11B). The vacuolar pH values of the open flowers in *P. hybrida* cv. Surfinia Purple Mini and Surfinia Violet Mini were reported to be around 5.2 and 5.6, respectively, and the recessive *ph1* and *ph2* mutants are known be higher than the wild type by about 0.5 unit of the pH value (de Vlaming et al. 1983, Fukui et al. 1998).

The biological roles of the NHX1 genes

The *NHX1* genes encode vacuolar Na^+/H^+ exchangers that catalyze the exchange of Na^+ for H^+ across the membrane in the vacuoles. In plants, the transport of Na^+ into the vacuoles is promoted by the NHX1 proteins that are energized by the H^+ gradient generated by the vacuolar H^+-ATPase and H^+-pyrophosphatase (Blumwald et al. 2000). The NHX1 protein is also involved in intracellular protein trafficking in yeast (Bowers et al. 2000). The *NHX1* genes were first isolated from yeast (Nass et al. 1997, Nass and Rao 1998) and subsequently obtained from Arabidopsis and rice (Apse et al. 1999, Fukuda et al. 1999, Gaxiola et al. 1999). These *NHX1* genes have been studied in the context of salt tolerance or responses to salinity stress. In plants, the *AtNHX1* and *OsNHX1* transcripts were reported to increase around 2–4-fold in response to the NaCl stress, and the induction appeared to occur more in leaves or shoots than in roots (Fukuda et al. 1999, Gaxiola et al. 1999, Quintero et al. 2000). However, Apse et al. (1999) could not detect such induction in wild-type Arabidopsis plants exposed to NaCl; even they were able to demonstrate that transgenic Arabidopsis plants overexpressing the *AtNHX1* gene were

shown to grow in the presence of 200 mM NaCl. Although no apparent increase in *InNHX1* mRNA level was observed after exposure of the *Pr-r* seedlings to 400 mM NaCl for 6 h (Fig. 6C), the induction of the *InNHX1* gene in response to NaCl stress could have occurred in some tissues under certain conditions of salinity stress.

Only one active copy of the *NHX1* gene is present in the genome of the Japanese morning glory, and the *InNHX1* gene is inactivated by insertion of *Tpn4* into the untranslated exon 1 sequence in the *pr* mutant (Fig. 5). Apart from the flower color, no apparent phenotype can be detected in the *pr* mutant, implying that the *InNHX1* gene is a nonessential gene for the plant growth. Alternatively, the *InNHX1* gene may be an essential gene but a residual expression of the *InNHX1* gene possibly from the inserted *Tpn4* element can be sufficient for sustaining the growth of the *pr* mutant since the *Pr* gene is expressed very scarcely in leaf, stem and root (Fig. 6). It would be interesting to ask whether the *InNHX1* gene is an essential gene or not by isolating an *InNHX1* mutant having an insertion into the *InNHX1* coding sequence.

We have shown here that the *NHX1* gene is employed for the vacuolar alkalization in epidermial cells of the open flowers in the Japanese morning glory, in order to display blue flower coloration presumably for attraction of pollinators (Harborne and Grayer 1994). The *InNHX1* gene appears to be expressed very scarcely, but significantly, in leaves, stems and roots (Fig. 6). The *InNHX1* gene is most abundantly expressed at 12 h before flower-opening in the limbs where the blue coloration occurs during flower-opening (Fig. 6, 7). This increase in the accumulation of the *InNHX1* transcripts appears to be much more drastic than the induction of the *AtNHX1* and *OsNHX1* mRNAs in response to NaCl stress (Fukuda et al. 1999, Gaxiola et al. 1999, Quintero et al. 2000). The transport of Na^+ into the vacuoles meditated by the NHX1 gene products should result in the vacuolar alkalization. The vacuolar pH in epidermial cells of the open blue flowers increases to near 8.0 (Table 1), and we do not know whether the Na^+ uptake into the vacuoles can be solely explained by the H^+ gradient generated by the vacuolar H^+-ATPase and H^+-pyrophosphatase. Alternatively, the Na^+ gradient formed by the accumulation of Na^+ in the cytosol generated by unknown mechanisms may drive the Na^+ transport into the vacuoles. Another puzzling fact is that the increase in the vacuolar pH for blue pigmentation consists of at least two components, (1) from reddish-purple in buds to purple open flowers and (2) from the purple to blue flowers (Table 1). The *InNHX1* gene is involved in the latter component and the molecular mechanism of the former component remains unknown. Not only Na^+ but also other cations may be involved in the vacuolar alkalization for the blue flower coloration.

Since the increase in the vacuolar pH occurs in the blue flowers of *I. tricolor* (Asen et al. 1977, Yoshida et al. 1995), the *ItNHX1* gene of *I. tricolor* is likely to play the same role. Indeed, we found that *ItNHX1* is also expressed predominantly in the flower limbs in the later stage of the flower buds (S. Fukada-Tanaka, unpublished). In petunia, however, no drastic increase of the *PhNHX1* transcripts was observed during flower development (Fig. 11A), indicating that not all flowering plants show abundant expression of the *NHX1* gene before blooming. The results in petunia may not be so surprising because the pH values of petal extracts in the wild-type *Ph* plants were reported to be about 5.3–5.5 whereas those in recessive *ph* mutants around 5.8–6.2 (de Vlaming et al. 1983, van Houwelingen et al. 1998), suggesting that the pH in the petunia vacuole is controlled to remain more acidic than that in the wild-type morning glory (Table 1). In addition, petunia transcriptional regulators for anthocyanin biosynthesis in flowers also control the vacuolar pH of flowers (Mol et al. 1998), whereas a mutation in the transcriptional regulator *C-1* does not affect the expression of the *InNHX1* gene (Fig. 7).

The coloration of blue flowers generally depends on the production of the suitable anthocyanin pigments, the presence of metal ions and co-pigments such as flavones and flavonols, and the pH in the vacuole (Davies and Schwinn 1997, Mol et al. 1998). While the majority of anthocyanin pigments found in the blue flowers are delphinidin derivatives, the blue pigment HBA of the morning glories is a cyanidin derivative (Goto and Kondo 1991, Lu et al. 1992). Since an increase of vacuolar pH causes a shift towards blue coloration, it is conceivable that the acquisition of the capability of the *NHX1* gene to be expressed abundantly before blooming is an important evolutionary step in the morning glories, which produce the cyanidin derivative HBA for blue flower coloration that attracts pollinators.

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