Identification and characterization of the *Pr* gene that is responsible for blue flower pigmentation in the Japanese morning glory.

Toshio Yamaguchi

Department of Molecular Biomechanics,
The Graduate University for Advanced Studies,
Okazaki, Aichi,444-8585,Japan and
Division of Gene Expression and Regulation I,
National Institute for Basic Biology,
Okazaki, Aichi, 444-8585, Japan

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Abbreviations

AMF; amplified restriction fragment length polymorphism-based mRNA fingerprinting
ANS; anthocyanidin synthase
AT; acyltransferase
CHI; chalcone isomerase
CHS; chalcone synthase
DFR; dihydroflavonol 4-reductase
F3H; flavanone 3-hydroxylase
F3'H; flavonoid 3'-hydroxylase
F3'5'H; flavonoid 3', 5'-hydroxylase
FLS; flavonol synthase
3GGT; UDP-glucose: flavonoid 3-*O*-glucoside glucosyltransferase
5GT; UDP-glucose: flavonoid 5-*O*-glucosyltransferase
HBA; heavenly blue anthocyanin
Mg; *magenta*
NHX1; vacuolar Na^+/H^+ exchanger protein
NHX1; vacuolar Na^+/H^+ exchanger gene
NOE; nuclear Overhauser effect
ORF; open reading frame
Pr; *purple*
Pr-r; *Pr-revertant*
pr-m; *pr-mutable*
Pr-w; *Pr* wild type
RT; reverse transcription
STDM; simplified transposon display method
Tpn; transposon in *Pharbitis nil*
TIR; terminal inverted repeat
UF3GT; UDP-glucose: flavonoid 3-*O*-glucosyltransferase
UTR; untranslated region

Introduction

Is producing "blue rose" just a dream? Since the rose employed as horticultural plant, many breeders have tried to produce the blue rose. However, there is no rose that blooms bright blue flower until now, 21 th century.

There are plenty of blue flowers in the world, however, horticulturists have had little success in producing blue varieties of flowers from normally blooms red or yellow flowers. The differences between blue and another colored flower had attracted not only breeders but also scientists, and as the consequence, the components and structures of the pigments had been revealed one after another. These accumulations of the findings made clear the relationships between coloration of the flowers and structure of the pigments to some extent. Furthermore, recent advancement of the molecular biology enabled us to identification of the genes that related with pigment biosynthesis, and advancement in biotechnology made route to producing novel colored flowers by using reverse-genetical techniques. Namely, now it is possible to produce blue flower by introducing the enzymes that are capable to produce the blue pigments into the normally red or yellow-flowered plants. Actually, blue variety of carnation was produced by introducing the gene that modifies the structure of the pigment toward blue (Tanaka *et al.* 1998). The worth of this work is not only exhibiting feasibility to employ the reverse-genetics into plant breeding, but also suggesting that producing the blue rose is not just a dreaming.

Anthocyanins and blue coloration

The pigments that are responsible for the blue color of the flower are anthocyanins. Anthocyanins are one of the flavonoid derivatives that are usually found as glucosylated form and sequestrated in plant vacuoles. Aglycones of anthocyanins are called as anthocyanidin and the number of hydroxyl groups on the anthocyanidin B ring defines their color variation to some extent (Fig. I-1, Goto and Kondo 1991). Most blue flowers contain delphinidins as a major aglycones, but there are several plants that contains cyanidins or peonidins as a major aglycones within blue flowers, indicating that hydoroxylation state of the anthocyanidin B ring is not an only factor to determine the flower color. pH of the solution also affects the color of the anthocyanins. Anthocyanins change their color sensitively in response to pH of the solutions, in strong acidic pH it appears orange to red, whereas in weakly acidic to neutral pH it appears

purple or violet, and neutral to alkaline, it appears blue to green. These color changes are due to the structural transformation of anthocyanin in response to the pH of solution (Fig. I-2, Brouillard and Dubois 1977a, 1977b, Chen and Hrazdina 1981, Goto and Kondo 1991). However, pH is not satisfactory explain the blue pigmentation of the flower because their colors are unstable and immediately disappear by hydration at neutral to alkaline pH in aqua, the usual range of the pH in the plant cells (Fig. I-2, Goto and Kondo 1991). Forming complex by chelating with metal like Mg^{2+} and Al^{3+} also have some effect to display blue color. However, chelating does not contribute for the stability of blue color of anthocyanin (Goto and Kondo 1991). Co-pigmentation has some effect to stabilize anthocyanins. Polyphenoles such as flavons and tannins have co-pigmentation effect which have long been attributed to hydrogen bonding between the host compound and the co-pigment used (Asen *et al.* 1972, Chen and Hrazdina 1981). Another stabilizing mechanisms were the self-association of anthocyanins (Asen *et al.* 1972), and intramolecular association (Goto *et al.* 1986) (Fig. I-3). These assumptions are based on the hydrophobic association between aromatic rings of anthocyanidin and other phenolic compounds or other anthocyanidins, and keep anthocyanidins from hydration.

The *Ipomoea tricolor* cv. Heavenly Blue is well-studied plant that has blue flowers. The blue pigment included in this cultivar is named as heavenly blue anthocyanin (HBA, Fig.I-4). HBA is complicated compound that is consisted by a peonidin, six molecule of glucoses, and three molecule of caffeic acids. HBA does not need chelation with Mg^{2+} and Al^{3+} to display blue flower (Yoshida *et al.* 1995), and NOE (nuclear Overhauser effect) data indicate that HBA keeps its stability by intramolecular association (Goto *et al.* 1986). Double-barreled pH microelectorodes study revealed that the vacuolar pH of the *I. tricolor* was weak alkaline (pH=7.7) (Yoshida *et al.* 1995). By keeping vacuolar pH at weak base and avoiding hydration with intramoleclar association, the Heavenly Blue displays its blue flower.

The 'blue' genes

Several genes that are important to display blue flowers, called 'blue' genes, had been characterized. The cytochrome P450 enzymes flavonoid 3'-hydroxylase (F3'H) and 3',5'-hydroxylase (F3'5'H) that catalyze hydroxylation on anthocyanin B ring are capable to produce more bluish pigments, cyanidin and delphinidin (Holton and Tanaka 1994). Introducing F3'5'H could successfully produce delphinidin in carnation, resulting in novel-colored carnation, bluish purple (Tanaka *et al.* 1998). The genes that

increase co-pigments such as tannins, certain flavone and flavonol glycoside also considered as 'blue' gene because co-pigmented anthocyanins are usually bluer than anthocyanins alone at a range of physiological pH values (Holton and Tanaka 1994). Flavonol synthase (FLS), that produce flavonol from dihydroflavonol (Fig. I-5, dihydrokaenpherol and dihydroquercetin) had applied to manipulate the flower color in *Petunia hybrida* (Holton *et al.* 1993). Glucosyltransferase and acyltransferases that is capable to produce polyacylated anthocyanins such as HBA and gentiodelphin (Goto and Kondo 1991), also susceptible to bluing flower due to its stablization effects. Vacuolar pH is also important to define flower color, thus the genes that affect vacuolar pH have the ability to be 'blue' genes. In *P. hybrida*, seven loci that affect vacuolar pH are defined as *ph* genes. Recessive mutations in these loci (*ph1-7*) cause shifting the flower color from red to blue due to failure in reducing vacuolar pH (Mol *et al.* 1998). Since the higher pH is capable of bluing of the flower, suppression of these genes may contribute to produce the novel blue flower. The genes that raise vacuolar pH can render the flower color to blue. However, no such genes are identified in the plant kingdom.

Blue coloration and *pr-mutable* allele in the Japanese morning glory

The Japanese morning glory, *Ipomoea nil* (or *Pharbitis nil*), is a traditional horticultural plant in Japan, and extensive physiological and genetical studies have been conducted (Yoneda 1990). A number of mutants related to the colors and shapes of its flowers and leaves have been isolated since the 17 th century in Japan, and more than 200 genetic loci have been localized for the 10 linkage group. They include several mutable loci that confer variegated flowers (Imai 1934).

The wild type *I. nil* displays blue flower and contains HBA as a major pigment in petals (Lu *et al.* 1992). Fig. I-5 shows the anthocyanin biosynthesis pathway assumed in *I. nil*. There are mutants that display white, pale yellow, red, magenta, and purple flower in *I. nil*. Some of them were characterized and revealed to have the mutation within the genes included in their anthocyanin biosynthesis pathway (Fig. I-5).

The blue petal color of *I. nil* flower was thought to be controlled by two genetic loci, *Purple* (*Pr*) and *Magenta* (*Mg*). When the *Pr* locus was mutated, their flowers display purple color, mutation occurs in the *Mg* locus confers magenta flowers, and mutations in both *Pr* and *Mg* loci confer red petals. Recent finding in our laboratory demonstrates the *Mg* gene encodes flavonoid 3'-hydroxylase (*F3'H*) that mediates hydroxylation at 3' of anthocyanidin B ring, results in producing red pigment,

pelargonidin (Fig. I-1), yet the function of the *Pr* gene is unknown.

The *pr* mutants are usually found as recessive and display mutable phenotype, named *purple-mutable* (*pr-m*). They confer purple flowers with blue sectors, the variegation is due to recurrent somatic mutation from the recessive *purple* to the *Purple-revertant* (*Pr-r*) allele. To date, seven mutable allele were characterized in *I. nil*, and found that the mutagens that were responsible for the variegated phenotype of them were *Tpn1*-related transposable elements in all case (Table I-1). Additionally, the stable recessive *duplicated* (*dp*) allele, that was found to be encoding MADS-box gene and confers their flower petals as duplicated form, carries the deleted form of *Tpn1*-related element, *Tpn-botan*. These evidences suggest that *Tpn1*-related elements may work as major mutagens in *I. nil*, not only in mutable plants, but also in stable mutants.

In this study, I describe details of the identification and characterization of the *Pr* gene and discuss the observations with regard to the blue flower coloration. Germinal revertant carrying *Pr-r* allele homozygously that displays blue flower were successfully obtained from *pr-m* line. No alteration were detected in the anthocyanin pigment compositions between the *pr-m* and *Pr-r* lines and that the vacuolar pH in the *pr-m* was significantly lower than that in *Pr-r*. By comparing the *pr-m* and *Pr-r* with simplified transposon display method (STDM), the *Pr* gene and also cDNA were successfully isolated. This gene was able to complement a deletion mutation in the *NHX1* gene for vacuolar Na^+/H^+ exchanger in yeast (*Saccharomyces cerevisiae*). The *Pr* gene was the most abundantly expressed at around 12 h before flower opening in the petals of *I. nil*, whereas no such increased expression was observed in the flower of *P. hybrida*. The concerning with the characteristic nature in expression and biological roles of the *Pr* gene were also discussed.

Materials and Methods

Plant materials

Three lines of the Japanese morning glory with blue flowers carrying the wild type *Pr* gene, KK/ZSK-2 (*Pr-w*, Inagaki *et al.* 1994), and three mutable *pr-m* lines bearing purple flowers with blue sectors are from the collections of our laboratory. From these *pr-m* lines, I obtained germinal revertants producing blue flowers. I selfed the mutants and revertants to obtain siblings with genotypes of *pr-m/pr-m* and *Pr-r/Pr-r*, and crossed the mutants and revertants to obtain *Pr-r/pr-m*. The heterogenotes (*Pr-r/pr-m*) and homogenotes (*Pr-r/Pr-r*) were assigned by the flower phenotypes of their selfed progeny. This revertant homogenote line was named as *Pr-r*, and their progeny was named as *pr-m*, respectively (Fig. 1).

The *c-1* mutant of *I. nil* displaying white flowers with red stems, 78ww*c-1* (Fig. 14B) is thought to be deficient in a transcriptional regulator because the accumulation of the mRNAs in the structural genes that included in anthocyanin biosynthesis pathway, *CHS-D*, *F3H*, *DFR-B*, *ANS* are reduced in the flower buds (Fukada-Tanaka *et al.* 1997, Hoshino *et al.* 1997, Abe *et al.* 1997).

The *P. hybrida* varieties cv. Surfinia Purple Mini and its derivative Surfinia Violet Mini bearing reddish purple and violet flowers, respectively, were described before (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.

HPLC analysis of the pigments of *pr-m* and *Pr-r* lines

The analysis of the pigments was performed according to Lu *et al.* (1992). Dried corolla (ca. 0.02 mg) of each cultivar were extracted with 20% MeOH contained 1.5% phosphoric acid. The quantitative analysis was performed on Waters C_{18} (46 ϕ x 250 mm) column at 40℃ with a flow rate of 1 ml/min monitoring at 530 nm for anthocyanins. Solvent systems used were as follows; a linear gradient elution for 30 min from 40 to 85% solvent B (1.5% H_3PO_4, 20% HOAc, 25% MeCN in H_2O) in solvent A (1.5% H_3PO_4 in H_2O).

Vacuolar pH estimation

To measuring the pH of epidermis homogenates, flower epidermis tissues were obtained by peeling mechanically from three flowers and pressed. After centrifugation (15,000 rpm. x 15 min), 50 μl of sap was measured with B-212 twin pH meter (Horiba).

To estimate the pH from the absorption spectrum, four flower petals was dissected and pressed. After centrifugation (15,000 rpm. x 15 min), 30 μl of supernatants were diluted with 1 ml of 100 mM potassium phosphate buffer series that adjusted at pH=7.1, 7.2, 7.3, 7.4, 7.5, 7.6, 7.7, 7.8, 7.9, 8.0, 8.1, 8.2. Intact flower petal was dissected and affixed to plastic cuvette directly. They were analyzed with scanning 660 to 400 nm by 557 spectrophotometer (Hitachi).

Simplified Transposon display Method (STDM)

Strategy for STDM was shown in Fig. 4. Genomic DNA was isolated from young leaves of the Japanese morning glory with Plant DNAZOL Reagent (GIBCO BRL). DNA (125 ng) was cleaved with *Mse*I (5 units) in 20 μl reaction mixture for 2 h at 37℃ and ligated to *Mse*I adapter (5'-GACGATGAGTCCTGAG-3' and 5'-TACTCAGGA CTCAT-3') by adding 3.5 μl of 25 μM *Mse*I adapter, 1 μl of 5 × ligation mix same as in Habu *et al.* (1997) and 175 units of T4 ligase (TaKaRa) for 2 h at 20℃. The reaction was stopped by incubating at 75℃ for 10 min.

The ligated DNA samples were 10-fold diluted with TE (10 mM Tris-HCl pH 7.5, 1 mM Na_2EDTA pH 8.0) and the pre-PCR amplification was performed in 20 μl reaction mixture containing 2 μl diluted ligated DNA, 240 nM TIR primer (5'-TGTGCA TTTTTCTTGTAGTG-3'), 240 nM *Mse*I primer (5'- GACGATGAGTCCTGAGTAA-3'), 200 μM dNTP, 1 unit of rTaq polymerase (TaKaRa) and 2 μl of 10 × PCR buffer (TaKaRa) for 20 cycles with the temperature profile: 30 sec at 94℃, 1 min at 56℃ and 1 min at 72℃.

The pre-PCR amplification reaction mixture is again 10-fold diluted with TE. To display bands in a sequencing gel, TIR+N primers (5'-TGTGCATTTTTCTTGTAGTGN -3' where N represents A, C, G or T) used were labeled at the 5' end either by rhodamine or fluorescein. The rhodamine-labeled primers were synthesized by TaKaRa Shuzo Co. Ltd., while fluorescein-labeling of the TIR+N primers were performed with Vistra fluorescence 5'-oligolabeling kit (Amersham Pharmacia Biotech). For the selective PCR amplification, either 120 nM rhodamine-labeled primers and 240 nM *Mse*I+N primer

(5'-GATGAGTCCTGAGTAAN-3' where N represents A, C, G or T) or 240 nM fluorescein-labeled TIR+N primer and 480 nM *Mse*I+N primer were used in 20 μl reaction mixture containing 2 μl of the diluted pre-PCR amplified DNA, 200 μM dNTP, 0.5 unit rTaq polymerase (TaKaRa) and 2 μl of 10× PCR buffer. The temperature profile for the selective PCR amplification is a touchdown protocol same as in Habu *et al.* (1997). The amplified DNA was precipitated by adding 20 μl of 2 mM $MgCl_2$ and 60 μl of EtOH. Either 5 μl (for rhodamine-labeled DNAs) or 3 μl (for the fluorescein-labeled DNAs) of the dye solution (83% (v/v) formamide, 4.2 mM Na_2EDTA pH 8.0 and 8.3 mg/ml blue dextran) was added to the precipitated samples, denatured at 90℃ for 3 min and quickly cooled to 4℃ and 1.5 μl of the mixture was loaded on a 5% Super Reading DNA Sequence Solution (Toyobo), 6 M urea sequencing gel. Electrophoresis was performed at 20 mA, 2500 V for 2 to 3 h, and bands in the gels were detected by FMBIO II Multi-View (TaKaRa) immediately after electrophoresis.

Cloning of amplified fragments obtained by STDM

A piece of the gel containing the band of interest was cut out and the DNA fragment was extracted and cloned as in Habu *et al.* (1997) with following modification. Re-amplification of the recovered fragment was performed in 20 μl of a reaction mixture containing 2 μl of the extracted DNA solution, 1 μM TIR primer, 1 μM *Mse*I primer, 250 μM dNTP, and 1 unit of rTaq polymerase (TaKaRa) in 1× PCR buffer. The amplified DNA fragment was cloned with a pGEM-T easy vector system TA cloning kit (Promega).

Southern and Northern blot analyses

For Southern blot analysis, 10 μg of genomic DNAs were digested with restriction enzymes, separated by 0.8 % (w/v) agarose gel electrophoresis and transferred to Hybond N membrane (Amersham Pharmacia Biotech). For Northern blot analysis, preparation of total RNAs from *I.nil* and Northern blot analysis were performed as described previously (Hoshino *et al.* 1997). In brief, 1.5 μg of mRNAs extracted from open flower petals were denatured, separated by 1.2% denatured agarose gel electrophoresis and transferred to a Hybond N membrane. The 130 bp DNA fragment obtained by STDM was labeled by PCR reaction (25 μl reaction containing TIR primer,

*Mse*I primer, 50 μM dATP, 50 μM dGTP, 50 μM dTTP, 5 μM dCTP, 1.85 MBq [α-^{32}P] dCTP, and 5 units rTaq (TaKaRa) in 1× PCR) for 30 cycles with the temperature profile; 30 sec at 94℃, 1 min at 56℃ and 1 min at 72℃. The hybridization was performed in a mixture of 5×SSC, 0.1% SDS, 5× Denhardt's, 20 μg/ml sonicated salmon sperm DNA and labeled probe at 60℃.

Cloning and sequencing of cDNA and genomic DNA

Using the 130 bp DNA fragment obtained by STDM as a probe, two positive λZapII (Stratagene) clones were isolated from approximately 6,000,000 recombinant plaques in a KK/ZSK-2 cDNA library (Inagaki *et al.* 1994) in the same way as described before (Habu *et al.*, 1997; Fukada-Tanaka *et al.*, 1997). One of them contained the entire open reading frame (ORF) for the *Pr* gene (accession No. AB033989).

Using the cDNA obtained that was labeled with Rediprime II DNA Labeling System (Amersham Pharmacia Biotech) as a probe, 58 positive λZAP Express (Stratagene) clones were isolated from about 4,000,000 recombinant plaques in an *Xba*I digested genomic library from the wild type KK/ZSK-2 plant in the same way as described before (Habu *et al.*, 1998). One of these λ clones λZExp:Pr-w1 carrying an approximately 7.5 kb fragment which contains all the exon region was cloned (accession No. AB033990). Using the same cDNA obtained as a probe, 12 positive λDASHII (Stratagene) clones were isolated from about 1,000,000 recombinant plaques in a *Sau*3AI partially digested genomic library from the mutable *pr-m* plant. One of these λ clones λDAII:pr-m1 containing a large insert was characterized further. DNA sequences were determined by the DNA sequencer ABI 377 (Applied Biosystems).

PCR amplification for examining the genotypes of the *Pr* gene

To examine the three different genotypes (*pr-m/pr-m, Pr-r/pr-m* and *Pr-r/Pr-r*) in the F2 progeny derived from hybrids between the revertant line *Pr-r* and the mutable line *pr-m*, genomic DNAs (50 ng) were subjected to PCR analysis using either one of the two primer sets: EX1FW (5'-GAAACAGAAAAAGAGAGAGTCACG-3') and EX2RV (5'-CAATGTCGTGGTTTCTGTTCACATA-3') or TIR and EX2RV (see Fig. 6D). The PCR amplification (50 ng of genomic DNA, 500 nM the primer EX2RV, 500

nM the primer EX1FW or TIR, 200 μM dNTP, 2.5 units rTaq polymerase (TaKaRa), in 1 × PCR buffer) was performed for 30 cycles with the temperature profile; 30 sec at 94℃, 1 min at 56℃ and 1 min at 72℃. After the reaction, a portion of the reaction mixture was analyzed on a 2% (w/v) agarose gel.

Southern blot analysis to determine copy number

Plant DNA was extracted from their leaves and purified by using Qiagen Genomic Tip (Qiagen) according to their protocol. General methods for Southern blot hybridization are described previously (Inagaki *et al.* 1994). For Southern blot hybridization, approximately 10 μg of DNA was digested with appropriate restriction enzyme and separated by 0.8% agarose gels. Washing conditions were as follows; for high stringency condition, final washing solution was 3 x SSC, 0.5% SDS and incubated for 45 min at 65℃, and for low stringency condition, same solution was used but incubation temperature was 42℃. The DNA probe of the *Pr* cDNA full length (2.2 kb) was described above. Probe A (corresponding to exon 1-2 of *Pr* cDNA, 470 bp) and probe B (exon 9-14, 555 bp) were generated by PCR amplification with *Pr* cDNA as a template and following primer sets; PrEX1F1 (5'- AGAATGTAGGCTACAGA-3') and PrEX2R2 (AATTATAAGGGCAGTAATGGATTC -3') were used for generating probe A, and PrEX9F1 (5'- GCACTCAACCGATCGTGA GG-3') and PrEX14R1 (5'- AACTGTGCTGAACAGAACAACCG-3') were used for generating probe B. The cycle of reaction were; initial denaturation (95℃ for 5 min), 30 cycles consisting of denaturation (95 ℃ for 30 sec), annealing (55℃ for 30 sec), extension (72℃ for 1 min) , and then final extension at 72℃ for 10 min.

Genomic cloning of the *Pr* homologue

General methods for DNA cloning were according to Sambrook *et al* (1989). Approximately 50 μg of *Pr-r* genomic DNA was completely digested with *Xba*I and fractionated by sucrose density gradient centrifugation. Collected the fraction containing 6-9 kb fragments, and cloned into λZAP Express cloning vector (Stratagene) according to manufacture's protocol. Using ^{32}P-labeled probe A, about 1,000,000 recombinant plaques were screened and 40 positive λZAP Express clones were isolated. From these λ clones, 4 clones that hybridized with both probe A and probe B, represented by

λZExp:Pr-r1, and 2 clones that hybridized with probe B but not with probe A, represented by λZExp:psPr-1, were characterized further. The clones λZExp:Pr-r1 and λZExp:psPr-1 carried approximately 7.5 kb and 6.9 kb *Xba*I fragments, and their nucleotide sequence were analyzed with ABI 377 (Applied Biosystems).

Functional complementation in yeast

The *Pr* cDNA was fused with the *GAL1* promoter in pINA147 which is a derivative of the multi-copy plasmid vector pYES2 (Invitrogen) 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 arginine. Although the APG medium used contains no galactose, the *GAL1* promoter in the high-copy vector appears to activate the *Pr* gene.

Northern blot analysis

Preparation of total RNAs from *I.nil* and Northern blot analysis were performed as described above. 10 μg of total RNA was applied for each lane and separated with 1.0 % agarose gel. The DNA probe of *I. nil Pr* was described above, and *CHS-D*, *F3H*, *DFR-B* were described previously (Fukda-Tanaka *et al.* 1997, Hoshino *et al.* 1997, Takahashi *et al.* 1999), and *I. nil ANS*, *CHI*, and *UF3GT* were amplified by RT-PCR method (unpublished) and γ-subunit cDNA of the sweet potato mitochondrial F1F0 ATP synthase was described elsewhere (Hoshino *et al.* 1997). I defined 0 h for opened flower as around 8:30 AM in summer, Okazaki, Japan, when the time that flower of *I. nil* completely opened and displays bright blue flower.

RT-PCR analysis

The reverse-transcription (RT) for synthesizing first strand cDNA was carried out with the oligo-dT adapter primer PrOlgadpr (5'-GCGGCTGAAGACGGCTATGT GGCC-3') in 20 μl reaction mixture containing 2 μl of Omniscript RT (Qiagen). To

examine the expression of the *Pr*-pseudogene at various organs, nested PCR was performed. 1 μl of RT reaction products were subjected to first amplification with the primers PrEX4F1 (within exon 4: 5'-ATTCATGACAATTATGTTGTTTGGAGC-3'), PrEX10R1 (exon 10: 5'-CTAGTAGTGACCCTTGAGCTC-3') in the 20 μl of reaction mixture. Temperature protocol was same as the condition that used to generate probe A and B as described above. 1 μl of resultant mixtures were subjected to nested amplification with primers PrEX9R1 (exon 9: 5'-AAGTAAGACATGAGCATCATAAG -3') and PrEX4F2 (exon 4: 5'-CTATTGGCACACTTATTAGCTGTTC-3') in 20 μl reaction mixtures.

For nested PCR to examine the expression of *Pr* gene at various organs, first amplification was performed with primers PrEX1F2 (exon1: 5'-CCATTTGTCTGAAG CTCTTCATC-3') and PrEX15R2 (exon 15: 5'-CATAGAGCCAAATTGATAATTCA GC-3'). The concentration of template and temperature profile was same as above. 1 μl of first amplified mix was subjected to nested amplification with PrEX2F1 (exon 2: 5'-AATTATAAGGGCAGTAATGGATTC-3'), PrEX15R1 (translation end of exon 15, underlined region was adapter sequence corresponding to the cutting site of *Not*I: 5'-GTGCGGCCGCTCATCTAGGGCTCTGCTCAACTGGT-3') in 20 μl reaction mixture. All of the detected bands were cloned and confirmed by the sequence analysis.

Cloning of *PhNhx1* gene and testing its expression patterns in *P. hybrida*

Using the *Pr* cDNA as a probe, 14 *PhNhx1* clones were isolated from approximately 200,000 λ ZapII (Stratagene) recombinant plaques in cDNA library prepared from petals of pigmented buds with emerging corolla of *Petunia hybrida* cv. Surfinia Purple Mini. For Northern blot analysis, total RNA 10 μg for each lanes were separated with 1.2% agarose gel and transferred to Hybond N+ membrane. Hybridization with the *Pr* cDNA probe was performed as described above.

Results

1. The role of *Pr* gene in the blue flower pigmentation

At first, to characterize the differences between *pr-m* and *Pr-r,* HPLC analysis of the pigment composition in *pr-m* (purple flower, Fig. 1A) and *Pr-r* (blue flower, Fig. 1B) flowers were performed. The resultant HPLC charts were almost identical and the ratios of pigments of *pr-m* and *Pr-r* are almost the same (Fig. 2, Table 1), indicating no differences in pigment compositions were seen between *pr-m* and *Pr-r* lines. In *I. tricolor*, the reddish-purple flower buds turn to blue flowers, these color changes are caused by raising pH of the flower vacuole that sequestrates pigments (Yoshida *et al.* 1995). Similar color changes are also observed in *I.nil* (Fig. 1). Thus, I estimated vacuolar pH by following two methods. First, pHs of the dissected petal homogenates were measured directly (Table 2, de Vlaming *et al.* 1983). The pHs of the opened *pr-m* and *Pr-r* flower homogenates were estimated as 5.9 and 6.2, thus the differences between *pr-m* and *Pr-r* was 0.3 (Table 2). I also measured the pH of the epidermis homogenates collected from opened *pr-m* and *Pr-r* flowers to further confirmation because the vacuolar pH of the parenchyma cells are more acidic than the epidermis cells even when the flower completely opened in *I. tricolor* (Yoshida *et al.* 1995). The pHs of *pr-m* and *Pr-r* epidermis homogenates were 6.4 and 7.0, and difference was 0.6 (Table 2). The pHs of the homogenates from both buds were also measured and estimated as 5.6 (Table 2), thus the differences between buds and opened flower were 0.3 for *pr-m* and 0.6 for *Pr-r*, respectively. Since epidermis tissues of flower buds couldn't obtain by a technical problem, the pHs of flower buds epidermis were not measured. However, the increasing of pHs were observed during flower opening in *pr-m* and *Pr-r* flowers as seen in *I. tricolor* (Yoshida *et al.* 1995). Although this procedure couldn't eliminate contamination of the parenchyma cells and some another compartment in the cells, these results clearly indicates that vacuolar pH of *pr-m* was lower than that of *Pr-r.*

Second, visible light spectrum (400 to 660 nm) of the intact petal was measured and compared with their homogenate solutions adjusted at various pH (Fig.3). In this condition, λmaxs of the petal homogenate solutions shift to longer wavelength as the pH of the solutions are raised to higher pH within 7.5-8.0 (Fig. 3A), and for under pH=7.4, λmaxs converged to 540 nm and also the shapes of the curve became similar. Thus, it was difficult to estimate the pH under 7.4. The shapes of the curves for *Pr-r* and *pr-m* homogenate solution at each pH were almost identical (Fig.3A), these observations

coincided with HPLC data that the pigment compositions were almost same between these two lines (Fig. 2). λmax of the intact opened *Pr-r* petal was 600 nm, and curve shape resembles to the curve for pH 8.0 of the homogenate solution at this condition, and for *pr-m* was 570nm, corresponding to the intermediate between 7.5-7.6, respectively. The λmaxs of both *pr-m* and *Pr-r* buds were identical (540 nm) thus pHs of the buds were not estimated. It should be noted that these λmaxs of homogenate solutions vary with concentration of the buffer (at lower concentration, λmax shifts to shorter wavelength), thus it is difficult to determine precise vacuolar pH by this method. However, difference between *pr-m* and *Pr-r* was clearly observed, this represented the difference of vacuolar pH because the pigment composition was almost identical at two flowers and pigments were sequestrated in the vacuoles. When this method was applied to *I. tricolor*, the pH of opened petal was estimated as 7.5-7.6 and was not estimated as in *I. nil*, respectively (Fig. 3B). Note that this value for opened flower petal is somewhat lower than previously measured by double-barreled pH microelectorodes (pH=7.7) (Yoshida *et al.* 1995).

Therefore, I concluded that the difference between these two flowers is, not structure or composition of the pigments, but pH of the vacuole that stores pigments. That is, the role of *Pr* gene is changing vacuolar pH when the flowers open.

2. Identification of the *Pr* gene

I revealed that the function of the *Pr* gene is to raise the pH of the flower vacuole in *I. nil*. There are no observations about the genes that affect the vacuolar pH of flower except *ph* genes found in *P. hybrida* (Mol *et al.* 1998). It is possible that the *Pr* gene is structural genes that affect directly to the vacuolar pH, and also the *Pr* gene encodes transcriptional regulator. Therefore, I choose exhaustive procedure to isolate the *Pr* genes.

Luckily, I could obtain the plant line that carries revertant allele (*Pr-r*) from *pr-mutable* (*pr-m*) plant as described in Materials and Methods. These two plants must be isogenic and the difference between these two lines was present only on the *Pr* locus.

In *I. nil*, several *mutable* loci that condition the variegated flower phenotype are known. Some of them were analyzed and revealed that their unstable phenotype were caused by the somatic excision of the *En/Spm*-related transposable elements, *Tpn1*-family transposons that inserted in corresponding loci (see Table I-1). Based on these findings, a simplified transposon display method (STDM) was employed to identify the

Pr gene. STDM is based on AMF procedure that is developed in our laboratory (Habu *et al.* 1997) and modified to detect only the franking fragment that is tagged by *Tpn1*-related elements by using terminal inverted repeat (TIR) sequence that is characteristic in *Tpn1* family as a primer for the amplification.

2-1. Simplified transposon display method (STDM)

Fig. 4 shows the strategy of STDM. The genomic DNA was cleaved with a 4-base cutter enzyme *Mse*I and the *Mse*I adapter was ligated to each end of the fragments. Other 4-base cutter enzyme which makes a 2-base sticky end (*ex. Taq*I, *Msp*I, *Bfa*I, *Csp*6I and *Hin*P1I) can also be used by changing the adapter and primer sequences. To enrich the fragment containing flanking sequences of integrated sites of the transposable element *Tpn1* and its relatives, we employed the 20 bp TIR primer which contained a part of the 28 bp TIR of the *Tpn1*-family elements. Two-step amplification was performed with selective primers of 16 combinations since the two primers (*Mse*I+N and TIR+N primers) used had a single selective nucleotide at their 3' ends. Only the selective TIR primer was labeled at its 5' end, which ensured PCR fragments containing the flanking sequences of the *Tpn1*-related elements to visualize on the sequencing gel. The combination of these primers allowed us to reduce the complexity of bands visualized and to screen the bands of interest systematically. As FMBIO II Multi-View can detect the rhodamine and fluorescein signals independently, two samples labeled with different dyes can be applied on the same well. I could thus analyze 96 samples simultaneously in a sequencing gel with 48 wells. Only 100-500 bp amplified DNA fragments could be displayed reproducibly because of the gel resolution and large DNA fragments might not amplify constantly in this PCR reaction. Approximately 40 visualized bands could be detected in each reaction. But as the genome carries 500-1000 copies of *Tpn*1-family elements which contains 1000-2000 TIR flanking region and one restriction enzyme can analyze approximately 640 bands (as I obtained approximately 40 visualized bands in each reaction). Therefore several kinds of different 4 base cutter enzymes might be necessary to be analyzed to display the *Tpn1*-family elements. Luckily one positive band were found in analyzing 2 kinds of 4-base cutter enzyme (*Mse*I and *Taq*I).

STDM had been successfully applied for identifying the mutable *pr-m* gene. A band that was present in plants carrying the *pr-m* allele and absent in plants with the homozygous *Pr-r* allele was searched. 13 homogenotes (*Pr-r/Pr-r*), 4 heterogenotes (*Pr-*

r/pr-m) and 16 mutable plants (*pr-m/pr-m*) were employed and also 5 wild-type (*Pr-w/Pr-w*) and 8 additional mutable (*pr-m/pr-m*) plants from other lines were employed to examine whether candidate bands conformed to their genotypes. STDM using *Mse*I and *Taq*I, only one band of about 130 bp, including the primer and adapter used, fulfilled all the criteria tested: present with the *pr-m* allele and absent in the homozygous *Pr-w/Pr-w* or *Pr-r/Pr-r* condition (Fig. 5).

2-2. cDNA and genomic cloning of corresponding region 130 bp fragment obtained by STDM

By Northern blot analysis using the 130 bp fragment as a probe, transcripts of around 2.3 kb were detected in the *Pr-r* petals but not in the mutable *pr-m* flowers, indicating that the 2.3 kb transcripts were associated with the *Pr* gene (Fig. 6A). Using the same 130 bp probe, a recombinant clone was isolated from a cDNA library prepared from flower buds of the wild-type *Pr-w* line, KK/ZSK-2 (Inagaki *et al.* 1994). The clone contained a 2,237 bp sequence carrying a 1,626 bp open reading frame. Its deduced amino acid sequence had high homology with vacuolar Na^+/H^+ exchangers. Comparison of the 130 bp sequence isolated by STDM with the sequence of cDNA clone indicated that a *Tpn1*-related element was integrated into an exon at the 5' untranslated region (5' UTR). And that there must be at least one intron between this untranslated exon and the exon containing the ATG initiation codon because the 130 bp fragment consists of the 20 bp TIR primer, the 69 bp sequence corresponding to the 5' UTR of isolated cDNA, a 22 bp sequence apparently derived from the intron and the 19 bp *Mse*I primer. To test this hypothesis, the genomic structures of the revertant (*Pr-r/Pr-r*) and the mutable (*pr-m/pr-m*) lines were compared by Southern blot analysis using the 130bp fragment as a probe. Clear restriction fragment length polymorphisms were seen in the *Eco*RI, *Xba*I and *Hind*III digests, suggesting that a large DNA rearrangement occurred at the *Pr* gene region (Fig. 6B). I cloned the 7.5 kb *Xba*I fragment from the wild-type *Pr-w* line KK/ZSK-2 containing the entire cDNA obtained and an approximately 15 kb segment from the mutable *pr-m* line which contains about 7 kb of a *Tpn1*-related element, *Tpn4*, and the complete coding region of the cDNA obtained. Comparison of the cDNA sequence with the entire genomic sequences from the *Pr-w* and *pr-m* lines indicated that the putative *Pr* gene comprised 15 exons and that *Tpn4* was integrated into the first exon (Fig. 6C). As expected, the 130 bp fragment contained both the 69 bp exon 1 and 22 bp intron 1 sequences. The junction sequences between the putative *Pr*

gene and *Tpn4* indicated that integration of *Tpn4* into the putative *Pr* gene generated a 3 bp target duplication. Sequences at both terminal regions of *Tpn4* confirmed that *Tpn4* is a *Tpn1*-related element of the *En/Spm* family because *Tpn4* contained the 28 bp terminal inverted repeats and long subterminal repetitive regions characteristic of the *Tpn1*-related elements (Inagaki *et al.* 1994, Hoshino *et al.* 1995).

To further confirmation, the linkage of genotypes of this putative *Pr* gene with phenotypes that blooms blue or purple flower was checked. The presence of *Tpn4* at the insertion site could be determined by the appearance of the PCR-amplified fragment of 375 bp using the primers TIR and EX2RV, and its absence by detecting about 390 bp fragments using the primers EX1FW and EX2RV (Fig. 6C, D). Characterization of the *Pr* gene genotypes were successfully achieved by combining these PCR amplifications in the selfed progeny of hybrids between a mutable *pr-m* line and a revertant *Pr-r* line (Fig. 6C). These results strongly supported that cDNA and putative *Pr* gene that was obtained based on 130 bp fragment was the part of *Pr* gene.

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

Cloning of the *Pr* gene revealed that it comprised 15 exons, and *Tpn4* was integrated into its first exon. Close examination of the southern blot analysis suggested that the genome of *I. nil* contained not only the 7.5 kb *Xba*I fragment including the *Pr* gene but also a second copy of 6.9 kb *Xba*I fragment that was hybridizable to the 3' region (probe B) but not to the probe A containing exon 1 and 2 (Fig. 7). This notion was also supported by the hybridization patterns in *Eco*RI. Therefore, I cloned both 7.5 kb and 6.9 kb *Xba*I fragments and performed the sequence analysis. Fig. 8 shows the structure of 7.5 kb fragment containing *Pr* gene and 6.9 kb of second copy. Sequence comparison of the *Pr* genes between the *Pr-r* line and the *Pr-w* plant previously sequenced revealed that 13 polymorphism occurred in these *Pr* genes and no alteration has occurred in their coding regions. The second copy lacked the regions corresponding to the exon 1 to 2 and 6 to 9, and the 3' half of the exon 11 of the *Pr* gene. The region corresponding to exon 6 to 8 contained putative transmembrane domain M5a to M6 in *S.cerevisiae*, highly conserved region among NHX1s (Nass *et al.* 1997 and also see Fig. 10). These structural deficiencies of the second copy suggested the lacking of ability to work as a NHX1. Indeed, no mRNA that was transcribed from the pseudogene could be detected by RT-PCR using appropriate primers (Fig. 9). The second copy had the insertions at the region corresponding to the intermediate between the exon 3 and 4.

Surprisingly, the regions homologous between two genes shared the extremely high identity (88-97%) at nucleotides. No further bands that suggested the presence of additional copy except the second copy were detected in southern blot analysis even low stringency conditions. Thus, I concluded that the *Pr* gene was the only active copy in the *I. nil*.

4. Functional analysis of the *Pr* gene products

The isolated 2.3 kb *Pr* cDNA has 1.7kb ORF encoding 542 aa polypeptide. The deduced amino acid sequence of *Pr* cDNA showed high homology with *NHX1*, Na^+/H^+ exchanger in *S.cerevisiae*, *A. thaliana*, and *O. sativa* (29.3, 73.4, and 75.7% identities, respectively, see Fig. 10). I also isolated cDNA of *Pr* homologue from *P. hybrida* flowers and this shared 75% identity with *Pr* cDNA (Fig. 10A). NHX1s are localized in tonoplast membrane in plant or prevacuolar membrane in yeast, and they mediate the exchanging of cytosolic Na^+ with vacuolar H^+ (Apse *et al.* 1999, Nass *et al.* 1997, 1998). The hydropathy plot (Kyte and Doolittle 1982) of the *Pr* cDNA indicated the presence of 12 hydrophobic regions (Fig. 10B) that could be thought as membrane spanning regions, as seen in other NHX1s. Phylogenetic analysis with various Na^+/H^+ exchangers (Fig. 11) showed that the *Pr* cDNA was included in the cluster shared by intracellular Na^+/H^+ exchangers, AtNHX1, OsNHX1, SpNHX1, ScNHX1, PtNHX1 and HsNHE6 (Fukuda *et al.* 1999), suggesting InNHX1 is localized in the intracellular membrane. These observations implied the possibility that InNHX1 might work as NHX1.

Since AtNHX1 protein suppressed the salt sensitivity in the *nhx1* mutant of yeast (Gaxiola *et al.* 1999), we tested whether the *Pr* gene product could complement the *S. cerevisiae nhx1* mutation (Fig. 12). As a result, introduction of the plasmid carrying *Pr* cDNA into the *S.cerevisiae nhx1* mutant could render to survive in the medium contains 400 mM NaCl, the condition *nhx1* mutant could not survive. This result strongly suggested that the *Pr* gene product had the ability to work as Na^+/H^+ exchanger. From these results, I defined the *InNHX1* (*Ipomoea nil* Nhx1) as a synonym for the *Pr* gene.

5. Spatial expression pattern of the *Pr* gene in *I. nil*

The expression of the *Pr* gene in various tissues was examined by Northern blot analysis (Fig. 13). For the flower tissues, I used pigmented flower buds at 12 h before flower opening when the maximum accumulation of the *Pr* transcript in the flower was seen (see Fig. 14A and next paragraph). The detectable amount of 2.3 kb *Pr* mRNA accumulation were seen only in flower organs (Fig. 13A), most abundantly in petals (lane 1), moderately in the tubes (lane 2) and stamens (lane 7), and weekly in the sepals (lane 3) and pistils (lane 8), but not in vegetative organs such as leaves (lane 4), stems (lane 5) and roots (lane 6). Further RT-PCR analysis showed the presence of *Pr* mRNA expression in roots, leaves and stems (Fig. 13B). These observations strongly indicated that the *Pr* gene expressed in the vegetative tissues, but the amount of them were extremely low comparing with floral organs.

6. Temporal expression pattern of the *Pr* gene and the relationship with anthocyanin biosynthesis

The temporal expression pattern of the *Pr* mRNA was shown in Fig. 14. The highest expression of the *Pr* gene was observed at 12 h before flowering in *Pr-r*. The longer transcripts in the *pr-m* flowers, probably due to insertion of *Tpn4* into *Pr* gene, were also seen at the same stage. Similar aberrant transcript had been characterized previously in the *DFR-B* gene having transposon *Tpn1* inserted (Takahashi *et al.* 1999). This aberrant transcription must read to deficiency in raising vacuolar pH because *pr-m* flower displayed purple flower due to lower vacuolar pH than that of *Pr-r*.

In *Antirrhinum majus*, the enzymes for the anthocyanin biosynthesis were controlled by the same regulatory mechanism, and their temporal expression patterns are parallel (Jackson *et al.* 1992). And in *P. hybrida*, the expression patterns of the genes downstream from flavonoid 3',5'-hidroxylase (F3'5'H) of the anthocyanin biosynthetic pathway were regulated same regulatory mechanism (Mol *et al.* 1998). Furthermore, *An2* and *An11*, regulators of the anthocyanin biosynthesis, also affected vacuolar pH of flower. And *ph6* gene was allele of *An1*, one of a regulatry genes of anthocyanin biosynthesis (Mol *et al.* 1999) These observations suggested that close relationships do exist in determination of flower colors between vacuolar pH regulation and anthocyanin biosynthesis in *P. hybrida*.

Northern blot analysis revealed that the expression patterns of chalcone synthase

(especially *CHS-D* in *I. nil*, Fukada-Tanaka *et al.* 1997), chalcone isomerase (*CHI*), flavonoid 3-hydoroxylase (*F3H*), dihydroflavonol 4-reductase (*DFR*), anthocyanidin synthase (*ANS*), UDP-glucose:flavonoid 3-*O*-glucosyltransferase (*UF3GT*) were parallel in both *Pr-r* and *pr-m* (Fig. 13). The expression of *Pr* gene was later than these genes that encoding the enzymes included in anthocyanin biosynthesis. These results strongly suggested that the *Pr* gene expression was regulated by different mechanism from genes for anthocyanin biosynthesis.

The *I. nil* 78wwc-*1*, which is believed as a mutant line on the regulatory gene of the anthocyanin biosynthesis pathway designated as *c-1* allele, sequestration of mRNAs for the genes includes in anthocyanin biosynthesis were significantly decreased (Abe *et al.* 1997, Hoshino *et al.* 1997). In this plant, the expression of the genes that involved in anthocyanin biosynthesis were significantly reduced than those of *Pr-r* and *pr-m*, and no differences were observed in the *Pr* gene expression pattern (Fig. 14), indicating that the *Pr* gene did not regulated by the *c-1* regulator.

These results strongly suggested that the *Pr* gene expression was regulated by independent pathway from other genes for anthocyanin biosynthesis in *I. nil*.

7. The *Pr* gene homologue in *P. hybrida*

Like *I. tricolor* (Yoshida *et al.* 1995), I showed that raising the vacuolar pH in the flowers of *I. nil* was responsible for blue coloration and that the recessive *pr* mutation in the *InNHX1* gene failed 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). Contrary to the morning glories, a recessive mutation in one of the seven loci failed to decrease pH in the vacuole, resulting in the bluing 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* was also expressed extensively in the flower limbs, we first cloned a petunia cDNA homologous to the *Pr* cDNA of *I. nil*.

Northern blot hybridization revealed that the *PhNHX1* gene was expressed in leaves, stems and flowers and that no dramatic increase of the mRNA accumulation was observed during flower development (Fig. 15). No significant differences of the mRNA accumulation at the beginning of flower opening stage were detected in several petunia cultivars available including the *ph1* and *ph2* mutants (Fig. 16). 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 were known to be higher than the wild type by about 0.5 (de Vlaming *et al.* 1983, Fukui *et al.* 1998). It is clear that the mode of expression of the *PhNHX1* gene was greatly different from that of the *InNHX1* gene.

Discussion

Plants develop the elegant mechanism toward bluing of their flowers, complicated pigment structure, forming complex with co-pigments, chelating with metal cation, and raising vacuolar pH, presumably for attraction of pollinators (Harborne and Grayer 1994). In this study, I analyzed the *pr-m* line that carries the *pr-mutable* allele blooming purple flower with blue sector and its germinal revertant that blooms fully blue flower carried *Pr-revertant* allele, and revealed that the variations of the flower color between two lines were due to the difference of pH in flower vacuole, that sequestrates anthocyanin pigments. Namely, the role of the *Pr* gene in the flower of the *I. nil* was the raising of the vacuolar pH during flower opening. The results from measurement of petal homogenates (Table 2) suggested that the raising of the vacuolar pH for blue pigmentation in *I. nil* consisted of two components, from reddish purple in buds to purple flowers (independent from *Pr* gene) and from the purple to blue flowers (*Pr*-dependent). The genes that are responsible for the first step remain to be elucidated.

By comparing the genomic DNA of *pr-m* and *Pr-r* lines with STDM and subsequent cDNA and genomic cloning, the candidate of the *Pr* gene was isolated. This candidate encoded Na^+/H^+ exchanger-like protein, and comprised with 15 exons. The *Tpn1*-related element, *Tpn4* insertion was seen in the exon 1 in *pr-m* line. From northern blot analysis, 2.3 kb of *Pr* mRNA were found only in *Pr-r* line, and the longer transcripts, this probably due to insertion of *Tpn4* into *Pr* gene were found in the *pr-m* flowers at the same stage. Since the *Pr-r* line came from *pr-m* line and these lines were isogenic, and their phenotypes (blue or purple flower) coincided with their genotype (*Pr-r*/*Pr-r*, *pr-m*/*Pr-r*, or *pr-m*/*pr-m*, see Fig. 6E), this putative Na^+/H^+ exchanger was concluded as the *Pr* gene. The presence of a second copy of this gene was also found in *I.nil* genome, and this copy did not express at any organ examined in *I. nil*. Thus I concluded that the *Pr* gene should be the only "active" *NHX1* gene in *I. nil*.

The *NHX1* genes encodes vacuolar Na^+/H^+ exchanger that mediate the exchange of Na^+ for H^+ across the vacuolar membrane, and the transport of Na^+ into the vacuoles promoted by the NHX1 proteins results in the vacuolar alkalization (Blumwald *et al.* 2000). This function of NHX1 protein coincided with the findings that the role of the *Pr* gene product was to raise the vacuolar pH. The result of complementation test with yeast *nhx1* mutant indicated that the *Pr* cDNA was able to work as Na^+/H^+ exchanger. From these evidences, I concluded the *Pr* gene, *InNHX1* was responsible for flower bluing in *I. nil*.

NHX1 is thought as electroneutral transporter that exchanges one vacuolar H^+

with one cytosolic Na^+. Although the driving force for operation is usually provided by H^+ gradient (vacuolar acidic) generated by H^+-pumps such as H^+-ATPase and H^+-PPiase (Blumward *et al.* 2000), the study of isolated tonoplast membrane showed that NHX1 could be also driven by higher Na^+ concentration of outer membrane (Blumward and Poole 1985). In the case of *I. nil* flower, it is difficult to think that motive force of InNHX1 was generated by H^+ pump because vacuolar pH is raised to >7.0 (Table 2) and usual cytosolic pH are considered as neutral. Therefore, inward-directed Na^+ gradient is conceivable as the motive force of InNHX1. The studies about the activity of vacuolar H^+-pumps and the contents of cytosolic also vacuolar Na^+ at flower opening may enables to discuss this hypothesis.

The expressions of the *InNHX1* were seen most abundantly in petal, and although scarcely, seen in leaves, stems, and roots (Fig. 13). It is interesting because the functional copy of *InNHX1* is considered as only one (Fig. 7) and the InNHX1 molecules included in opened petals and other vegetative organs must be come from a same copy. The findings that the transcripts found in these organs contained complete ORF of *InNHX1* is conceivable that InNHX1 is active in these organs.

The study in the cell suspension culture of sugar beat showed that the activation of Na^+/H^+ exchange was due to the changing of V_{max} but no alteration were seen in K_m, indicating the increasing amount of Na^+ sequestration was caused by the addition of more exchanger molecules (Blumward and Poole 1987). The *InNHX1* gene was the most abundantly expressed at 12 h before flower opening in the petals where the blue coloration occurred during flower opening (Figs. 13, 14). Although the rate of producing *InNHX1* mRNA remains elusive, this finding indicated that the increasing amount of InNHX1 was responsible to the raising of vacuolar pH during flower opening, resulted in blue flower.

The organ specific increasing of the expression of *InNHX1* gene during flower opening was seen in *I. nil*. Contrary to this, in petunia, however, no dramatic increase of the *PhNHX1* transcripts was observed during flower development (Fig. 14). The results indicated that the abundant expression of the *NHX1* gene before blooming was not common in all flowering plants. The results in petunia might 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 was controlled to remain more acidic than that in the wild-type morning glory (Table 2).

As described above, the coloration of blue flowers 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 the pH in the vacuole 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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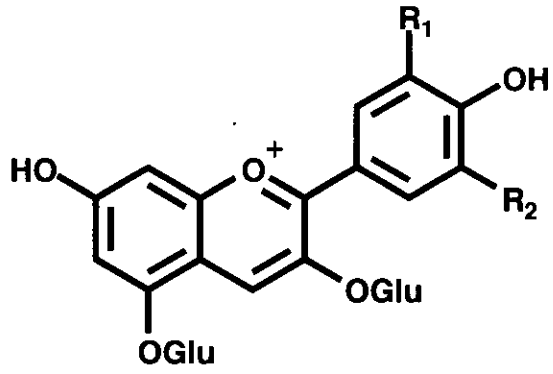

R_1	R_2	Anthocyanin	Anthocyanidin
H	H	pelargonin	pelargonidin
OH	H	cyanin	cyanidin
OCH_3	H	peonin	peonidin
OH	OH	delphin	delphinidin
OCH_3	OH	petunin	petunidin
OCH_3	OCH_3	malvin	malvidin

Fig. I-1 Structure of common anthocyanins. Glu: glucosyl residues.

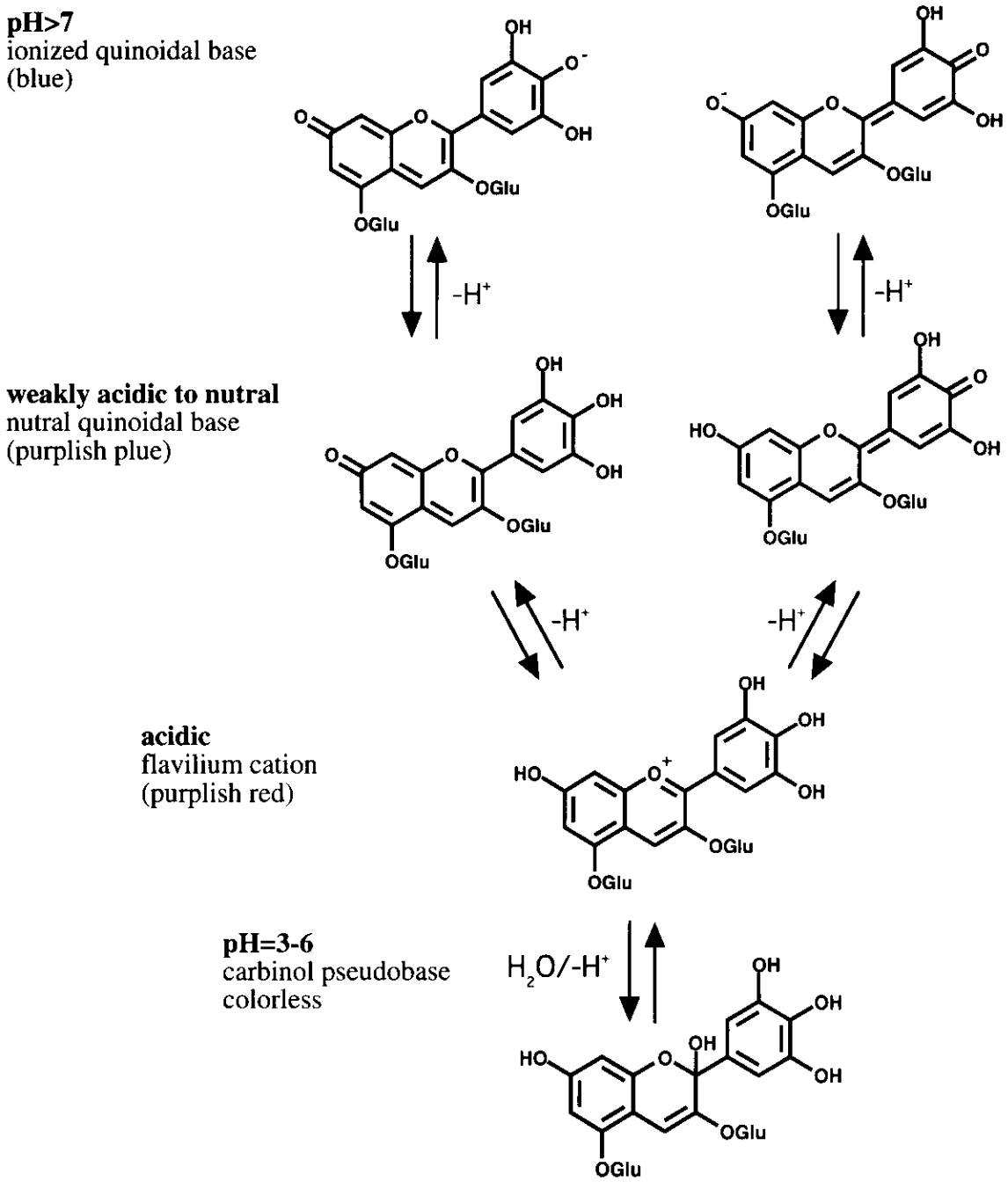

Fig. I-2 The structure of an anthocyanin and pH of the solution. Glu : suger residue

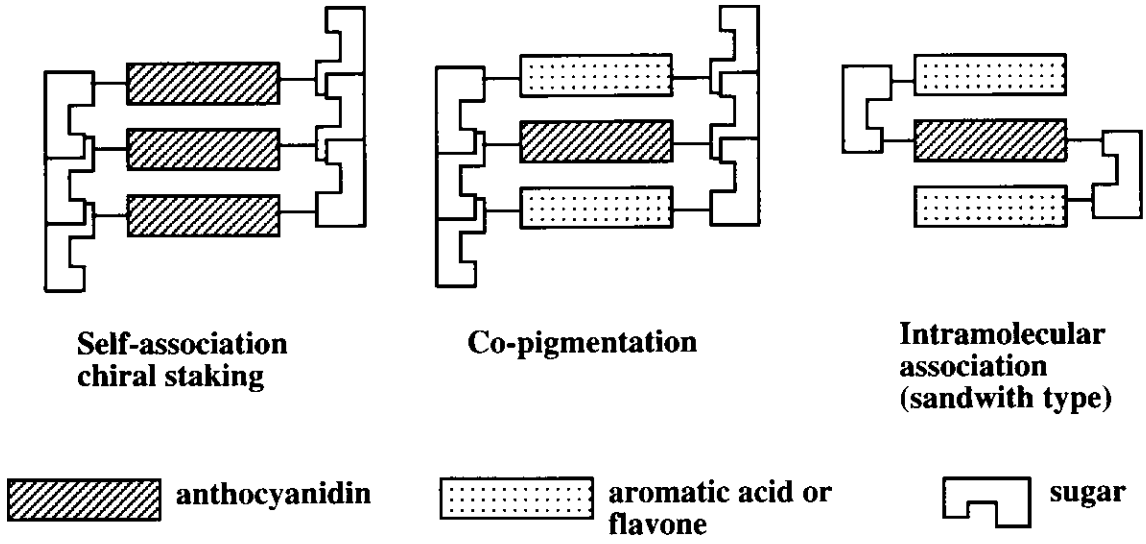

Fig. I-3 Schematic representation of three stacking types.

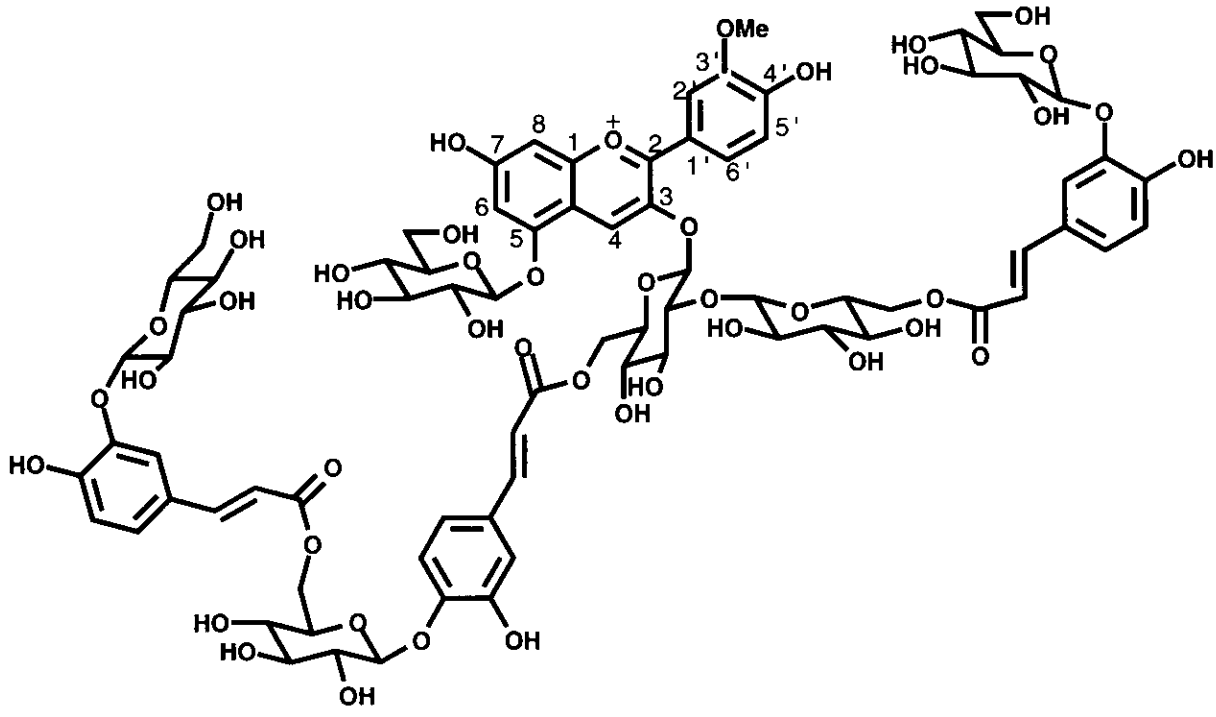

Fig. I-4 Structure of the heavenly blue anthocyanin (HBA).

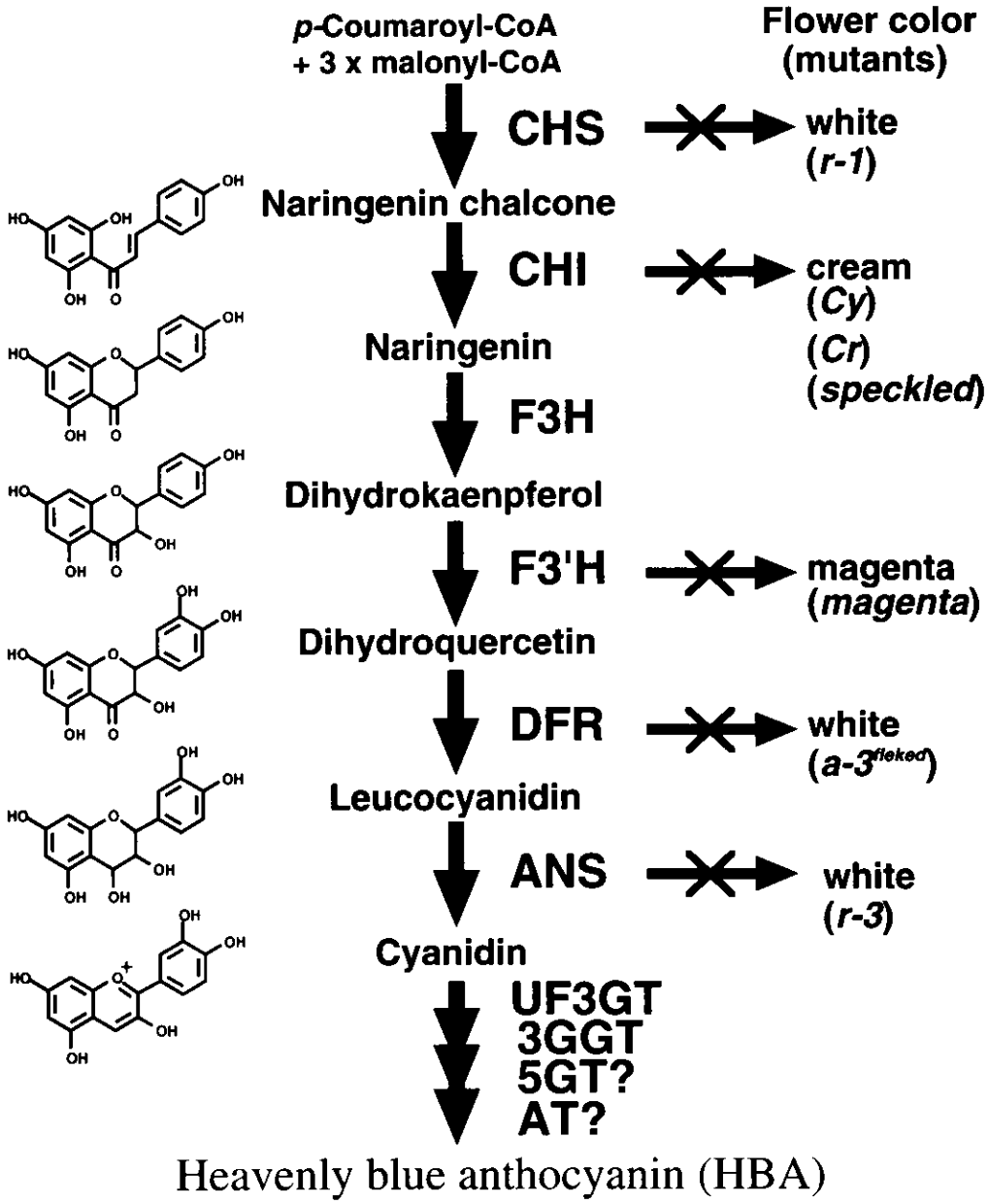

Fig. I-5 Anthocyanin biosynthesis pathway in *I. nil*.
CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone 3-hydroxylase; F3'H, flavonoid 3'-hydroxylase; DFR, dihydroflavonol 4-reductase; ANS, anthocyanidin synthase; 3GT, UDP-glucose:flavonoid 3-*O*-glucosyltransferase; 3GGT, UDP-glucose;flavonoid 3-*O*-glucoside glcosyltransferase; 5GT, flavonoid 5-*O*-glucosyltransferase; AT; acyltransferase. Note that 5GT and AT molecules had not been identified in *I. nil*.

Table I-1 *Tpn1*-family transposon found in characterized alleles of *I. nil*.

allele	gene products	transposon	phenotype
a-3^{flecked}	DFR	*Tpn1*	flecked with white background
r-1	CHS	*Tpn3*	white, rarely spotted
r-1.2	CHS	*Tpn6*	white
speckled	CHI	*Tpn2*	spotted with cream background
blizzard	DFR	*Tpn5*	white sectors
duplicated	MADS-box (C type)	*Tpn-botan*	duplicated petals

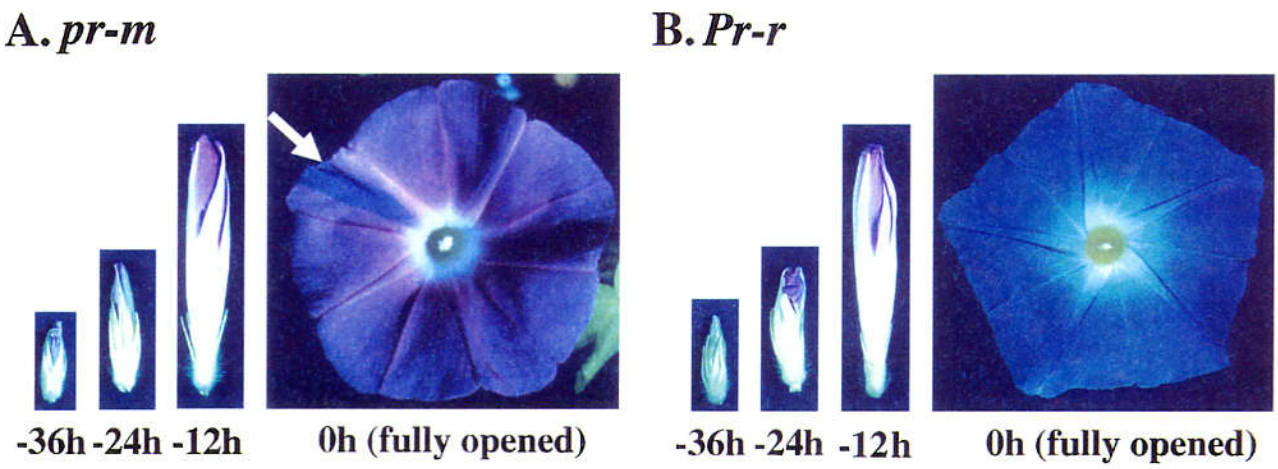

Fig. 1 *pr-m* and *Pr-r* flower.
A, *pr-m* flower bud at 36h, 24h, 12h before opening and fully opened flower. White arrow indicates blue sector which due to somatic reversion from *pr-m* to *Pr-r*. B, *Pr-r* flower bud 36h, 24h, 12h before opening and fully opened flower.

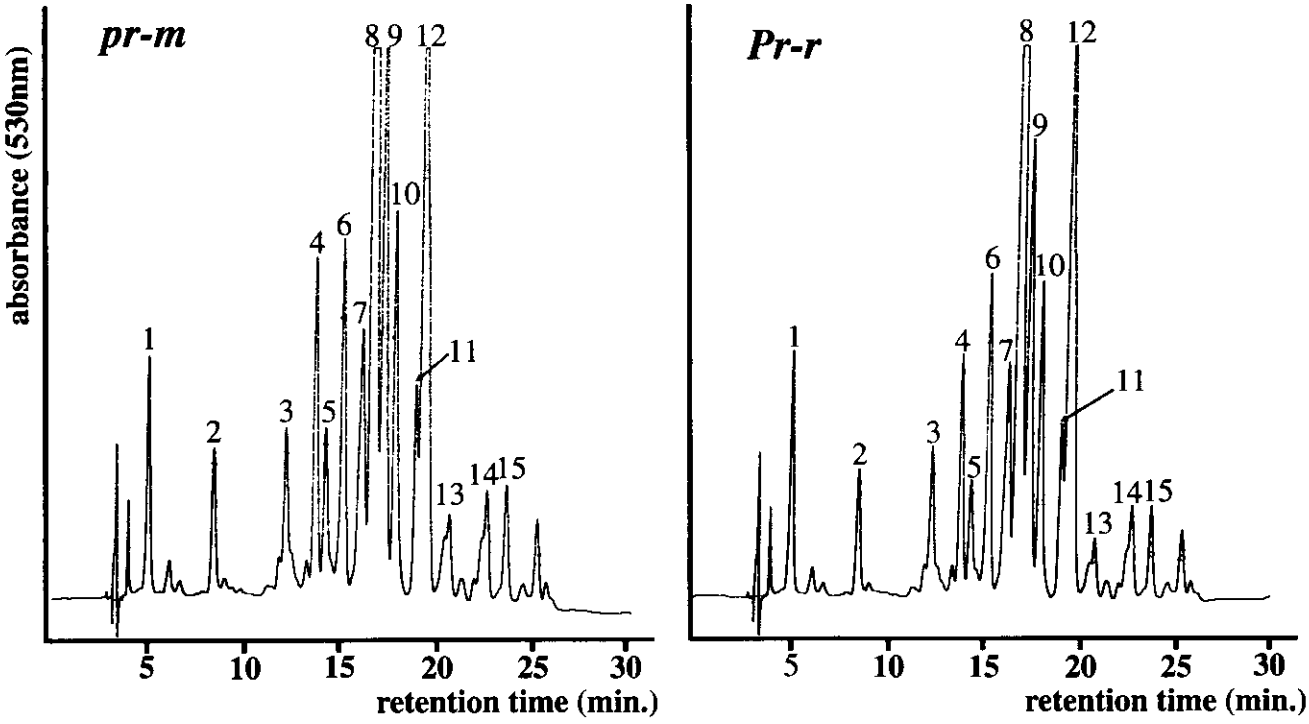

Fig. 2 HPLC analysis of the pigments in *Pr-r* and *pr-m* flowers.
The pigments were extracted from *pr-m* and *Pr-r* flower and subjected to HPLC analysis, and absorbance at 530 nm were measured. Another analytical conditions are described in "Materials and Methods". The numbers of each peaks are identical with Table 1.

Table 1 Pigments composition in *pr-m* and *Pr-r*.

	Anthocyanins (as %) (retention time:min)														
lines	1 (5.0)	2 (8.4)	3 (12.1)	4 (13.6)	5 (14.1)	6 (15.0)	7 (16.0)	8 (16.5)	9 (17.1)	10 (17.7)	11 (18.9)	12 (19.3)	13 (20.7)	14 (22.6)	15 (23.6)
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
Pr-r	2.2	1.5	3.3	4.5	2.2	5.8	5.7	26.7	8.4	5.3	-	18.4	1.9	2.8	2.0

The peak numbers are same as Fig. 2. Each anthocyanin contents are described as per cent value of total absorbance of detected anthocyanins at this condition. The pigment 8 is heavenly blue anthocyanin (HBA), and the other pigments identified were: 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; pigment 7, peonidin 3-[2-(glucosylcaffeoylglucosyl)-6-(caffeoyl)-glucoside]-5-glucoside.

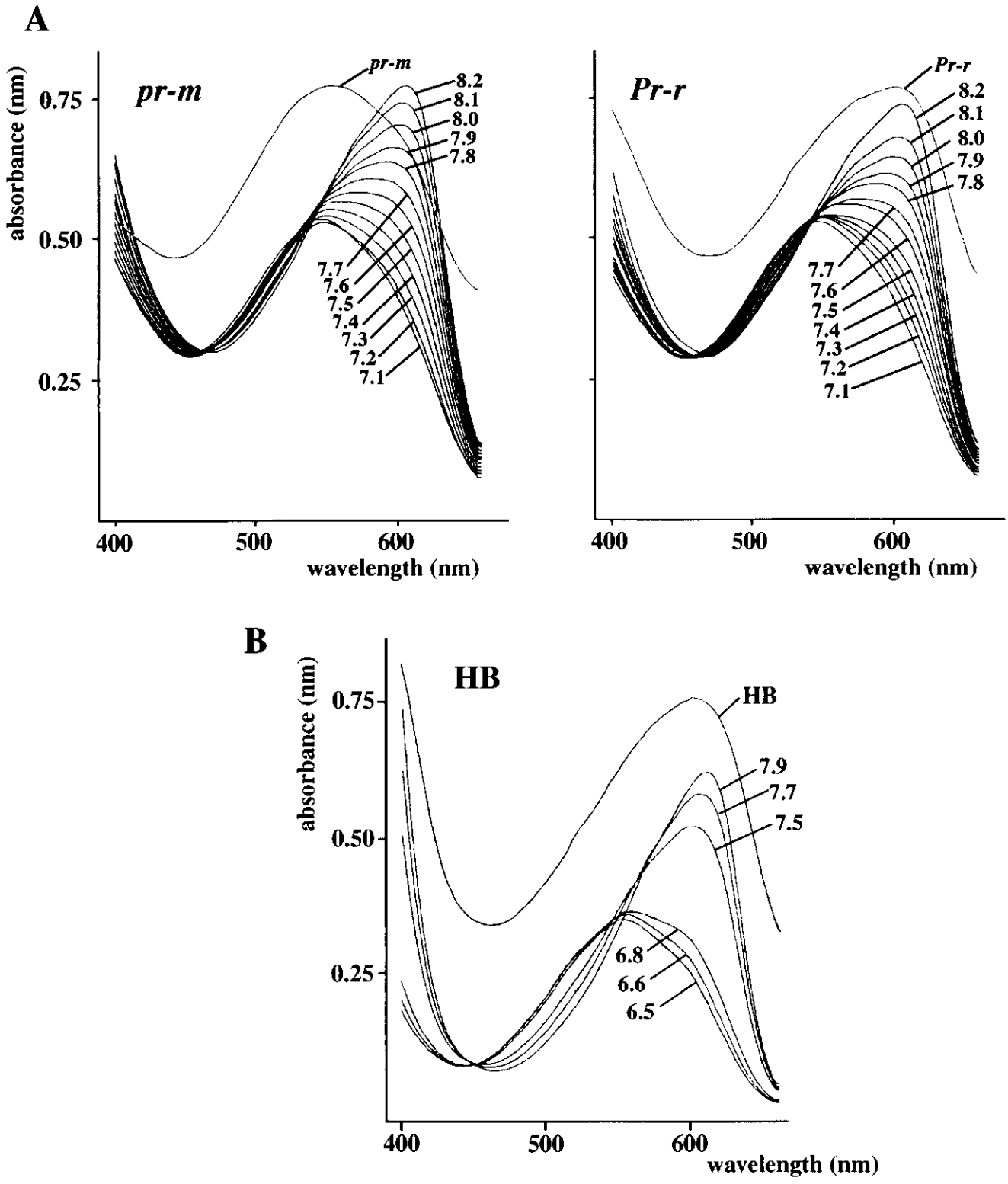

Fig.3 Absorption spectra of homogenate solutions and intact petal.
Detailed experimental conditions are described in "Material and Methods". A, Absorption spectra of *pr-m* and *Pr-r*. Note that the absorption spectra for the homogenates of *pr-m* and *Pr-r* that were dissolved in various pH buffers were identical. 7.1, 7.2, 7.3, 7.4, 7.5, 7.6, 7.7, 7.8, 7.9, and 8.0 indicate the pH of the solution and the spectra of intact *pr-m* and *Pr-r* petals are indicated with bar. B, Absorption spectrum of Heavenly blue (HB). 6.5, 6.6, 6.8, 7.5, 7.6, 7.9 indicate the pH of the solution. The spectrum for intact petal of Heavenly Blue is denoted as HB.

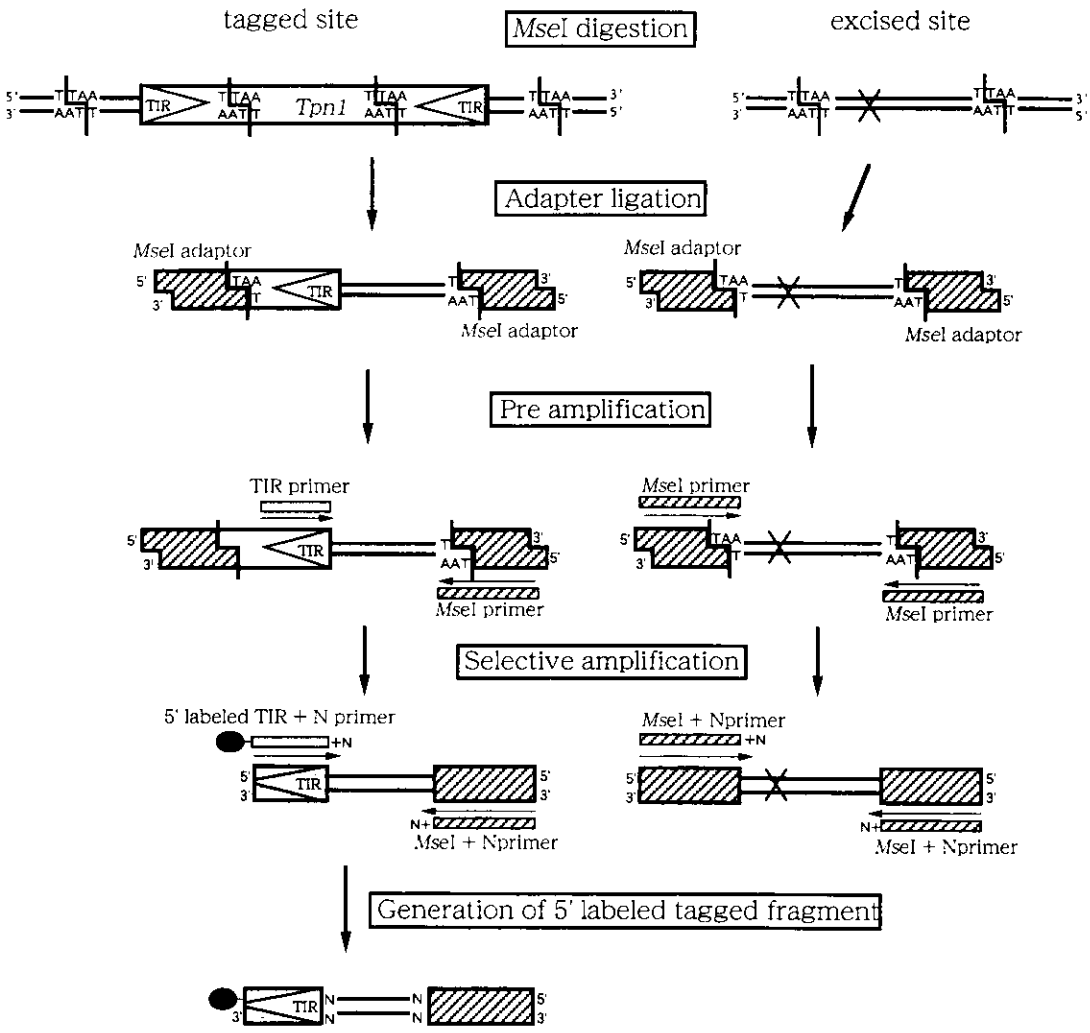

Fig. 4 Schematic representation of the strategy for analysis by STDM.
The transposon *Tpn1* is integrated into the tagged site and excised from excised site. The large X represents a footprint sequence generated by the excision of *Tpn1*. Since the TIR+ N primers were 5'-labeled with rhodamine or fluorescein, only fragments derived from the tagged sites were expected to be visualized. Note that two tagged fragments can be visualized from a single tagged site because the 5'-labeled TIR+N primers are hybridizable to both termini of *Tpn1*.

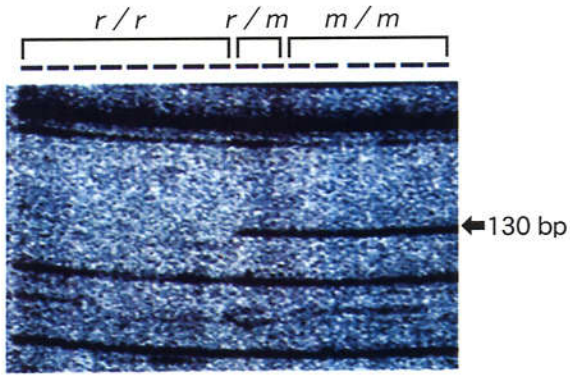

Fig. 5 Display of the fragment derived from the *pr-m* gene in a sequence gel.
The genotypes of the individual plants are: *r/r*, *Pr-r/Pr-r*; *r/m*, *Pr-r/pr-m*; *m/m*, *pr-m/pr-m*. The arrow points to the 130bp fragment.

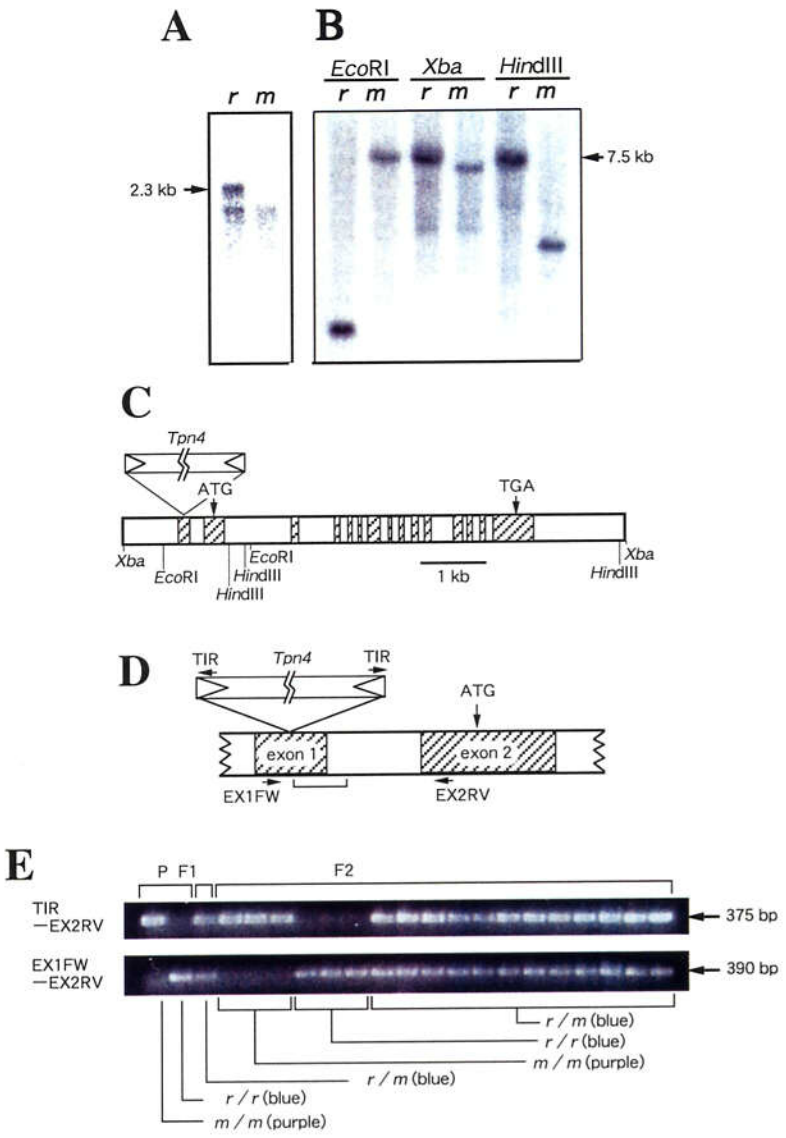

Fig. 6 Characterization of the *Pr* gene.

A, Northern blot analysis. mRNA (1.5 μg) extracted from open flower petals were hybridized with the 130 bp DNA fragment obtained by STDM as a probe. Symbols *r* and *m* indicate the plants *Pr-r* and *pr-m*, respectively. The arrow points to the 2.3 kb *Pr* mRNA.

B. Southern blot analysis. Genomic DNA (10 μg) digested with *Eco*RI, *Xba*I or *Hind*III were hybridized with the 130 bp probe. The arrow points to the 7.5 kb *Xba*I fragment containing the entire *Pr* gene. The symbols are as in A.

C. The genomic structure of the *Pr* gene. The open box and hatched areas represent the 7.5 kb *Xba*I fragment and the exons of the *Pr* gene, respectively. The small vertical arrows with ATG and TGA indicate the positions of the initiation and termination codons, respectively. The *Tpn4* insertion site is indicated above the map.

D. The enlarged physical map of the 5' region of the *Pr* gene. The hatched boxes represent the untranslated exon 1 and exon 2 carrying the ATG initiation codon of the *Pr* gene. The small horizontal arrows and the bracket below the map indicate the positions of the primers and the 130 bp fragment obtained by STDM, respectively.

E. PCR analysis for the *Pr* genotypes in the selfed progeny of a hybrid between a mutable *pr-m* line (*pr-m/pr-m*) and a revertant homozygote (*Pr-r/Pr-r*). Two parental plants (*Pr-r/Pr-r* and *pr-m/pr-m*), an F1 hybrid (*Pr-r/pr-m*), and its selfed F2 progeny are indicated by P, F1 and F2, respectively. The symbols for genotypes are as in Fig. 2, and the flower phenotypes are indicated in parenthesis.

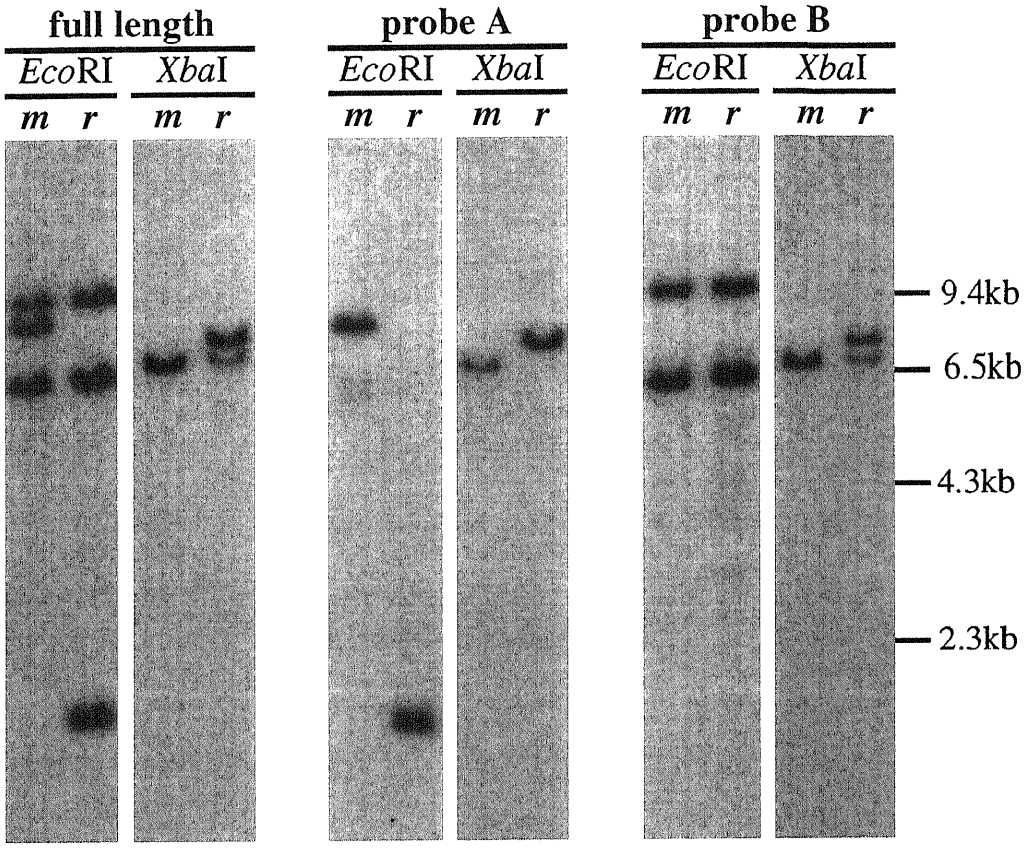

Fig. 7 Southern blot analysis of the *Pr* gene in the *pr-m* and *Pr-r*.
Genomic DNA which prepared from leaves were digested with *Eco*RI or *Xba*I and were hybridized with full length *Pr* cDNA, exon1-2 of *Pr* cDNA (probe A) and exon 9-14 (probe B), respectively. "*m*" indicates *pr-m* and "*r*" indicates *Pr-r*.

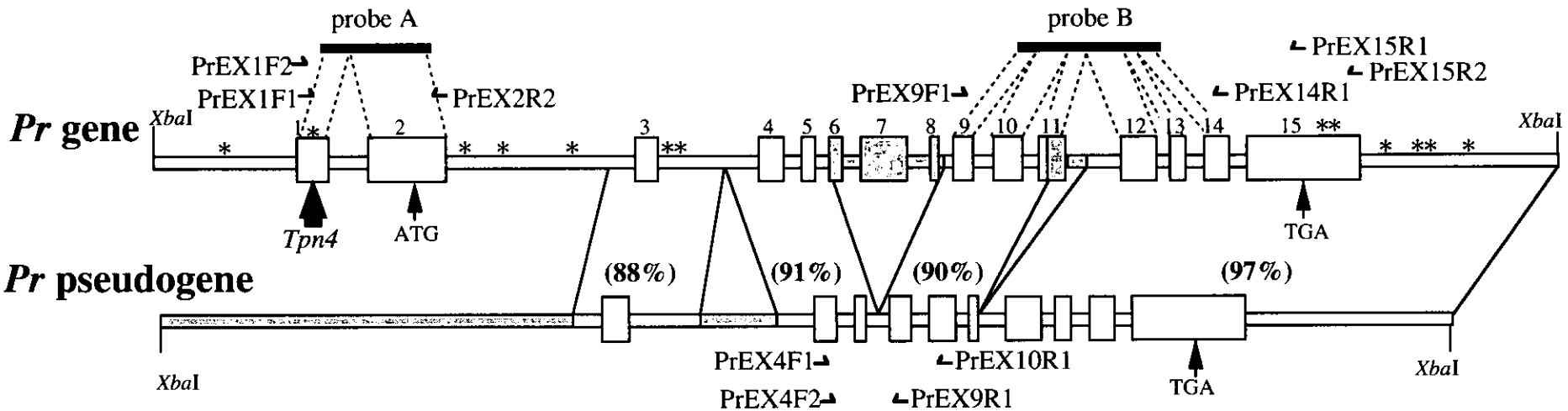

Fig. 8 Comparison of the genomic structures of the *Pr* gene (above) and the pseudogene (below).
The open boxes with the numerals indicate exons (*ex.* 1 indicates exon 1). The open areas represent homologous regions between these compared segments whereas the shadowed parts indicate non-homologous regions. The small vertical arrows with ATG and TGA indicate the positions of the initiation and termination codons, respectively. The large vertical arrow with *Tpn4* indicates the *Tpn4* insertion site, and the asterisks indicate the positions where polymorphisms were detected between the *Pr-w* and *Pr-r* lines. The lines with probe A or Probe B indicate the probes used for Southern blot analysis. The small horizontal arrows above and below the map indicate the positions of the primers used to generate the probes or RT-PCR (Figs. 9, 13), respectively. Nucleotide identities (%) between the *Pr* gene and the pseudogene are indicated in parentheses.

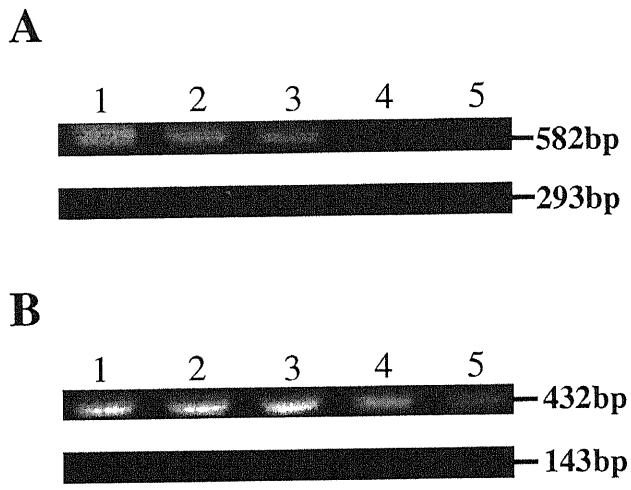

Fig. 9 RT-PCR for detection of the *Pr* gene and *Pr pseudogene* transcripts.
A, Ethidium bromide stained gel of PCR products that were amplified with the primer set PrEX4F1 and PrEX10R1 using total RNAs of petals (1), tubes (2), sepals (3), leaves (4), stems (5), respectively. Predicted sizes for *Pr* gene (582 bp) and *Pr pseudogene* (293 bp) are indicated. B, Ethidium bromide stained gel of nested PCR products that were amplified with the primer set PrEX4F2 and PrEX9R1. Predicted sizes for *Pr* gene (432bp) and *Pr pseudogene* (143 bp) are indicated.

A

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InNHX1      --------MAFGLSSLLQN--S----------------------------DLFTSDHAS
OsNHX1      --------MGMEVAAARLG--A----------------------------LYTTSDYAS
PhNHX1      --------MAFDFGTLLGN--VD---------------------------RLSTSDHQS
AtNHX1      --------MLDSLVSKLPS-------------------------------LSTSDHAS
ScNHX1      MLSKVLLNIAFKVLLTTAKRAVDPDDDDELLPSPDLPGSDDPIAGDPDVDLNPVTEEMFS

InNHX1      VVSMNLFVALLCACIVLGHLLEEN--RWVNESITALIIGLCTGVVILLLSGGKSSHLLVF
OsNHX1      VVSINLFVALLCACIVLGHLLEEN--RWVNESITALIIGLCTGVVILLMTKGKSSHLFVF
PhNHX1      VVSINLFVALICACIVIGHLLEEN--RWMNESITALVIGSCTGIVILLISGGKNSHILVF
AtNHX1      VVALNLFVALLCACIVLGHLLEEN--RWMNESITALLIGLGTGVTILLISKGKSSHLLVF
ScNHX1      SWALFIMLLLLISALWSSYYLTQKRIRAVHETVLSIFYGMVIGLIIRMSPGHYIQDTVTF
                      M2                      M3

InNHX1      SEDLFFIYLLPPIIFNAGFQVKKKQFFVNFMTIMLFGAIGTLISCSIISFG-AVKIFKHL
OsNHX1      SEDLFFIYLLPPIIFNAGFQVKKKQFFRNFMTITLFGAVGTMISFFTISIA-AIAIFSRM
PhNHX1      SEDLFFIYLLPPIIFNAGFQVKKKSFFRNFSTIMLFGALGTLISFIIISLG-AIGIFKKM
AtNHX1      SEDLFFIYLLPPIIFNAGFQVKKKQFFRNFVTIMLFGAVGTIISCTIISLG-VTQFFKKL
ScNHX1      NSSYFFNVLLPPIILNSGYELNQVNFFNNMLSILIFAIPGTFISAVVIGIILYIWTFLGL
                  M4                              M5

InNHX1      DIDFLDFGDYLAIGAIFAATDSVCTLQVLSQ-DETPLLYSLVFGEGVVNDATSVVLFNAI
OsNHX1      NIGTLDVGDFLAIGAIFSATDSVCTLQVLNQ-DETPFLYSLVFGEGVVNDATSIVLFNAL
PhNHX1      NIGSLEIGDYLAIGAIFSATDSVCTLQVLNQ-DETPLLYSLVFGEGVVNDATSVVLFNAI
AtNHX1      DIGTFDLGDYLAIGAIFAATDSVCTLQVLNQ-DETPLLYSLVFGEGVVNDATSVVVFNAI
ScNHX1      ESIDISFADAMSVGATLSATDPVTILSIFNAYKVDPKLYTIIFGESLLNDAISIVMFETC
                      M5a                             M5b

InNHX1      QSFDMTSFDPKIGLHFIGNFLYLFLSSTFLGVGIGLLCAYIIKKLYFGRHSTDREVALMM
OsNHX1      QNFDLVHIDAAVVLKFLGNFFYLFLSSTFLGVFAGLLSAYIIKKLYIGRHSTDREVALMM
PhNHX1      QNFDLSHIDTGKAMELVGNFLYLFASSTALGVAAGLLSAYIIKKLYFGRHSTDREVAIMI
AtNHX1      QSFDLTHLNHEAAFHLLGNFLYLFLLSTLLGAATGLISAYVIKKLYFGRHSTDREVALMM
ScNHX1      QKFHGQPATFSSVFEGAGLFLMTFSVSLLIGVLIGILVALLLKHTHIRRYP-QIESCLIL
                          M6

InNHX1      LMSYLSYIMAELFYLSGILTVFFCGIVMSHYTWHNVTESSRVTTRHSFATLSFVAETFIF
OsNHX1      LMAYLSYMLAELLDLSGILTVFFCGIVMSHYTWHNVTESSRVTTKHAFATLSFIAETFLF
PhNHX1      LMAYLSYMLAELFYLSAILTVFFSGIVMSHYTWHNVTESSRVTTKHTFATLSFIAEIFIF
AtNHX1      LMAYLSYMLAELFDLSGILTVFFCGIVMSHYTWHNVTESSRITTKHTFATLSFLAETFIF
ScNHX1      LIAYESYFFSNGCHMSGIVSLLFCGITLKHYAYYNMSRRSQITIKYIFQLLARLSENFIF
                  M7                                                  M8

InNHX1      LYVGMDALDIEKWKFVK---NSQGLSVAVSSILVGLILVGRAAFVFPLSFLSNLAKKNSS
OsNHX1      LYVGMDALDIEKWEFAS---DRPGKSIGISSILLGLVLIGRAAFVFPLSFLSNLTKKAPN
PhNHX1      LYVGMDALDIEKWKFVS---DSPGISVQVSSILLGLVLVGRAAFVFPLSFLSNLTKKTPE
AtNHX1      LYVGMDALDIDKWRSVS---DTPGTSIAVSSILMGLVMVGRAAFVFPLSFLSNLAKKNQS
ScNHX1      IYLGLELFTEVELVYKPLLIIVAAISICVARWCAVFPLSQFVNWIYRVKTIRSMSGITGE
                                        M9

InNHX1      -----DKISFRQQIIIWWAGLMRGAVSIALAYNKFTTSGHTSLHENAIMITSTVTVVLFS
OsNHX1      -----EKITWRQQVVIWWAGLMRGAVSIALAYNKFTRSGHTQLHGNAIMITSTITVVLFS
PhNHX1      -----AKISFNQQVTIWWAGLMRGAVSMALAYNQFTRGGHTQLRANAIMITSTITVVLFS
AtNHX1      -----EKINFNMQVVIWWSGLMRGAVSMALAYNKFTRAGHTDVRGNAIMITSTITVCLFS
ScNHX1      NISVPDEIPYNYQMMTFWAG-LRGAVGVALALG--IQGEYKFTLLATVLVVVVLTVIIFG
                          *                                       M10
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                .  ...  ...                                  ..  ...                  .    .
InNHX1          TVVFGLMTKP----------------------LINLLLPPHKQMPSGHSSMTTSEPSSP
OsNHX1          TMVFGMMTKP----------------------LIRLLLP-----ASGHP--VTSEPSSP
PhNHX1          TVVFGLMTKP----------------------LIRILLPSHKH----LSRMISSEPTTP
AtNHX1          TVVFGMLTKP----------------------LISYLLPHQN-----ATTSMLSDDNTP
ScNHX1          GTTAGMLEVLNIKTGCISEEDTSDDEFDIEAPRAINLLNGSSIQTDLGPYSDNNSPDISI

                .    ...     .                                  .  .  .  ...  .
InNHX1          KHFTVPLLDNQPDSESDMIT---------GPEVARPT-------ALRMLLRTPTHTVHRY
OsNHX1          KSLHSPLLTSMQGSDLESTT---------N--IVRPS-------SLRMLLTKPTHTVHYY
PhNHX1          KSFIVPLLDSTQDSEADL-----------ERHVPRPH-------SLRMLLSTPSHTVHYY
AtNHX1          KSIHIPLLD--QDSFIEPSG---------NHNVPRPD-------SIRGFLTRPTRTVHYY
ScNHX1          DQFAVSSNKNLPNNISTTGGNTFGGLNETENTSPNPARSSMDKRNLRDKLGTIFNSDSQW

                .. ..   ... ....... .  ...
InNHX1          WRKFDDSFMRPVFGGRGFVPFVAGSPVEQSPR----------------
OsNHX1          WRKFDDALMRPMFGGRGFVPFSPGSPTEQSHGGR--------------
PhNHX1          WRKFDNAFMRPVFGGRGFVPFAPGSPTDPVGGNLQ-------------
AtNHX1          WRQFDDSFMRPVFGGRGFVPFVPGSPTERNPPDLSKA-----------
ScNHX1          FQNFDEQVLKPVFLDN-VSPSLQDSATQSPADFSSQNH----------
```

B

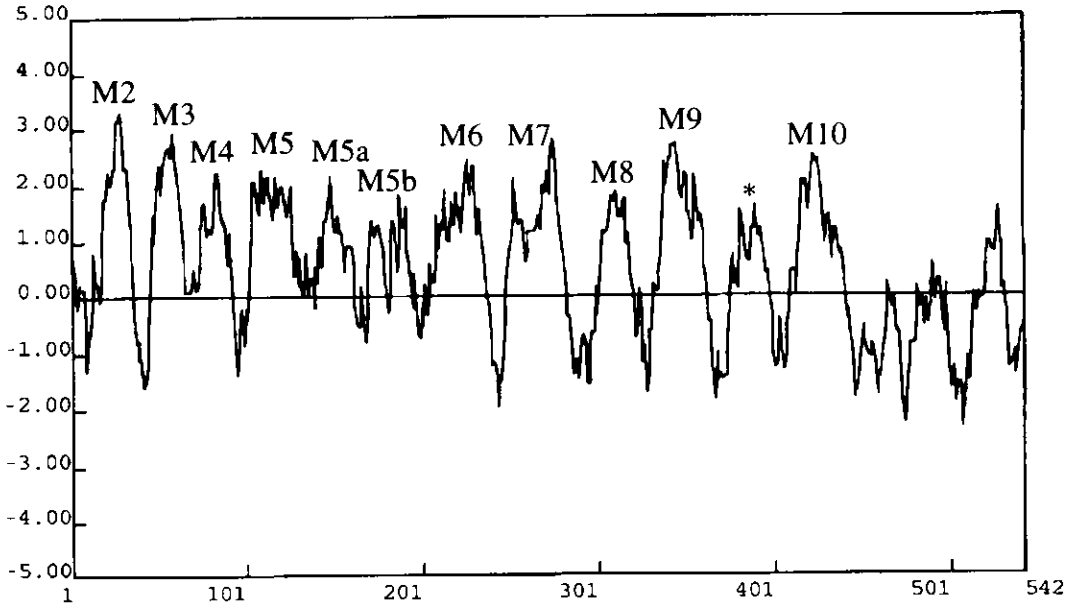


Fig. 10 Deduced amino acid sequence of *InNHX1*.
A, The deduced amino acid sequences of NHX1 from *I. nil* (*InNHX1*, accession No.BAB16380.1), *A. thaliana* (*AtNHX1*, AAD16946.1), *O. sativa* (*OsNHX1*, BAA83337.1), *P. hybrida* (*PtNHX1*), and *S. cerevisiae* (*ScNHX1*, NP_010744) were aligned by using ClustalW. Dots on the alignment indicated homologous region within plants used for alignment. Hydrophobic regions predicted from hydropathy prot (B) are underlined and predicted membrane spanning regions are indicated acccording to Nass *et al.* (1997) and Wells *et al.* (2000). B, Hydropathy plot (Kyte and Doolittle 1982) of deduced amino acid sequence of the *Pr* gene. Numerals indicate hydrophobic regions described above.

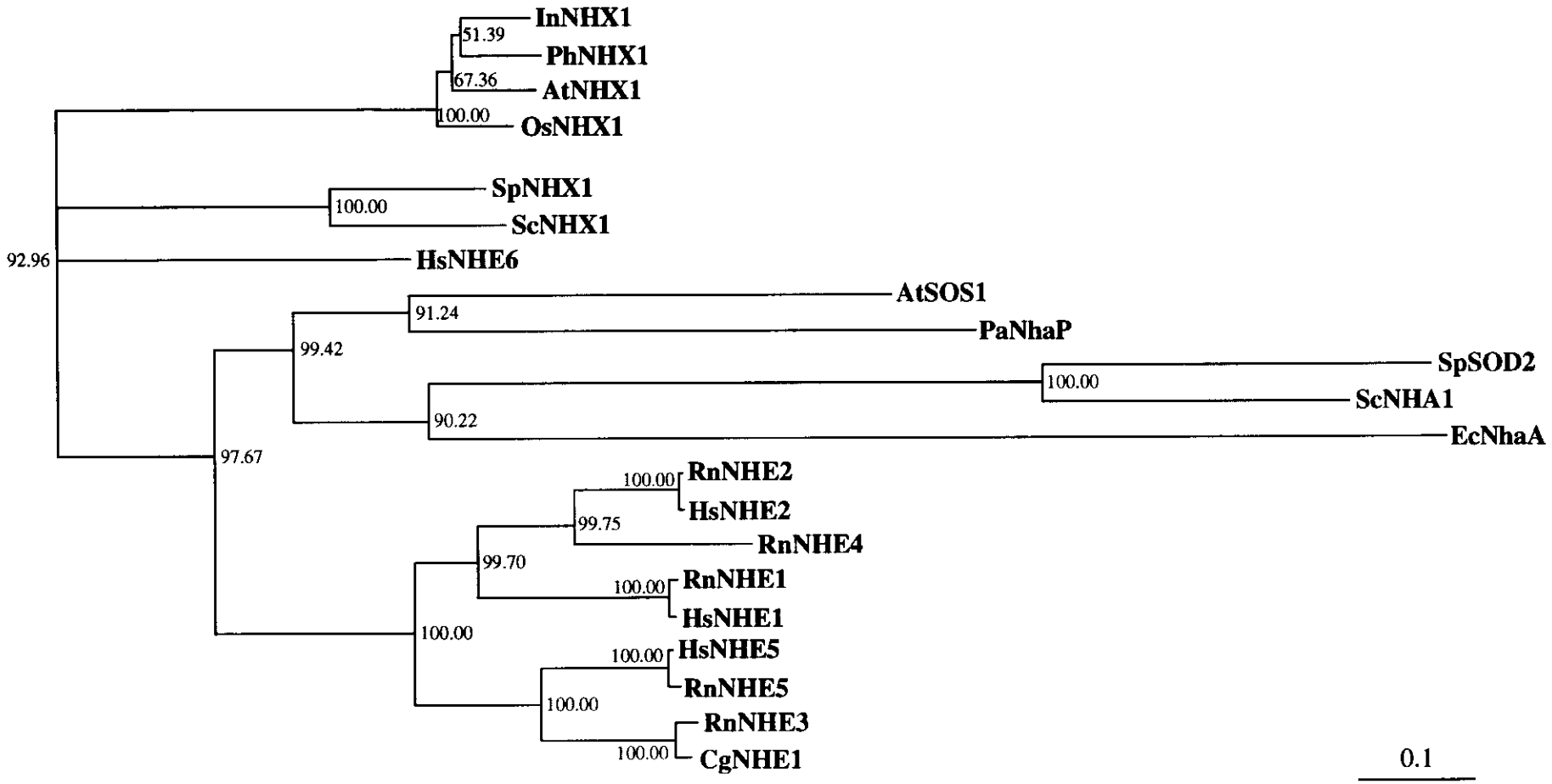


Fig. 11 Phylogenic analysis of Na^+/H^+ exchengers.

Multiple sequence alignment and generation of phylogenic tree were performed with Clustal W and GENETYX MAC software. Gapped regions of aligned sequences were deleted, and evolutional distances were calculated by Neighbor Joining methods and expressed with length of the lines. Bootstrap values (10,000 replicates) were expressed as percentage. InNHX1 (accession No. BAB16380.1), *Ipomoea nil*; PhNHX1, *Petunia hybrida*; AtNHX1(AAD16946.1), AtSOS1 (AAF76139), *Arabidopsis thaliana*; OsNHX1 (BAA83337.1), *Oriza Sativa*; SpNHX1 (CAB10103.1), SpSOD2 (CAA77796.1), *Schizosaccharomyces pombe*; ScNHX1 (NP_010744), ScNHA1 (NP_013239), *Saccharomyces cerevisiae*; HsNHE1 (P19634), HsNHE2 (AAD41635), HsNHE5 (AAC98696.1), HsNHE6 (NP_006350), *Homo sapiens*; RnNHE1 (AAA98479), RnNHE2 (AAA98479.1), RnNHE3 (AAA41702.1), RnNHE4 (P26434), RnNHE5 (AAD 16413.1); *Rattus. norvegicus*; PaNhaP (BAA31695); *Pseudomonas aeruginosa*; EcNhaA (P13738), *Escherichia coli*; CgNHE1 (P48761), *Cricetulus griseus*.

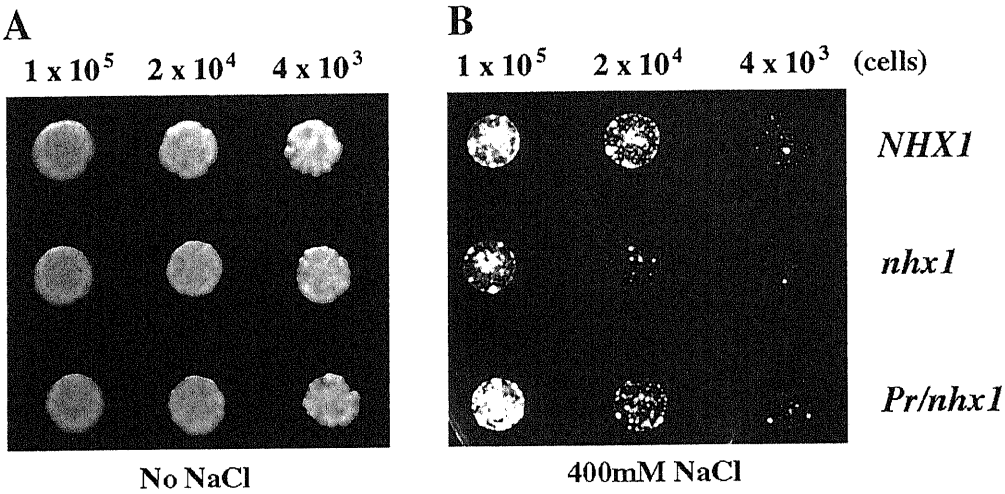


Fig. 12 Complementation of NaCl tolerance by the *Pr* gene in the *nhx1* yeast mutant. Serial dilutions (starting at 10^5 cells) of the yeast strains indicated were grown at 30°C for 3 days on APG medium without NaCl (A) or for 4 days on APG supplemented with 400 mM NaCl (B). *NHX1*, *nhx1*, and *Pr/nhx1* indicate K601 carrying pINA147, R100 containing pINA147, and R100 harboring pINA151, respectively.

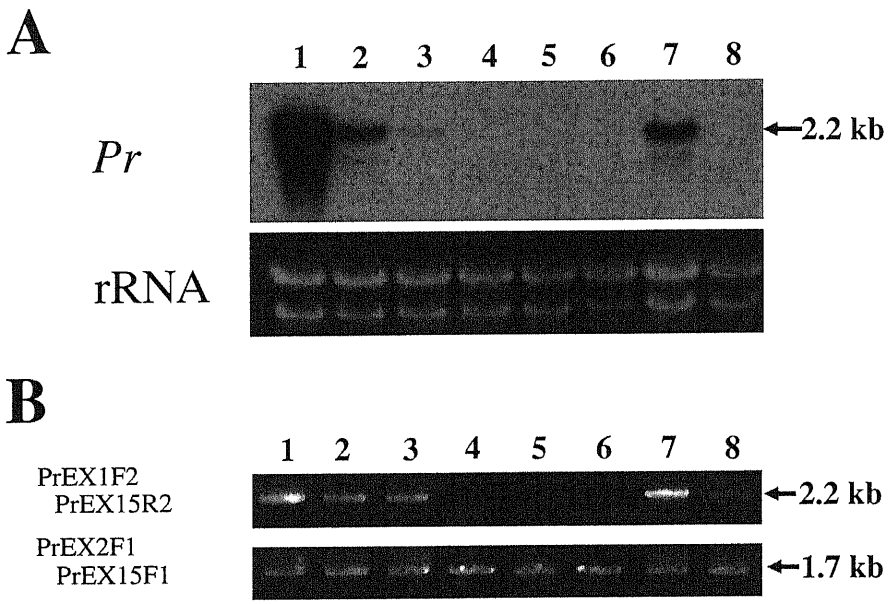
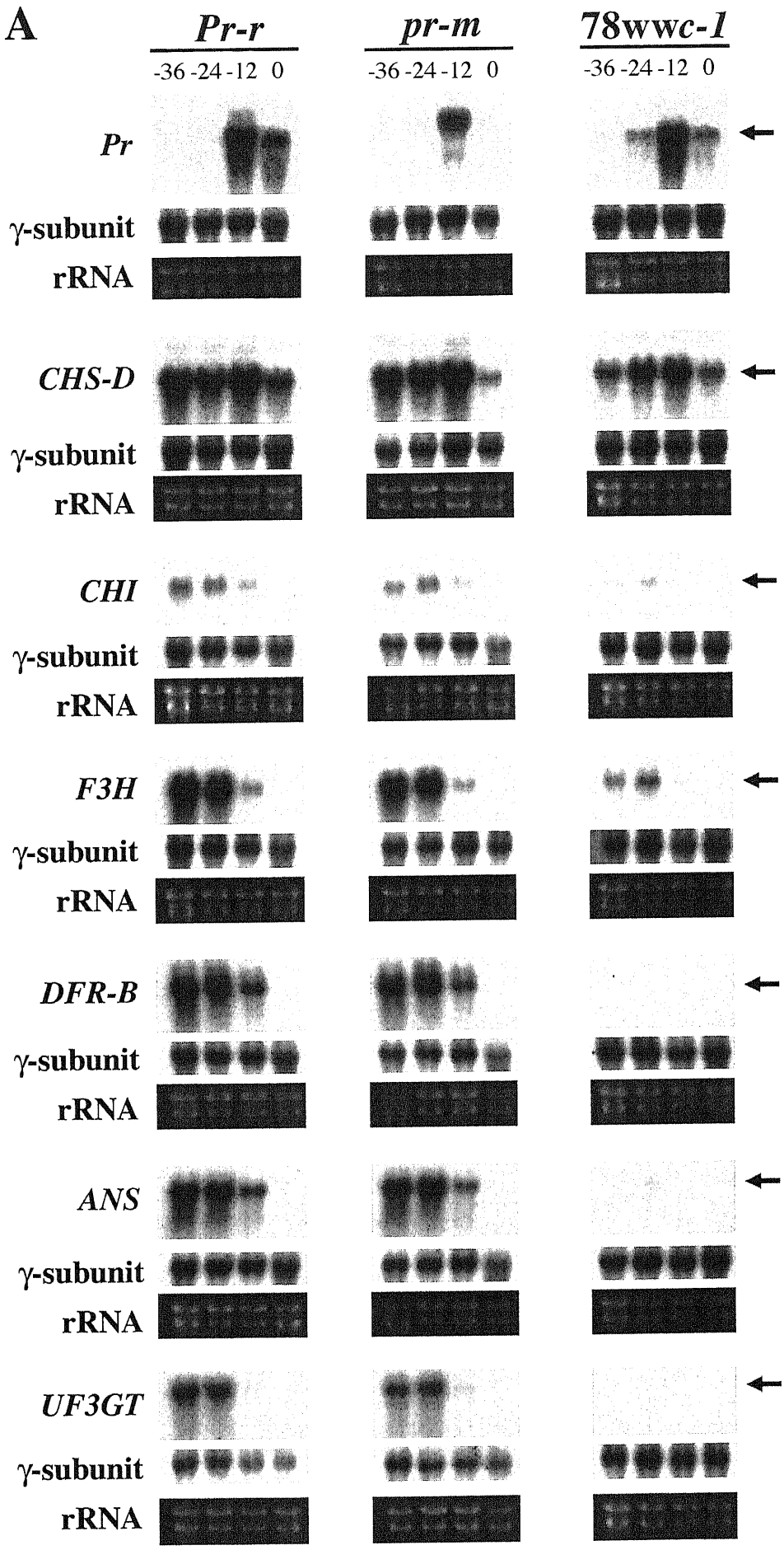


Fig. 13 Expression of the *Pr gene* in various tissues.
A, Northern blot analysis (above). Total RNAs (10 μg) prepared from flower limbs (1), flower tubes (2), sepals (3), leaves (4), stems (5), roots (6), stamens (7) and pistils (8) of the *Pr-r* line were subjected to Northern blot analysis using the *Pr* cDNA as a probe. The RNAs from floral tissues were obtained at 12 h before flower opening. The ethidium bromide-stained rRNA bands are shown as a loading control (below).
B, RT-PCR analysis. RT-PCR amplification (above) was performed with the primers PrEX1F2 and PrEX15R2, and subsequent nested PCR amplification (below) was with the primers PrEX2F1 and PrEX15R1 described in Fig. 8. The arrows indicate the molecular size of detected bands.


A
Pr-r
pr-m
78wwc-1
-36 -24 -12 0
-36 -24 -12 0
-36 -24 -12 0
Pr
γ-subunit
rRNA
CHS-D
γ-subunit
rRNA
CHI
γ-subunit
rRNA
F3H
γ-subunit
rRNA
DFR-B
γ-subunit
rRNA
ANS
γ-subunit
rRNA
UF3GT
γ-subunit
rRNA

B

Fig. 14 Temporal expression of the *Pr* gene and its regulation.

A, Total RNAs (10 μg) prepared from the flower petals of the *I. nil* lines indicated were subjected to Northern blot analysis using the probes indicated. The arrows point to the mRNAs examined. –36, -24, -12, 0 indicate that 36 h, 24 h, 12 h before flower opening and opened flower, respectively. The γ-subunit probe of the sweet potato mitochondrial F_1F_0 ATP synthase was used as an internal control. The ethidium bromide-stained rRNA bands are shown as a loading control.

B, Fully opened flower of 78ww*c-1*.

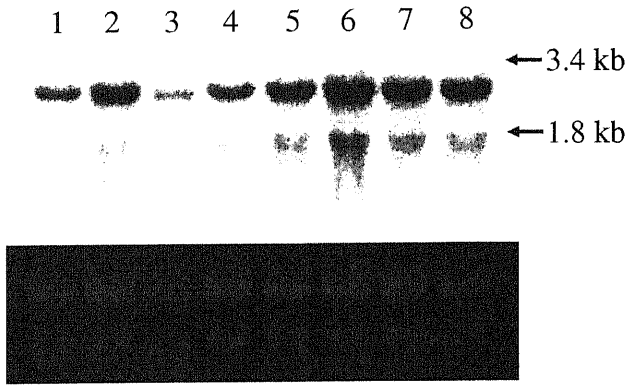


Fig. 15 Spatial and temporal expression of the *PhNHX1* gene in *P. hybrida*.
Total RNAs (10 μg) from *P. hybrida* cv. *Surfinia Purple mini* were analyzed by Northern blot hybridization using the *PhNHX1* cDNA as a probe (above). RNAs used were: leaves (1), stems (2), sepals (3), petals of closed buds 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), opened flowers with all anthers dehisced (8). The ethidium bromide-stained rRNA bands are shown as a loading control (below).

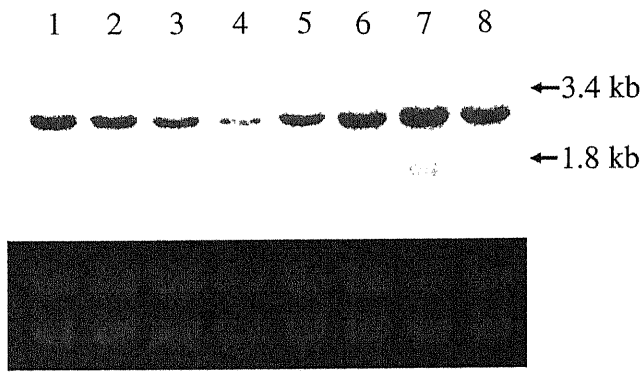


Fig. 16 Expression of the *PhNHX1* gene in various petunia lines.
Total RNAs (10 μg) from petals of pigmented buds with emerging corolla were subjected to Northern blot analysis using the *PhNHX1* cDNA as a probe (above). Plants used were: MO59 (*ph1*) (1), V26 (*ph1*) (2), Pr57 (*ph2*) (3), Rw14 (*ph2*) (4), Titan Red (5), Surfinia Purple Mini (6), Surfinia Violet Mini (7), Surfinia Light Blue Mini (8). The ethidium bromide-stained rRNA bands are shown as a loading control (below).

Genes encoding the vacuolar Na^+/H^+ exchanger and flower coloration

Toshio Yamaguchi[1,2], Sachiko Fukada-Tanaka[1], Yoshishige Inagaki[1], Norio Saito[3], Keiko Yonekura-Sakakibara[4], Yoshikazu Tanaka[4], Takaaki Kusumi[4] and Shigeru Iida[1,2,5]

[1] *National Institute for Basic Biology, Okazaki, 444-8585, Japan*

[2] *Department of Molecular Biomechanics, The Graduate University for Advanced Studies, Okazaki, 444-8585, Japan*

[3] *Chemical Laboratory, Meiji-gakuin University, Totsuka-ku, Yokohama, 244-8539, Japan*

[4] *Institute for Fundamental Research, Suntory Ltd., Wakayamadai, Shimamoto-cho, Mishima-gun, Osaka, 618-8503, Japan*

[5] Corresponding author: E-mail, shigiida@nibb.ac.jp, Fax, +81-564-55-7685

Abstract

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.

Footnotes:

Corresponding author: Shigeru Iida
National Institute for Basic Biology, Okazaki, 444-8585, Japan
E-mail, shigiida@nibb.ac.jp, Fax, +81-564-55-7685

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

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 (3bp CAG insertion) generated by the excision of *Tpn4*, respectively.

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 (*Saccharomyses 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 described before (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 the 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 described before (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 described before (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 described previously (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 no. 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 sec), annealing (55°C for 30 sec) 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 described before (Habu et al. 1998, Fukada-Tanaka et al. 2000, Fukada-Tanaka et al. 2001). From these λ clones, 4 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 2 clones hybridized with the probe B but not with the 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 homologues 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 reverse-transcription (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 subsequent 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℃ for 1 min, 32 cycles consisting of denaturation (94°C for 15 sec), annealing (55°C for 30 sec) 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'-GTATGTTTTCGGGAGGGATTGGAATGG-3') and PrEX15R1 (5'-CTGCGGCCGCTCATCTAGGGCTCTGCTCAACTGGT-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-bp 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'-CTATTGGCACACTTATTAGCTGTTC-3') and PrEX9R1 (5'-AAGTAAGACATGAGCATCATAAG-3'), 293-bp and 143-bp fragments are produced from the 582-bp 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 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 values (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 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 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-kb 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 no. 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 no. 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 the exons 1, 2, 6, 7 and 8, and 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 no. 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 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 characterized previously (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 the probe A but also the 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 two-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, *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 an 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 grow 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 homologues 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 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 homologues 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 discrete insertions, and 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 (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 two- to four-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 showed 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 (Figs. 6 and 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 the *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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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 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).

Figure legends

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.

Fig. 2 HPLC analysis of the pigments in the *pr-m* **(A)** and *Pr-r* **(B)** flowers. The analysis was performed as described previously (Lu et al. 1992). The pigment **8** is heavenly blue anthocyanin (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, 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.

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-m*, respectively. The arrows with *Pr* and *psd-Pr* point to the 7.5-kb 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.

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-kb and 1.7-kb amplified fragments, respectively.

(C) Northern blot analysis. Poly(A)$^+$ RNAs (1 μg) obtained from two-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 h **(1)**, at 24 h **(2)**, at 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.

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 no. AB054979), *AtNHX1* (Gaxiola et al. 1999, Darley et al. 2000), *OsNHX1* (Fukuda et al. 1999), *PhNHX1* (this study, Accession no. AB051817), *NcNHX1* (this study, Accession no. AB051818), *ThNHX1* (this study, Accession no. AB051819), *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 shadowed boxes with the numerals or the asterisk under the *ScNHX1* map indicate the regions encoding hydrophobic putative transmembrane domains described in *ScNHX1* before (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.

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). 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)**, 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)**, 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.

Fig. 1 Yamaguchi *et al.*

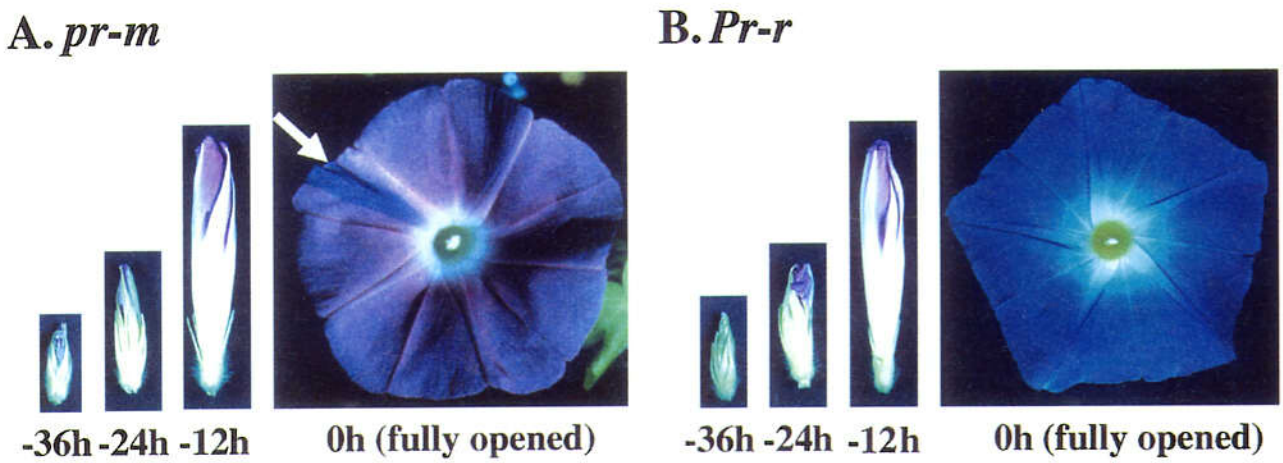
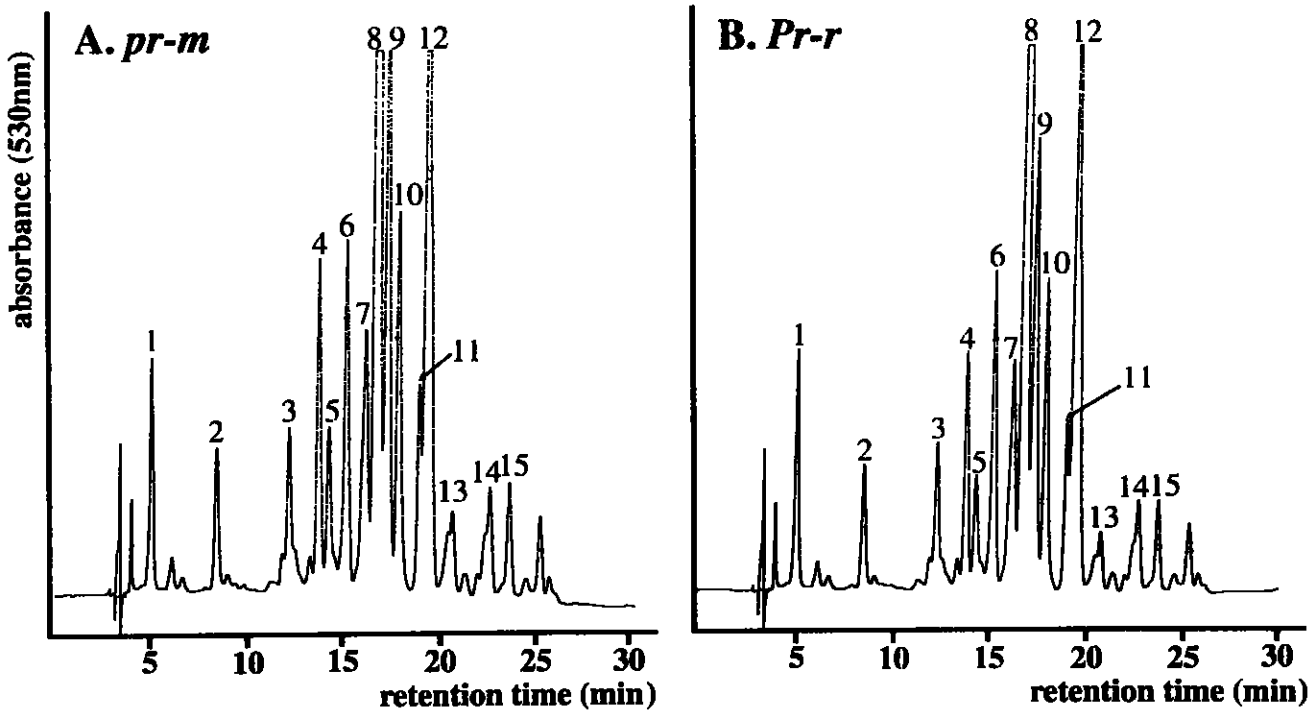


Fig. 2 Yamaguchi *et al.*



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. 3 Yamaguchi *et al.*

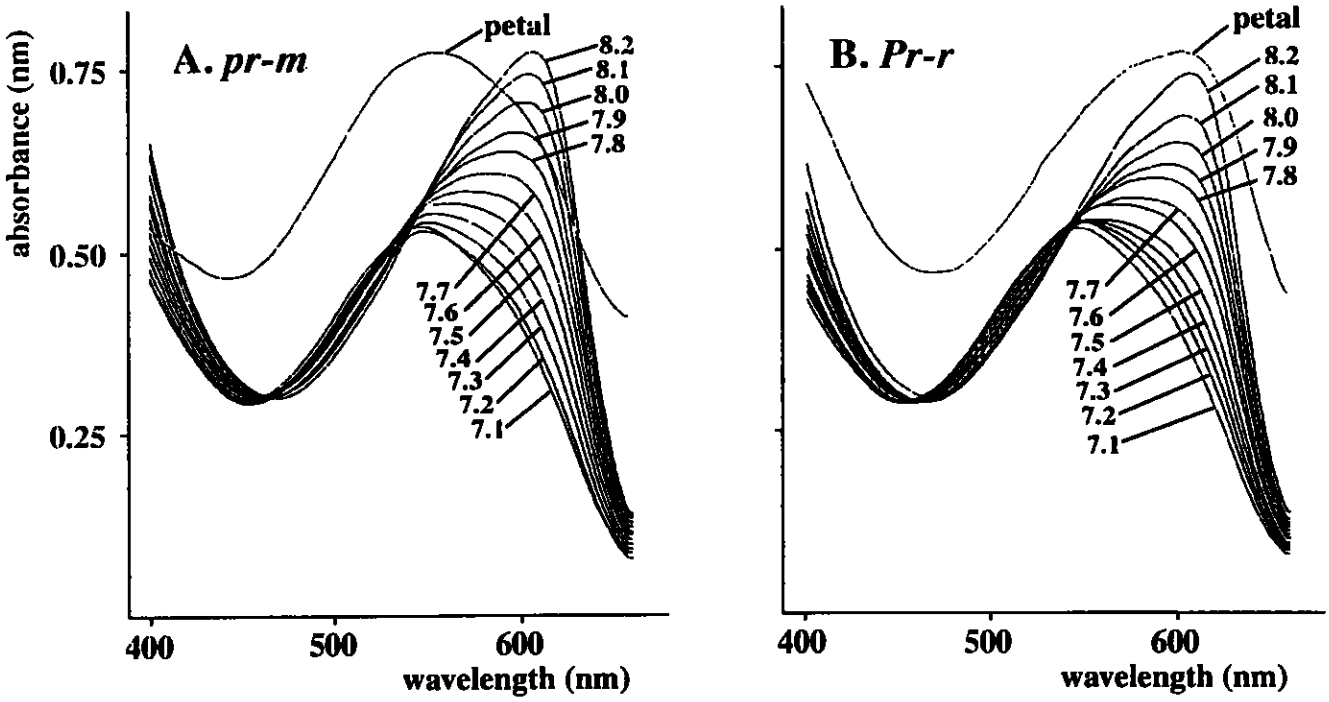


Fig. 4 Yamaguchi *et al.*

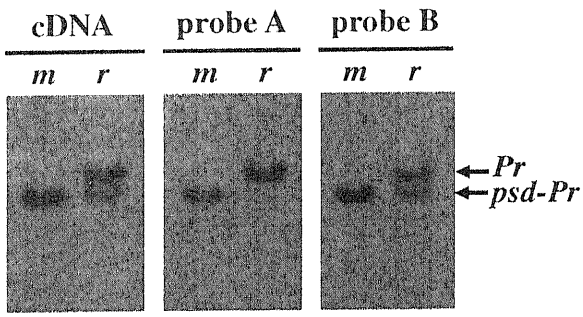


Fig. 5 Yamaguchi *et al.*

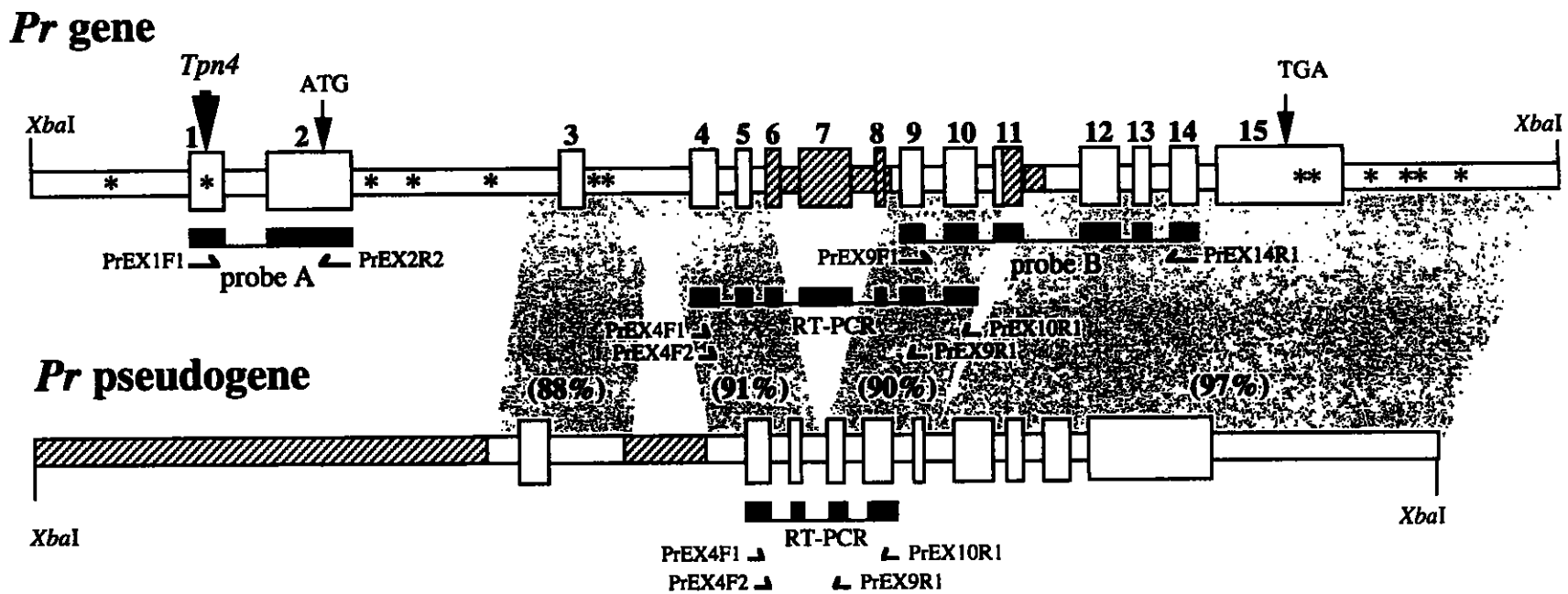


Fig. 6 Yamaguchi *et al.*

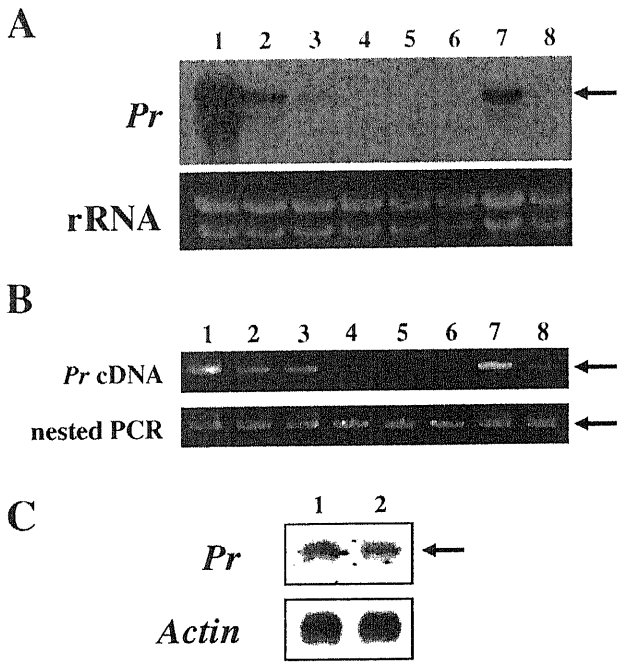


Fig. 7 Yamaguchi *et al.*

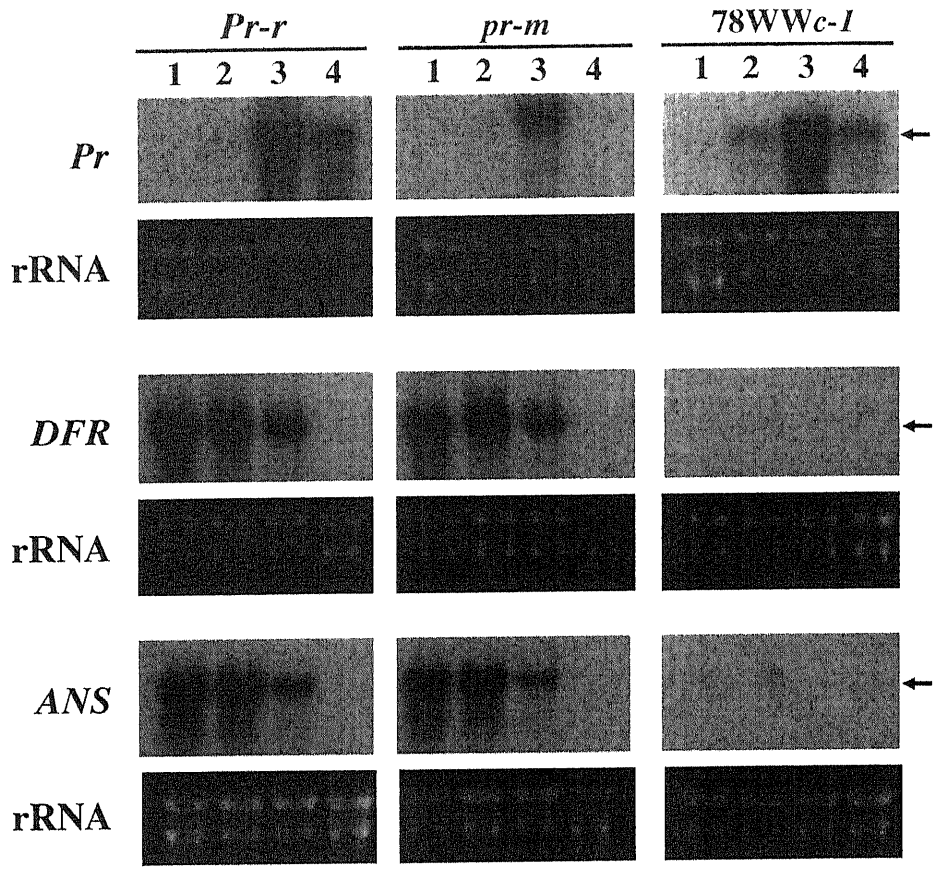


Fig. 8 Yamaguchi *et al.*

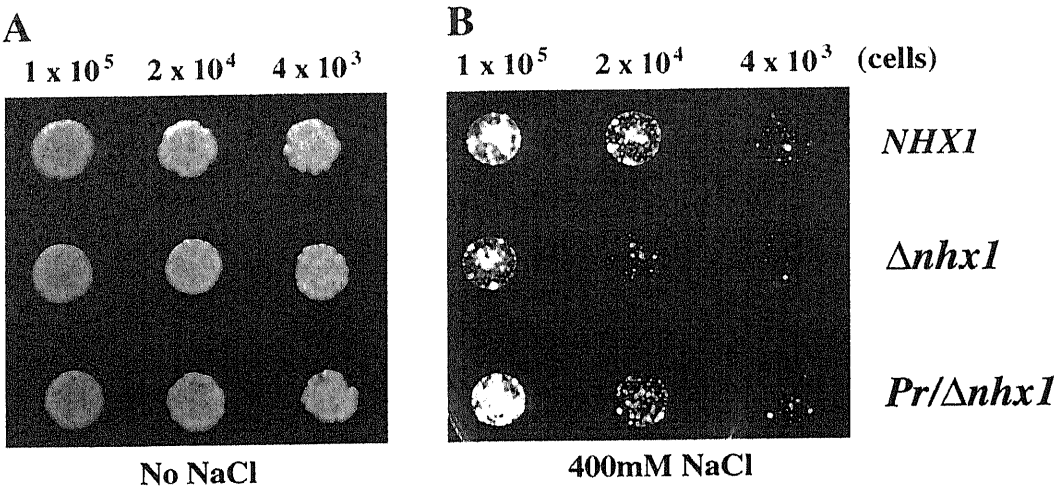


Fig. 9 Yamaguchi *et al.*

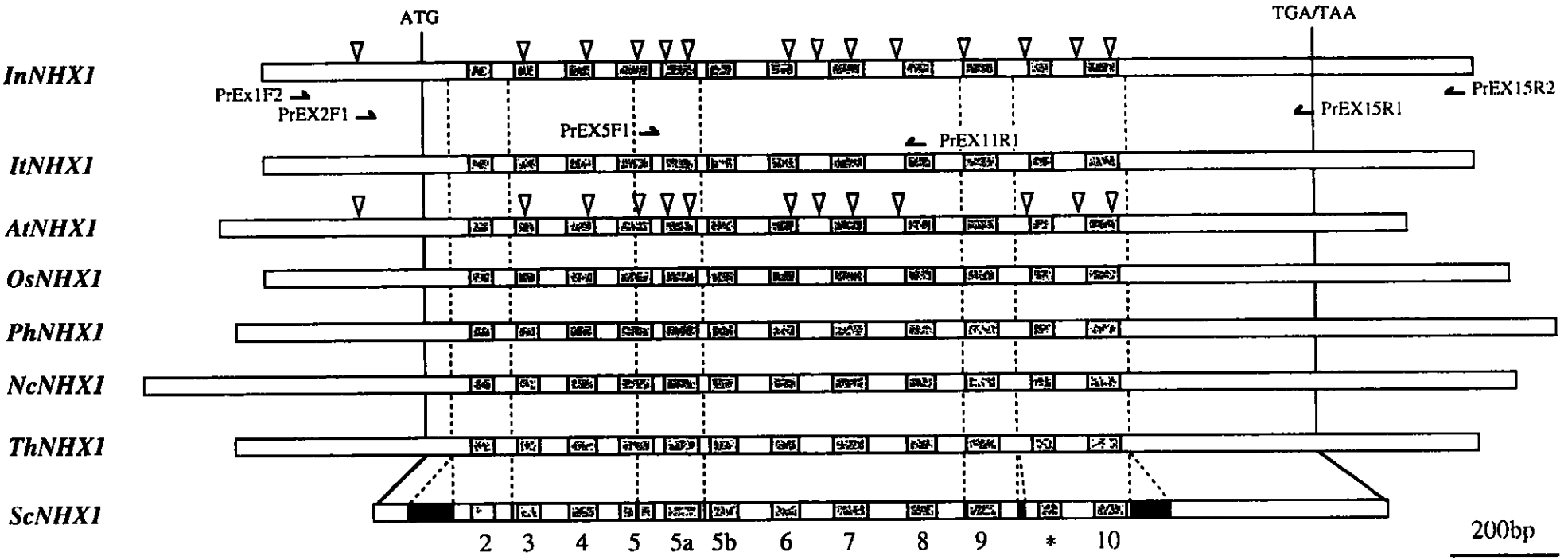


Fig. 10 Yamaguchi *et al.*

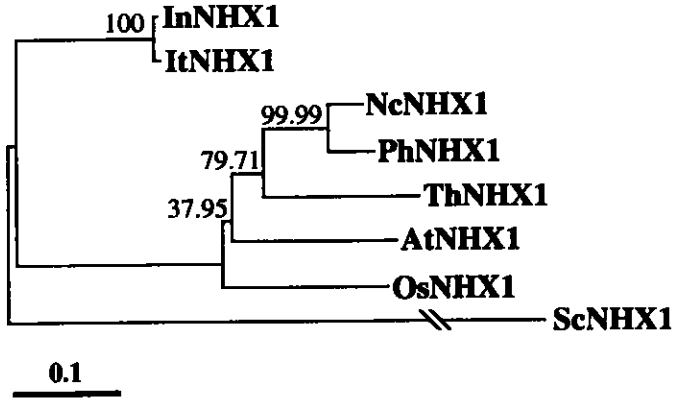
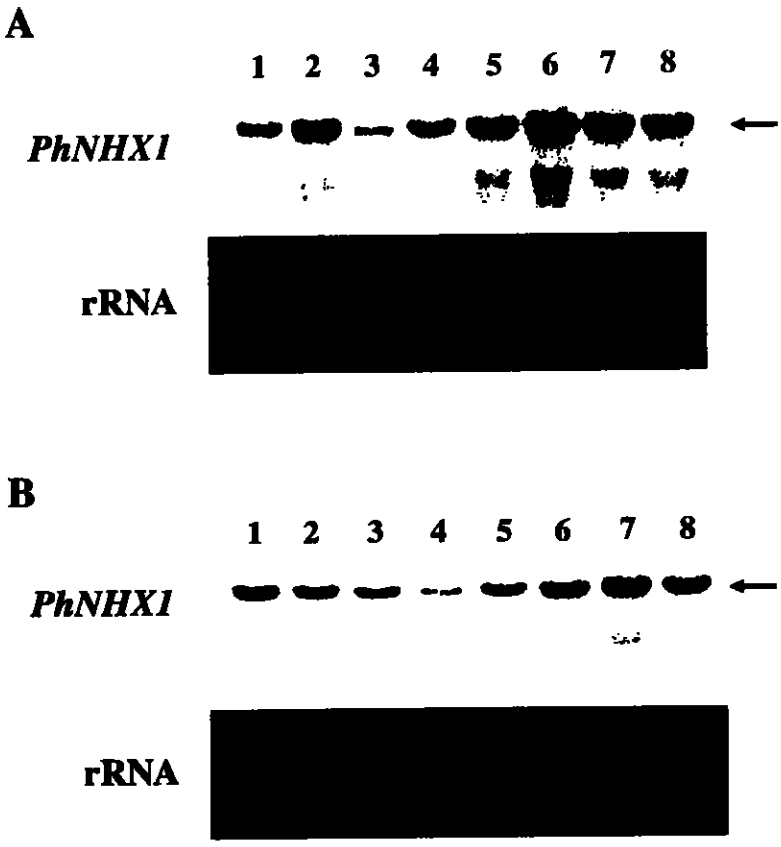


Fig. 11 Yamaguchi *et al.*



Plant Biotechnology: An Original Paper

Simplified Transposon Display (STD): a new procedure for isolation of a gene tagged by a transposable element belonging to the *Tpn1* family in the Japanese morning glory.

Sachiko FUKADA-TANAKA[1], Yoshishige INAGAKI[1], Toshio YAMAGUCHI[2] and Shigeru IIDA[1,2,*]

[1] *National Institute for Basic Biology, Okazaki, 444-8585, JAPAN*

[2] *Department of Molecular Biomechanics, The Graduate University for Advanced Studies, Okazaki, 444-8585, JAPAN*

Running title: Simplified transposon tagging procedure

*Corresponding author:
National Institute for Basic Biology, Okazaki, 444-8585, JAPAN
Tel: 0564-55-7680; Fax: 0564-55-7685; E-mail: shigiida@nibb.ac.jp

(3 Figures, No Table)

Abstract

Transposable elements are regarded as a powerful mutagen and as an effective tool to isolate genes tagged by transposon insertions. In the Japanese morning glory, a number of spontaneous mutants related to the colors and shapes of the flowers have been isolated. The plant contains around 500-1000 copies of an *En/Spm*-related element *Tpn1* and its relatives, which act as major spontaneous mutagens. We have developed a new protocol for identifying genes tagged by insertion of *Tpn1*-related elements. The procedure, named simplified transposon display (STD), is simple and requires neither biotinylated oligonucleotides nor streptavidin-capturing which are essential in other transposon display methods published recently. Here we describe the details of STD used for identification of the *Purple* (*Pr*) gene that encodes a vacuolar Na^+/H^+ exchanger for increasing vacuolar pH responsible for blue flower coloration.

1. Introduction

The Japanese morning glory (*Ipomoea nil* or *Pharbitis nil*) is a traditional floricultural plant in Japan, and a number of spontaneous mutants related to the colors and shapes of its flowers have been isolated since the 17th century (Imai, 1927; Iida *et al.*, 1999). Several lines of evidence suggest that an *En/Spm*-related element *Tpn1* and its relatives, which we termed *Tpn1*-family elements, are major source of these spontaneous mutations (Iida *et al.*, 1999). *Tpn1* and its relatives carrying 28-bp terminal inverted repeats (TIRs) are present at about 500-1000 copies per haploid genome of the Japanese morning glory (Inagaki *et al.*, 1994; Kawasaki and Nitasaka, 1998). We have previously developed an amplified restriction fragment length polymorphism (AFLP)-based mRNA fingerprinting (AMF) procedure which is based on the systematic comparison of differently expressed transcripts in the same tissue in different lines (Habu *et al.*, 1997). We have succeeded in applying AMF for the identification of a new mutable allele caused by integration of a transposable element into an anthocyanin biosynthesis gene (Fukada-Tanaka *et al.*, 1997; Habu *et al.*, 1998). Since transposon mutagenesis has become a powerful tool for the isolation of genes of interest (Kunze *et al.*, 1997), we have attempted to develop a new protocol for identifying tagged genes by insertion of *Tpn1*-related elements in the Japanese morning glory. Our transposon tagging method was based on our AMF procedure (Habu *et al.*, 1997), and we chose the mutable gene, *purple-mutable* (*pr-m*), which confers purple flowers with blue sectors (Imai, 1934). The flower

variegation is regarded to be due to recurrent somatic mutation from the recessive purple to the blue revertant allele, *Purple-revertant* (*Pr-r*) and we assumed that the *pr-m* allele is caused by insertion of a *Tpn1*-family element. Indeed, the mutation *pr-m* was recently found to be caused by integration of a *Tpn1*-related element, *Tpn4*, into the *Pr* gene (Fukada-Tanaka *et al.*, 2000).

While we were characterizing the *Pr* gene which encodes a vacuolar Na^+/H^+ exchanger for increasing vacuolar pH and is responsible for blue flower coloration (Fukada-Tanaka *et al.*, 2000), similar methods to identify genes tagged by transposon insertions were reported (Frey *et al.*, 1998; Van den Broeck *et al.*, 1998; Yephremov and Saedler, 2000). Important and critical steps in all of these published methods are the use of biotinylated linkers or biotinylated primers for polymerase chain reaction (PCR) amplification and streptavidin-capturing for increasing selectivity of fragments derived from transposon flanking sequences containing the tagged genes. Contrary to these methods, the procedure we developed is simple, reproducible and requires neither biotinylated oligonucleotides nor streptavidin-capturing to improve the selectivity of amplification reactions. We would thus like to call our procedure simplified transposon display (STD). This paper describes details of STD used for identification of the mutable *Pr* gene.

2. Materials and Methods

2.1 Plant material

Three lines of the Japanese morning glory with blue flowers carrying the wild-type *Pr* gene, *Pr-w*, and three mutable *pr-m* lines bearing purple flowers with blue sectors were from our collection. From these *pr-m* lines, we obtained germinal revertants producing blue flowers. Since such revertants are usually heterozygotes (*Pr-r/pr-m*), we selfed and obtained siblings with different genotypes (*pr-m/pr-m*, *Pr-r/pr-m* and *Pr-r/Pr-r*). The heterozygotes (*Pr-r/pr-m*) and homozygotes (*Pr-r/Pr-r*) were assigned by the flower phenotypes of their selfed progeny. Siblings with three different genotypes (*pr-m/pr-m*, *Pr-r/pr-m* and *Pr-r/Pr-r*) were also obtained in the selfed progeny of hybrids between a mutable *pr-m* line (*pr-m/pr-m*) and a revertant homozygote (*Pr-r/Pr-r*).

2.2 Simplified transposon display (STD)

The strategy for STD is outlined in Fig. 1. Genomic DNA was isolated from young leaves of the Japanese morning glory with Plant DNAZOL Reagent (GIBCO BRL). DNA (125 ng) was cleaved with *Mse*I and subsequently ligated to 88 pmol *Mse*I adapter (5'-GACGATGAGTCCTGAG-3' and 5'-TACTCAGGACTCAT-3') as described before (Habu *et al.*, 1997). The ligated DNA sample was 10-fold diluted with TE (10 mM Tris-HCl pH 7.5, 1 mM Na_2EDTA pH 7.5). We employed the TIR primer (5'-TGTGCATTTTTCTTGTAGTG-3'), and the *Mse*I primer (5'-GACGATGAGTCCTGAGTAA-3') for pre-amplification by PCR. The pre-amplification was performed in 20 µl reaction mixture containing 2 µl of 10-fold diluted DNA

sample, 4.8 pmol TIR primer and 4.8 pmol *Mse*I primer for 20 cycles consisting of denaturation (94°C for 30 sec), annealing (56°C for 60 sec) and extension (72°C for 60 sec). The reaction mixture was again 10-fold diluted with TE. For subsequent selective PCR amplification, we used the TIR+N primers (5'-TGTGCATTTTTCTTGTAGTGN-3', where N represents A, C, G or T) labeled at their 5'-ends with rhodamine or fluorescein and the non-labeled *Mse*I+N primers (5'-GATGAGTCCTGAGTAAN-3'). The rhodamine-labeled primers were synthesized by TaKaRa Biomedicals, and the fluorescein-labeled primers were prepared by Vistra fluorescence 5'-oligolabeling kit (Amersham Pharmacia Biotech). We employed FMBIO II Multi-View (Hitachi Software Engineering) to detect these labeled dyes. Selective PCR amplification was carried out in 20 µl reaction mixture containing 2 µl of 10-fold diluted DNA sample, and either 2.4 pmol rhodamine-labeled TIR+N primers and 4.8 pmol *Mse*I+N primers or 4.8 pmol fluorescein-labeled TIR+N primers and 9.6 pmol *Mse*I+N primers were added in the reaction mixture. We used a higher concentration of the fluorescein-labeled primers because they gave weaker signals when analyzed by FMBIO II. The TIR+N primers labeled with ^{32}P at their 5' ends can also be used as described in the AMF procedure (Habu *et al.*, 1997). The first cycle consisted of denaturation (94°C for 30 sec), annealing (65°C for 30 sec) and extension (72°C for 60 sec). For the following 12 cycles, the annealing temperature was reduced by 0.7°C per cycle. The final 20 cycles included denaturation (94°C for 30 sec), annealing (56°C for 60 sec) and extension (72°C for 60 sec). The reaction products were

concentrated by ethanol precipitation and analyzed on a 5% Super Reading DNA Sequence Solution (Toyobo), 6 M urea sequencing gel, and the bands were detected by FMBIO II Multi-View. To isolate a band from the gel, reamplification of the recovered fragment by PCR with the TIR and *Mse*I primers and cloning of the reamplified fragment were performed as described by Habu *et al.* (1997).

2.3 Characterization and cloning of the Pr gene

Southern and Northern blot analyses were carried out as described before (Habu *et al.*, 1997; 1998) with the 130 bp DNA fragment obtained by STD as a probe. The probe was labeled by PCR amplification with TIR and *Mse*I primers in 25 µl reaction mixture containing 50 µM dATP, 50 µM dGTP, 50 µM dTTP, 5 µM dCTP, and 0.7 µM [α-^{32}P] dCTP (1.85 MBq) in the same way as in the pre-amplification described above.

Using the same 130 bp probe, two positive λZapII (Stratagene) clones were isolated from approximately 6,000,000 recombinant plaques in a cDNA library prepared from flower buds of the *Pr-w* line in the same way as described previously (Habu *et al.*, 1997; Fukada-Tanaka *et al.*, 1997). One of them contained the entire open reading frame (ORF) for the *Pr* gene (accession No. AB033989).

Using the *Pr* cDNA labeled with Rediprime II DNA Labeling System (Amersham Pharmacia Biotech) as a probe, we isolated 58 positive λZAP Express (Stratagene) clones from about 4,000,000 recombinant plaques in an *Xba*I-digested genomic library from a wild-type *Pr-w* plant as described before (Habu *et al.*, 1998). One of these λ clones, λZExp:Pr-

w1 carrying an approximately 7.5 kb fragment which contains all the exon region, was cloned (accession No. AB033990). Using the same *Pr* cDNA as a probe, we isolated 12 positive λDASHII (Stratagene) clones from about 1,000,000 recombinant plaques in a partially *Sau*3AI-digested genomic library from the mutable *pr-m* plant. One of these λ clones, λDAII:pr-m1 containing a large insert, was characterized further. DNA sequences were determined with the DNA sequencer ABI 377 (Applied Biosystems).

2.4 PCR amplification for examining the genotypes of the Pr *gene.*

To distinguish among the three different genotypes (*pr-m/pr-m, Pr-r/pr-m* and *Pr-r/Pr-r*) in the F2 progeny derived from a hybrid between a mutant (*pr-m/pr-m*) and a revertant (*Pr-r/Pr-r*), we employed PCR amplification using either one of the two primer sets: EX1FW (5'-GAAACAGAAAAAGAGAGAGTCACG-3') and EX2RV (5'-CAATGTCGTGGTTTCTGTTCACATA-3') or TIR and EX2RV (see Fig. 3d). The PCR amplification was performed in 20 μl reaction mixture containing 50 ng of genomic DNAs, 10 pmol. each of appropriate primers for 30 cycles in the same way as in the pre-amplification described above. After the reaction, a portion of the reaction mixture was analyzed on a 2% (w/v) agarose gel.

3. Results and discussion

3. 1 General outline of STD.

Fig. 1 shows the strategy of STD. The genomic DNA was cleaved with a 4-base cutter enzyme *Mse*I and the *Mse*I adapter was ligated to each end of the fragments. Other 4-base cutter enzymes that produce 2-base sticky ends (e.g. *Taq*I, *Msp*I, *Bfa*I and *Hin*P1I) can also be used with appropriate adapters and primers. To enrich the fragment containing flanking sequences of the integrated transposable element *Tpn1* and its relatives, we employed the 20 bp TIR primer which corresponds to the outermost 20 bp sequence of the 28 bp TIR of *Tpn1* (Inagaki *et al.*, 1994; Hoshino *et al.*, 1995). Like our AMF procedure (Habu *et al.*, 1997), we employed two-step amplifications, pre-amplification and selective amplification with selective primers of 16 combinations since the primers used (*Mse*I+N and TIR+N primers) have a single selective nucleotide at their 3' ends. Only the selective TIR primers were labeled at the 5' ends, which ensures visualization of PCR-amplified fragments containing the flanking sequences of the *Tpn1*-related elements on the sequencing gel. The combination of these primers allowed us to reduce the complexity of bands to be visualized and to screen the bands of interest systematically. As FMBIO II Multi-View can detect the rhodamine and fluorescein signals independently, two samples labeled with different dyes can be applied on the same well. We can thus analyze 96 samples simultaneously in a sequencing gel with 48 wells.

Only around 100-500 bp amplified DNA fragments could be displayed reproducibly because of the gel resolution, and the efficiency of amplification in larger DNA fragments might vary in the PCR

amplification conditions used. Approximately 40 visualized bands could be detected in each reaction. We also carried out similar experiments using another 4-base cutter enzyme, *Taq*I, and about 40 visualized bands were detected in each reaction. Using selective primers in 16 different combinations, we were able to monitor approximately 640 bands that must correspond to 640 different flanking sequences of integrated *Tpn*1-family elements. It should be noted that two tagged fragments were generated from a single tagged site because the TIR+N primers could be hybridized to both termini of the *Tpn1*-related elements (Fig. 1). It would thus increase the probability of generating a tagged fragment of around 100-500 bp, which can be visualized in the sequencing gel. Indeed, the tagged fragment produced from the other end of *Tpn4* was too small to be detected in the gel system used. Since the genome of the Japanese morning glory is thought to carry around 500-1000 copies of *Tpn*1-related elements, or 1000-2000 TIR flanking sequences (Kawasaki and Nitasaka, 1998), it would be advisable to employ several different 4-base cutter enzymes. Although we used the selective primers with a single selective nucleotide at their 3′ ends (e.g. *Mse*I+N and TIR+N), the number of the selective nucleotides to be used may depend upon the copy numbers of the transposable elements employed: the higher the copy number of the elements in the genome, the larger the number of the selective nucleotides at the 3′ ends that should be considered.

3. 2 Application of STD for identifying the mutable pr-m gene.

We have successfully applied STD to identifying the mutable *pr-m* gene. We searched for a band present in the plants carrying the *pr-m* allele and absent in the plants with the homozygous *Pr-r* allele. We first employed 13 homozygotes (*Pr-r/Pr-r*), 4 heterozygotes (*Pr-r/pr-m*) and 16 mutable plants (*pr-m/pr-m*) derived from one mutable line. Only one band of about 130 bp, including the 20 bp primer and 19 bp adapter used, fulfilled all the criteria tested: present with the *pr-m* allele and absent with the homozygous *Pr-r/Pr-r* (Fig. 2). We also used 5 wild-type (*Pr-w/Pr-w*) plants from the 3 different lines and 8 additional mutable (*pr-m/pr-m*) plants from another line and found that the presence or absence of the candidate 130 bp band conformed to their genotypes (data not shown). Sequence analysis revealed that all of the 19 clones examined contained the identical 130 bp fragment.

By Northern blot analysis using the 130 bp fragment as a probe, we detected transcripts of around 2.3 kb in the *Pr-r* petals but not in the mutable *pr-m* flowers, indicating that the 2.3 kb transcripts are associated with the *Pr* gene (Fig. 3a). Using the same 130 bp probe, we isolated a recombinant clone from a cDNA library prepared from flower buds of the wild-type *Pr-w* line, KK/ZSK-2 (Inagaki *et al.*, 1994). The clone contained a 2,237 bp sequence carrying a 1,626 bp open reading frame. Its deduced amino acid sequence has high homology with vacuolar Na^+/H^+ exchangers in plants (Fukuda *et al.*, 1999; Gaxiola *et al.*, 1999; Fukada-Tanaka *et al.*, 2000). Comparison of the 130 bp sequence isolated by STD with the *Pr* cDNA sequence indicated that a *Tpn1*-related element is integrated into an exon at the 5′ untranslated

region (5′ UTR) and that there must be at least one intron between this untranslated exon and the exon containing the ATG initiation codon, because the 130 bp fragment consists of the 20 bp TIR primer, the 69 bp sequence corresponding to the 5′ UTR of the *Pr* cDNA, a 22 bp sequence apparently derived from the intron and the 19 bp *Mse*I primer. To test this hypothesis, we first compared the genomic structures of the revertant (*Pr-r/Pr-r*) and the mutable (*pr-m/pr-m*) lines by Southern blot analysis using the 130 bp fragment as a probe. Clear restriction fragment length polymorphism were seen in the *Eco*RI, *Xba*I and *Hin*dIII digests, suggesting that a large DNA rearrangement occurred at the *Pr* gene region (Fig. 3b). We cloned the 7.5 kb *Xba*I fragment from the wild-type *Pr-w* line containing the entire *Pr* gene and an approximately 15 kb segment from the mutable *pr-m* line which contains about 7 kb of a *Tpn1*-related element, *Tpn4*, and the complete coding region of the *Pr* gene. Comparison of the cDNA sequence with the entire genomic sequences from the *Pr-w* and *pr-m* lines indicated that the *Pr* gene comprises 15 exons and that *Tpn4* is integrated into the first exon (Fig. 3c). As expected, the 130 bp fragment contains both the 69 bp exon 1 and 22 bp intron 1 sequences (Fig. 3d). Sequences at both terminal regions of *Tpn4* confirmed that *Tpn4* is a *Tpn1*-related element of the *En/Spm* family because *Tpn4* contains the 28 bp TIRs and long subterminal repetitive regions characteristic of the *Tpn1*-related elements (Inagaki *et al.*, 1994; Hoshino *et al.*, 1995).

3. 3 Characterization of genotypes in the Pr *gene by PCR amplification*

Since the mutable *pr-m* gene has an insertion of *Tpn4*, it becomes easy to distinguish the heterozygotes (*Pr-r/pr-m*) from homozygotes (*Pr-r/Pr-r*), both of which produce blue flowers, by PCR amplification with appropriate primers. The presence of *Tpn4* at the insertion site can be determined by the appearance of the PCR-amplified fragment of 375 bp using the primers TIR and EX2RV, and its absence by detecting about 390 bp fragments using the primers EX1FW and EX2RV (Fig. 3d). By combining these PCR amplifications, we were able to assign three different genotypes of the siblings, *Pr-r/Pr-r*, *Pr-r/pr-m* and *pr-m/pr-m*, in the selfed progeny of the germinal revertant used for STD as well as the other two germinal revertants isolated independently (data not shown). We also characterized the genotypes of the *Pr* gene in the selfed progeny of a hybrid between a mutable *pr-m* line and a revertant *Pr-r* homozygote (Fig. 3e). All three mutable *pr-m* lines examined carry the identical *pr-m* allele with *Tpn4* insertion, indicating that they are derived from a common founder mutant.

3. 4 Comparison of STD with other procedures.

Transposable elements are regarded as a powerful mutagen and as an effective tool to isolate genes tagged by transposon insertions (Kunze *et al.*, 1997; Maes *et al.*, 1999). We have developed an AFLP-based transposon tagging procedure using the *Tpn1*-family elements in the Japanese morning glory, which carries 500-1000 copies of *Tpn*1-related

elements, and succeeded in isolating the *Pr* gene encoding a vacuolar Na^+/H^+ exchanger (Fukada-Tanaka *et al.*, 2000; this study). Recently, similar methods for identifying genes tagged by transposon insertions have been reported: (1) transposon display (TD) employing petunia *dTph1*-family elements that are present about 100-200 copies in the petunia genome (Van den Broeck *et al.*, 1998), (2) amplification of insertion mutagenized sites (AIMS) using maize *Mu* elements (Frey *et al.*, 1998), and (3) transposon insertion display (TID) utilizing maize elements *En/Spm*, *Mu1* and *Cin4* (Yephremov and Saedler, 2000). Important and critical steps in all these published methods are the use of biotinylated linkers ligated specifically to the transposon sequences used or biotinylated primers for PCR amplification which corresponded to the transposon sequences employed, and to enrich transposon-flanking sequences containing the tagged genes by streptavidin-capturing. To ensure that the fragments to be detected were derived from transposon-flanking sequences, nested primers containing either TIR or internal sequences of the transposon sequences were used for subsequent PCR amplification.

Contrary to these published methods, the STD procedure we developed is simple, reproducible and requires neither biotinylated oligonucleotides nor streptavidin-capturing. The difference between STD and other procedures may partly stem from the lengths and sequences of TIRs carried by the transposon employed. Most of the transposons used for TD, AIMS and TID carry around 11-13 bp TIR sequences (Kunze *et al.*, 1990), whereas the *Tpn1*-family elements

contain 28 bp TIR (Inagaki *et al.*, 1994; Hoshino *et al.*, 1995). We thus used the TIR primers containing the outermost 20 bp sequence instead of employing the nested primers corresponding to internal sequences of the *Tpn1* family. Actually, we avoided using the internal *Tpn1* sequences near TIR as a primer because the subterminal repetitive regions of around 650-800 bp from both ends of *Tpn1* contain multiple direct and/or inverted repeats of the characteristic 10 bp sequence motif. We are currently applying STD to identifying several spontaneous mutations in the Japanese morning glory, presumably caused by insertion of the *Tpn1* family.

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Figure legends

Fig. 1 Schematic representation of the strategy for analysis by STD.

The transposon *Tpn1* is integrated into the tagged site and excised from the excised site. The large X represents a footprint sequence generated by the excision of *Tpn1*. Since the TIR+N primers were 5'-labeled with rhodamine or fluorescein, only fragments derived from the tagged sites were expected to be visualized. Note that two tagged fragments can be visualized from a single tagged site because the 5'-labeled TIR+N primers are hybridizable to both termini of *Tpn1*.

Fig. 2. Display of the fragment derived from the *pr-m* gene in a sequencing gel.

The genotypes of the individual plants are: *r/r*, *Pr-r/Pr-r*; *r/m*, *Pr-r/pr-m*; *m/m*, *pr-m/pr-m*. The arrow points to the 130 bp fragment.

Fig. 3. Characterization of the *Pr* gene.

(a) Northern blot analysis. mRNAs (1.5 μg) extracted from open flower petals were hybridized with the 130 bp DNA fragment obtained by STD as a probe. Symbols *r* and *m* indicate the plants *Pr-r* and *pr-m*, respectively. The arrow points to the 2.3 kb *Pr* mRNA.

(b) Southern blot analysis. Genomic DNAs (10 μg) digested with *Eco*RI, *Xba*I or *Hin*dIII were hybridized with the 130 bp probe. The arrow points to the 7.5 kb *Xba*I fragment containing the entire *Pr* gene. The symbols are as in **(a)**.

(c) The genomic structure of the *Pr* gene. The open box and hatched areas represent the 7.5 kb *Xba*I fragment and the exons of the *Pr* gene, respectively. The small vertical arrows with ATG and TGA indicate the positions of the initiation and termination codons, respectively. The *Tpn4* insertion site is indicated above the map.

(d) The enlarged physical map of the 5′ region of the *Pr* gene. The hatched boxes represent the untranslated exon 1 and exon 2 carrying the ATG initiation codon of the *Pr* gene. The small horizontal arrows and the bracket below the map indicate the positions of the primers and the 130 bp fragment obtained by STD, respectively.

(e) PCR analysis for the *Pr* genotypes in the selfed progeny of a hybrid between a mutable *pr-m* line (*pr-m/pr-m*) and a revertant homozygote (*Pr-r/Pr-r*). Two parental plants (*Pr-r/Pr-r* and *pr-m/pr-m*), an F1 hybrid (*Pr-r/pr-m*), and its selfed F2 progeny are indicated by P, F1 and F2, respectively. The symbols for genotypes are as in Fig. 2, and the flower phenotypes are indicated in parentheses.

Fig. 1 Fukada-Tanaka *et al.*

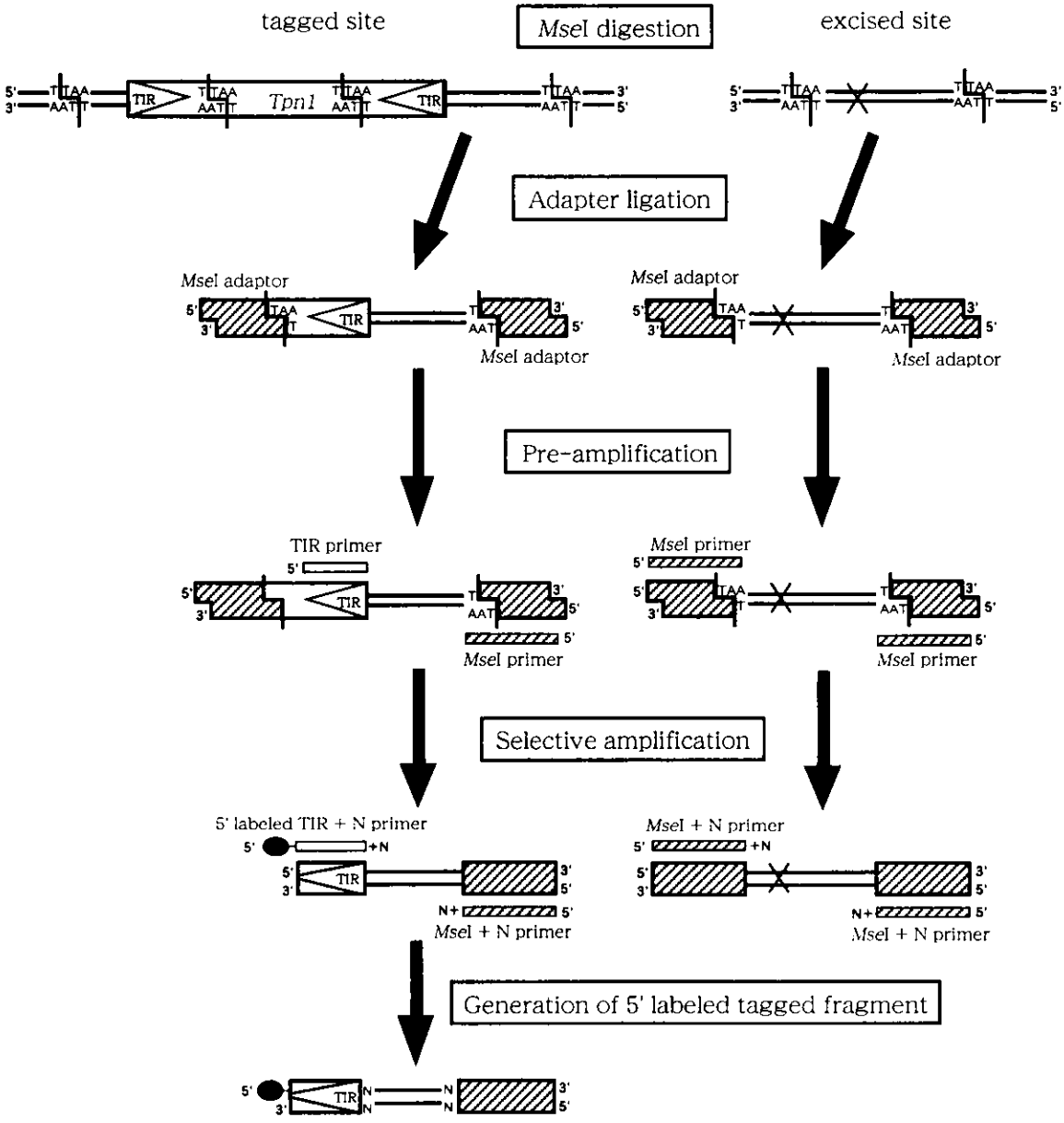


Fig. 2 Fukada-Tanaka *et al.*

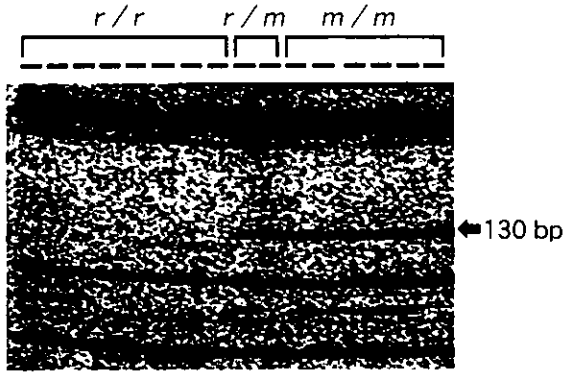
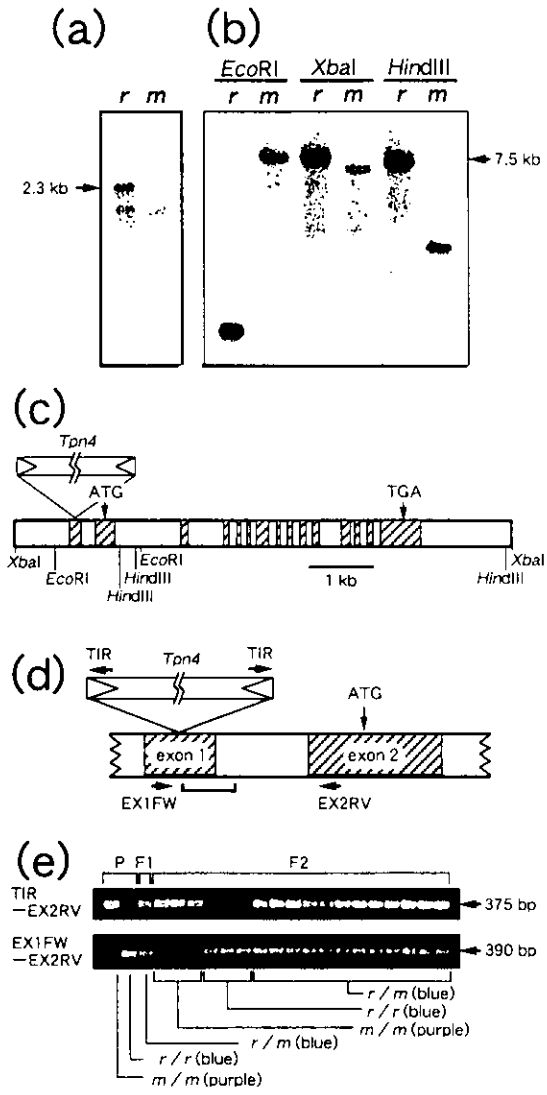


Fig. 3 Fukada-Tanaka et *al.*



（別紙様式　2）

博士論文目録

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出　願　者	生命科学研究科　分子生物機構論　専攻 氏（ふりがな）名　山口（やまぐち）　利男（としお）　（学籍番号：983210）

博士論文

（1）題　目

Identification and characterization of the *Pr* gene that is responsible for blue flower pigmentation in the Japanese morning glory.

（2）発表論文リスト

Sachiko Fukada-Tanaka, Yoshishige Inagaki, Toshio Yamaguchi, Norio Saito and Shigeru Iida (2000) Colour-enhancing protein in blue petals. *Nature* **407**; 581

Sachiko Fukada-Tanaka, Yoshishige Inagaki, Toshio Yamaguchi, and Shigeru Iida Simplified transposon display (STD): a new procedure for isolation of a gene tagged by a transposable element belonging to the *Tpn1* family in the Japanese morning glory. *Plant Biotech.* in press

（3）その他

Toshio Yamaguchi, Sachiko Fukada-Tanaka, Yoshishige Inagaki, Norio Saito, Keiko Yonekura-Sakakibara, Yoshikazu Tanaka, Tkaaki Kusumi and Shigeru iida, Genes encoding the vacuolar Na^+/H^+ exchanger and flower coloration. in press

Toshio Yamaguchi, Fumiya Kurosaki, Dae-Yeon Suh, Ushio Sankawa, Mizue Nishioka, Takumi Akiyama, Masaaki Shibuya and Yutaka Ebizuka (1999) Cross-reaction of chalcone synthase and stilbene synthase overexpressed in *Escherichia coli*. *FEBS Lett.* **460**: 457-461

Toshio Yamaguchi, Yoshishige Inagaki, Sachiko Fukada-Tanaka, and Shigeru Iida (1999) Molecular cloning of a cDNA sequence encoding the H^+/Ca^{2+} antiporter 2 from the Japanene morning glory. *Plant Physiol.* **119**: 806

（別紙様式　3）

博士論文の要旨

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出願者
生命科学研究科　分子生物機構論 専攻
氏名（ふりがな）　山口（やまぐち）　利男（としお）
（学籍番号：983210 ）

博士論文

題目: Identification and characterization of the *Pr* gene that is responsible for blue flower pigmentation in the Japanese morning glory.

The coloration of blue flowers depends on the production of the appropriate anthocyanin pigments, the presence of metal ions and co-pigments, and vacuolar pH. An increase in vacuolar pH enhances blue coloration, however, little is known about the proteins that are responsible for raising the vacuolar pH. The Japanese morning glory (*Ipomoea nil*) normally displays bright blue flower. One of the mutable allele, named *purple-mutable* (*pr-m*) confers purple flowers with blue sectors owing to recurrent somatic mutation from recessive *pr-m* allele to the blue *Pr-r* (*Purple-revertant*) allele. In this study, I described detail of the identification and characterization of the *Pr* gene, that is necessary for displaying blue flower in the *I. nil*, and discussed the observations with regard to the blue flower coloration. Germinal revertant carrying *Pr-r* allele homozygously that displays blue flower was successfully obtained from *pr-m* line, and compared with *pr-m* line. No alterations were found in the anthocyanin pigment compositions between the *pr-m* and *Pr-r* lines whereas the vacuolar pH in the *pr-m* was significantly lower than that in *Pr-r*. These results indicate that the function of the *Pr* gene is to raise vacuolar pH. By comparing the *pr-m* and *Pr-r* with simplified transposon display methods (STDM), the *Pr* gene also cDNA were successfully isolated. A *Tpn1*-related transposon, *Tpn4* insertion was found within the exon 1 of the *Pr* gene in *pr-m* plant. This gene contained an open reading frame that is similar to the vacuolar Na^+/H^+ exchanger found in yeast, *Arabidopsis*, and rice. The *Pr* gene cDNA was able to complement the *nhx1* mutation for vacuolar Na^+/H^+ exchanger

in yeast (*Saccharomyces cerevisiae*). A second copy for *Pr* gene was found in *I. nil* genome, but this copy was found to be pseudogene. The *Pr* gene was the most abundantly expressed in flower petals, and extensively found at around 12 h before flower opening, whereas no such increased expression was observed in the flower of *Petunia hybrida*. This result indicates the abundant expression of *NHX1* genes before flower blooming was not common in all flowering plants. Since the *NHX1* genes that promote the transport of Na^+ into the vacuoles has been known to be involved in salt tolerance, we can add another important role for blue flower coloration in the Japanese morning glory by the vacuolar alkalization.