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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.

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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.

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.

Materials and Methods

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*).

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 ℃ for 30 sec), annealing (56 ℃ for 60 sec) and extension (72 ℃ 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 ℃ for 30 sec), annealing (65 ℃ for 30 sec) and extension (72 ℃ for 60 sec). For the following 12 cycles, the annealing temperature was reduced by 0.7 ℃ per cycle. The final 20 cycles included denaturation (94 ℃ for 30 sec), annealing (56 ℃ for 60 sec) and extension (72 ℃ 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).

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 λ ZAPII (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*

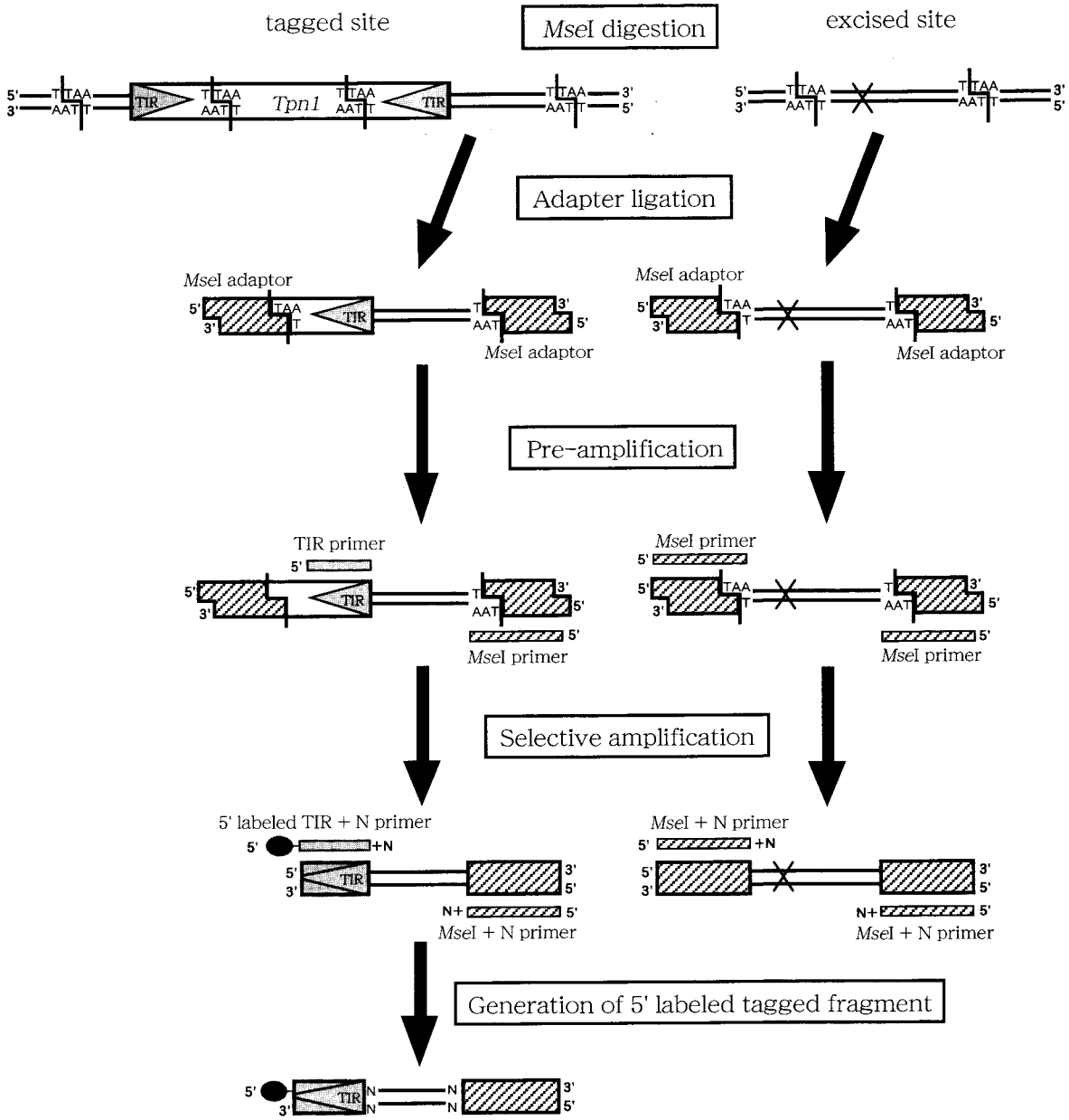

Fig. 1 Schematic representation of the strategy for analysis by STD.

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*.

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, λ ZExp: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 λ DASHII (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).

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 F_2 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'-GAAACA-GAAAAAGAGAGAGTCACG-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.

Results and discussion

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 *Tpn1*-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 *Tpn1*-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.

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

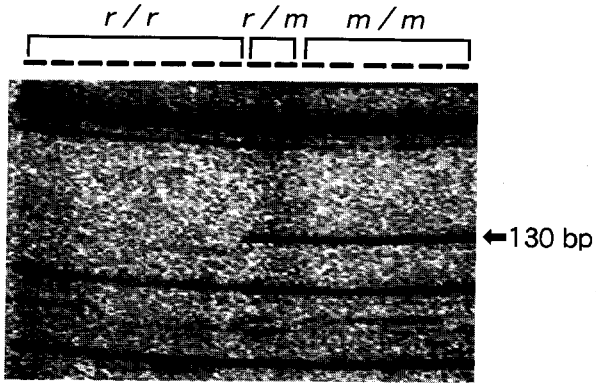

Fig. 2 Display of the fragment derived from the *pr-m* gene in a sequencing gel.
The genotypes of the individual plants are: *r/r, Pr-r/Pr-r; r/m, Pr-r/pr-m; m/m, pr-m/pr-m.* The arrow points to the 130 bp fragment.

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).

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.

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 *Tpn1*-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

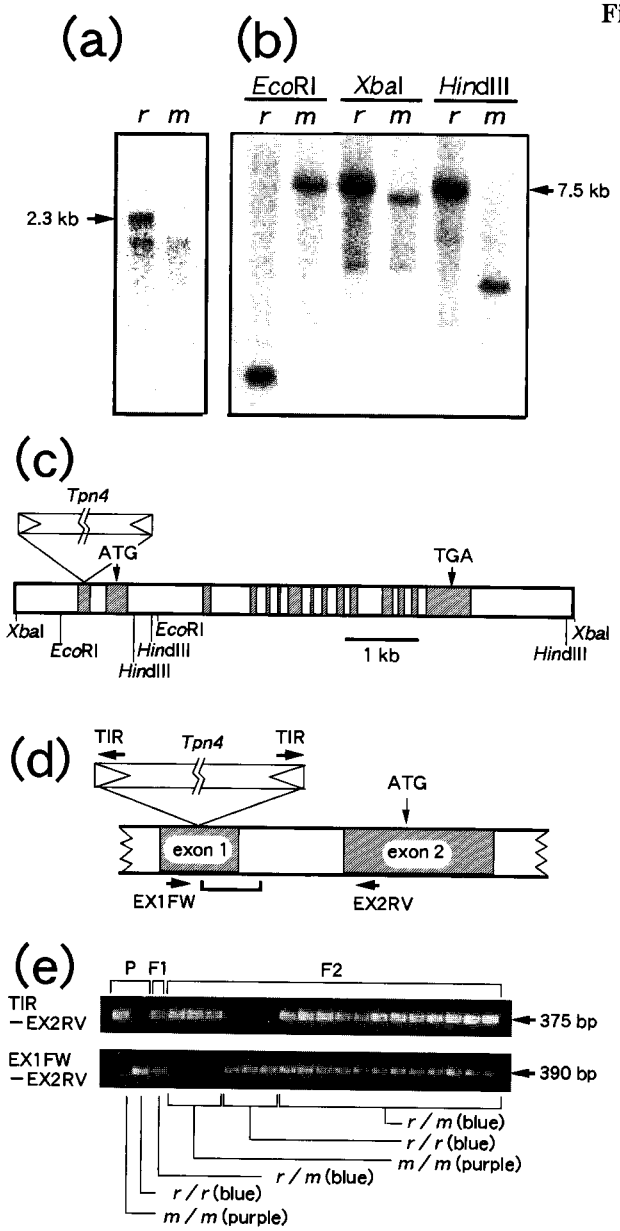

Fig. 3 Characterization of the *Pr* gene.

(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 F_1 hybrid (*Pr*-*r*/*pr*-*m*), and its selfed F_2 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.

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.

Acknowledgments

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