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***Original Paper***

# Role of miniature inverted repeat transposable elements inserted into the promoter region of a carrot phenylalanine ammonia-lyase gene and its gene expression

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**Abstract** The expression of the phenylalanine ammonia-lyase gene (*PAL*) was induced in anthocynanin-producing suspension-cultured carrot cells. In our previous study, we isolated two *PAL* genes, *DcPAL3* and *DcPAL4*, from a carrot genomic library, the nucleotide sequences of which are highly similar. Here, the complete nucleotide sequence of *DcPAL4* was determined and compared with that of *DcPAL3*, revealing two miniature inverted repeat transposable elements (MITEs), here designated MITE1 and MITE2, inserted into the proximal promoter region of *DcPAL3*, but not into that of *DcPAL4*. The expression of *DcPAL4* was not detectable by RT–PCR in cultured carrot cells or the tissues of carrot plants, whereas *DcPAL3* expression was detected. Transient expression experiment in carrot protoplasts showed that the *DcPAL4* promoter actively produces transcripts, as does the *DcPAL3* promoter. To characterize the roles of MITE1 and MITE2 (MITE1/2) in the transgenes of stable transformants, they were inserted into a plant transformation vector containing the 35S–*β*-glucuronidase (*GUS*) reporter gene and selectable antibiotic-resistance marker genes, and then introduced into tobacco BY-2 cells using an *Agrobacterium* method. More regenerated calli carrying the MITE1/2 construct survived on medium containing kanamycin than calli lacking MITE1/2. Regenerated calli containing MITE1/2 were more numerous having stronger GUS activity than those without MITE1/2. These results suggest that MITE1/2 enhances the expression of adjacent transgenes introduced into stable transformants and possibly into the endogenous *DcPAL3* gene.

**Key words:** Anthocyanin, carrot, miniature inverted repeat transposable elements, phenylalanine ammonia-lyase.

Transposable elements occur widely in the genomes of many organisms, not only plants, but also animals and microorganisms. They are divided into two types according to the intermediate molecules in the transposition event: RNA-mediated and DNA-mediated transposable elements (Flavell et al. 1994). Representatives of the former consist of retrotransposons, long interspersed nuclear elements (LINEs) and short interspersed nuclear elements (SINEs). The latter group, the DNA transposable elements, has common and typical features, including terminal inverted repeat (TIR) sequences at both ends and target-site duplications (TSDs) at the insertion site in the host genome sequence. The major plant DNA elements belong to three types, the *En*/*Spm*, *Ac*/*Ds*, and *Mu* types, classified according to their common characteristic TIR and TSD sequences. Elements with similar features to those of the *En*/*Spm* type have been found in animals (Handler and Gomez 1996). Recent advances in genome-wide sequencing projects have identified other DNA elements, miniature inverted repeat transposable elements (MITEs), in plant genomes. MITEs have imperfect direct repeat sequences as TIRs and two to nine nucleotide sequences as TSDs, and most of them are less than 1,000 bps long, with no remarkable coding sequences such as those for transposases. Genome projects in several organisms have revealed that the MITE copy numbers in these genomes are quite high (Santiago et al. 2002; Feschotte et al. 2003; Quesneville et al. 2006). The majority of MITEs belongs to two major superfamilies, the *Stowaway* (Bureau and Wessler 1994) and *Tourist* families (Bureau and Wessler 1992). Although the elements are found in these genomes in high copy numbers, their transposition events have not been demonstrated. Recently, the movement of *miniature Ping* (*mPing*), which belongs to the *Tourist* superfamily, was reported by three independent laboratories (Jiang et al. 2003; Kikuchi et al. 2003; Nakazaki et al. 2003).

[a] Deceased on July 2, 2004.
This article can be found at http://www.jspcmb.jp/

Nakazaki et al. (2003) showed that *mPing* inserted into a structural gene to disrupt its function results in a recessive phenotype and that excision from the gene recovers the function, resulting in a reversion to the original phenotype. This result suggests that MITEs including *mPing* act as disrupters of gene functions in the genome, as do other elements belonging to the *En*/*Spm*, *Ac*/*Ds*, and *Mu* families.

We established a system of carrot suspension cell cultures in which anthocyanin synthesis is induced as "metabolic differentiation" (Ozeki and Komamine 1981). The carrot cells in the suspension cultures grow in medium containing 2,4-dichlorophenoxyacetic acid (2,4-D). Anthocyanin synthesis is induced when the cells are cultured in medium lacking 2,4-D (Ozeki and Komamine 1981). We studied the regulatory mechanism of the phenylalanime ammonia-lyase genes (*PAL*) in this system, because PAL is a well-known key enzyme at the branching point from primary metabolism to phenylpropanoid metabolism (Dixon and Paiva 1995; Weisshaar and Jenkins 1998). *PAL* genes comprise a small gene family in many plant species (Wanner et al. 1995). Their expression is regulated by environmental stimuli and in accordance with differentiation and development of tissues and organs to synthesize lignins, flavonoids, and anthocyanins. Environmental stimuli such as ultraviolet irradiation, elicitor treatments, and the dilution effect (osmotic shock) on the carrot cells in the suspension cultures rapidly and transiently induce the accumulation of *PAL* transcripts, which is unaffected by 2,4-D (Ozeki et al. 1990; Takeda et al. 2002). The slow accumulation of transcripts for *PAL*, together with chalcone synthase gene, was observed in cells four to five days after their transfer to and culture in medium lacking 2,4-D, at the time of induction of anthocyanin synthesis (Ozeki et al. 2000; Ozeki et al. 2003).

Two independent cDNA libraries were prepared from *PAL* transcript-enriched poly(A)$^+$ RNA fractions of either the dedifferentiated cells subject to the dilution effect by transferring fresh medium containing 2,4-D or the anthocyanin-synthesizing cells cultured in the medium lacking 2,4-D by sucrose density-gradient centrifugation. The nucleotide sequence of *PAL* cDNAs was identical in 12 independent clones isolated from the library for the cells treated with the dilution effect; these were designated TRN-PAL. In contrast, the sequence of 20 clones from anthocyanin-synthesizing cells, designated ANT-PAL, differed from the sequence in TRN-PAL. The nucleotide sequence of the coding region of TRN-PAL shares 82% similarity with that of ANT-PAL (Ozeki et al. 1990). Two genomic sequences, *DcPAL1* and *2*, corresponding to TRN-PAL, were isolated from a carrot genomic library. It has been demonstrated that an *En*/*Spm* transposable element, *dTdc1*, had inserted into the promoter region of the *DcPAL1* gene, giving rise to *DcPAL2*, in one or several cultured cells during successive subculturing under "genomic stress" (McClintock 1984; Ozeki et al. 1997; Itoh et al. 2003). The promoter activity of the *DcPAL2* gene was reduced by the *dTdc1* insertion relative to that of *DcPAL1* (Ozeki et al. 1997). Further analysis of *DcPAL1* revealed that the expression of *DcPAL1* is induced by environmental stimuli other than the dilution effect, such as ultraviolet irradiation and elicitor treatments, but that this does not occur during anthocyanin synthesis (Ozeki et al. 2000; Takeda et al. 2002). Screening for the genomic sequence corresponding to ANT-PAL in a carrot genomic library isolated two genomic fragments, *DcPAL3* and *4*, whose nucleotide sequences show strong similarity. Because the nucleotide sequence of the coding region of *DcPAL3* is identical to that of ANT-PAL and because the sequence of *DcPAL4* has more than 98% similarity to ANT-PAL, our previous studies focused on *DcPAL3*, which is expected to be responsible for induction of anthocyanin synthesis (Ozeki et al. 2003). We noticed during the determination of the nucleotide sequence of *DcPAL3* that two MITEs, here designated MITE1 and MITE2, were inserted into the promoter region of *DcPAL3* but not into that of *DcPAL4*. In this study, the structure of *DcPAL3* with the MITE1 and MITE2 insertions is compared with the nucleotide sequence of *DcPAL4* and the role of these MITEs in the expression of *DcPAL3* is investigated.

## Materials and methods

### *Carrot suspension-cultured cells and carrot plants*

Carrot (*Daucus carota* L cv. Kurodagosun) cells subcultured in medium containing 2,4-D under undifferentiated conditions, without anthocyanin synthesis, were maintained as described previously (Ozeki and Komamine 1981). The dedifferentiated cells were cultured and harvested five days after their transfer to medium containing 2,4-D. The cells were induced to synthesize anthocyanin by culture for seven days in medium lacking 2,4-D and were harvested and frozen in liquid nitrogen until use. Carrot plants were grown on soil in a green house and their leaves, stems, roots, and flowers were separately harvested and frozen in liquid nitrogen until RNA extraction.

### *Determination of the complete nucleotide sequence of DcPAL4*

A DNA fragment of *DcPAL4*, which had been isolated from positive clones of a carrot genomic library using ANT-PAL (Ozeki et al. 1990) as the probe, was digested with the appropriate restriction enzymes and subcloned into pBluescript SK. The double-stranded nucleotide sequence was determined with the preparation of serial deletion mutants in a similar way to that used for *DcPAL3* (Ozeki et al. 2003; accession number of *DcPAL4* is AB435640).

### RT–PCR

The preparation of total RNA from the cultured cells and tissues of carrot plants and poly(A)$^+$ RNA purification were as previously described, except for an additional treatment with RNase-free DNase (Ozeki et al. 1990). The first-strand cDNA was synthesized from 1 $\mu$g poly(A)$^+$ RNA in 10 $\mu$l of reaction mixture using the First-Strand cDNA Synthesis Kit (TaKaRa Bio Inc., Shiga, Japan), according to the supplier's manual. After the reaction, 40 $\mu$l of water was added to the mixture, which was then incubated at 65°C for 10 min, and 2 $\mu$l of the mixture were used as the template for PCR. A PCR primer pair was designed to specifically amplify a DNA fragment from the region near the C-terminal coding region of *DcPAL3*, but not from *DcPAL4*: 5′-CACGCACTAAACAATGGC-3′ and 5′-TAAAATAACCAACACTCCG-3′. Another primer pair that amplified *DcPAL4* but not *DcPAL3* was also designed: 5′-CACGCATTGAATAATGGT-3′ and 5′-AAAAATAAACATT-ACTCCTG-3′. The PCR reaction mixture consisted of 2 $\mu$l of the cDNA mixture, 1×Ex *Taq* PCR buffer (TaKaRa Bio Inc.), 2 mM $MgCl_2$, 0.25 mM dNTPs, and 0.2 $\mu$M each primer. The mixture was made up to a total volume of 10 $\mu$l with water, and 30 $\mu$l of mineral oil was layered on top of the mixture. The reaction tubes were placed into a thermocycler (MiniCycler™, MJ Japan Ltd., Tokyo, Japan) and incubated at 98°C for 1 min, after which the temperature was lowered to 92°C. Ex *Taq* DNA polymerase (0.25 units in 1 $\mu$l; TaKaRa Bio Inc.) was added directly to the bottom of the reaction mixture with a micropipette, and the PCR proceeded for 30 cycles of denaturing at 92°C for 30 s, annealing at 60°C for 40 s for the *DcPAL3* primer pair or at 55°C for 40 s for the *DcPAL4* primer pair, and extension at 72°C for 1 min. To verify the specificity of the *DcPAL3* and *DcPAL4* primer pairs, 1 ng of plasmid containing a genomic DNA fragment encoding the proximal region of the second exon of *DcPAL3* or *DcPAL4* was added to the PCR reaction mixture instead of the cDNA reaction mixture. After the PCR reaction, the amplified products were analyzed by 1.5% agarose gel electrophoresis.

### Transient expression assay in carrot protoplasts

The promoter regions of *DcPAL3* (−2443 to +229) and *DcPAL4* (−1397 to +231) were introduced into p*luc*-SK, which contains the firefly luciferase (*luc*) cDNA as the reporter gene and the nopaline synthase (*NOS*) terminator, as described previously (Ozeki et al. 1993). To determine the terminator activities of *DcPAL3* and *DcPAL4*, the 3′ noncoding region of a genomic DNA fragment corresponding to either *DcPAL3* or *DcPAL4* was amplified by PCR with the appropriate primers, and was then introduced instead of the *NOS* terminator (see Figure 5). Furthermore, no-terminator constructs were prepared by removing the *NOS* terminator sequence from the plasmids described above (see Figure 5).

Carrot protoplasts were isolated from the suspension-cultured cells grown in medium containing 2,4-D; the constructs prepared above were introduced together with the 35S–*GUS* construct into the carrot protoplasts by electroporation; the LUC activity derived from the reporter gene was measured; and the GUS activity was measured to normalize the electroporation efficiency after protoplast culture, as reported previously (Ozeki et al. 1993).

### Construction of a plant transformation vector harboring MITE1 and MITE2 and its transformation into tobacco BY-2 cells

Because MITE1 and MITE2 have imperfect TIR sequences that give rise to inaccurate PCR products, we surveyed the appropriate inserts containing MITE1 and MITE2 in 3′ serial deletion mutants of the genomic DNA of the *DcPAL3* promoter region subcloned into pBluescript SK, which was used to determine the double-stranded nucleotide sequence. To prepare the MITE1 construct, the mutant *DcPAL3* promoter region deleted to −1581 from the 3′ end of the *DcPAL3* genomic DNA, near the 3′ end of MITE1, was digested with *Kpn*I adjacent to the deleted site, and then digested with *Sca*I at the 5′ end of the TIR sequence of MITE1 (see Figure 2, left). This was followed by a fill-in reaction with T4 DNA polymerase to produce a 322-bp fragment, which was subcloned into pBluescript SK with *Hin*cII, to produce MITE1/SK. The DNA fragment corresponding to the cauliflower mosaic virus 35S promoter region (35S), prepared from pBI221 digested with *Hin*dIII and *Sma*I, was introduced into pBluescript SK doubly digested with *Hin*dIII and *Sma*I, to produce 35S/SK. The MITE1 DNA fragment prepared from MITE1/SK doubly digested with *Kpn*I and *Hin*dIII was inserted into 35S/SK doubly digested with *Kpn*I and *Hin*dIII, to produce MITE1–35S/SK. To prepare the MITE2 construct, 3′ serially deleted mutant carrying the plasmid including −388 from the 3′ end of the *DcPAL3* promoter region was digested with *Kpn*I adjacent to the deleted site, followed by a fill-in reaction with T4 DNA polymerase. It was then digested with *Dde*I at nucleotide −1,184, 28 bp upstream from the 5′ end of MITE2 (see Figure 2, right). The 797-bp DNA fragment of MITE2, treated with T4 DNA polymerase, was introduced into the *Hin*cII site of pBluescript SK, resulting in MITE2/SK, which was introduced into 35S/SK in a similar way to MITE1 to produce MITE2–35S/SK. The MITE2 fragment was introduced into MITE1–35S/SK digested with *Cla*I followed by T4 DNA polymerase treatment, to produce MITE1/2–35S/SK. MITE1–35S/SK, MITE2–35S/SK, and MITE1/2–35S/SK were doubly digested with *Xho*I and *Xba*I. The fragments were then introduced into the *Xho*I/*Xba*I sites of the plant binary vector pAB, which contains the *GUS* reporter gene and the neomycin phosphotransferase II (*nptII*) and hygromycin phosphotransferase (*hygB*) genes as antibiotic-resistance markers (see Figure 6A).

The plasmids were transformed into *Agrobacterium tumefaciens* EHA 101 by electroporation, and then cultured at 28°C for two days on plates containing hygromycin (50 mg l$^{-1}$). The method for the transformation of tobacco BY-2 cells was according to Ito et al. (1998). *Agrobacterium tumefaciens* carrying the plasmids were cultured, resuspended in water to an optical density of $OD_{600}$=0.25, and mixed with $10^6$ tobacco BY-2 cells for 10 min. The cells were washed with fresh liquid Murashige–Skoog (MS) medium by centrifugation. Several series of different numbers of cells per plate were spread onto plates of solid MS medium containing 100 $\mu$g ml$^{-1}$ or 300 $\mu$g ml$^{-1}$ kanamycin together with 500 $\mu$g ml$^{-1}$ cefotaxime. After one month in culture, the numbers of regenerated transformant calli were counted. The regenerated calli on the medium containing 100 $\mu$g ml$^{-1}$ kanamycin and 500 $\mu$g ml$^{-1}$ cefotaxime were randomly picked and transferred to fresh MS

medium containing 100 $\mu$g ml$^{-1}$ kanamycin and grown to clonal calli. The GUS activity in the crude extracts prepared from individual calli was measured, as previously described (Jefferson et al. 1987), and the amount of protein in the extracts was measured according to Bradford (1976).

## Results and discussion

### *Characterization of two types of MITEs in the promoter region of DcPAL3 but not in that of DcPAL4*

Although *DcPAL3* encodes a transcript with a nucleotide sequence completely identical to that of ANT-PAL, analysis of the nucleotide sequence of *DcPAL4* revealed that the sequence of its open reading frame has *ca.* 98% similarity to that of *DcPAL3*, and that the sequences of their promoter regions and introns share *ca.* 95% identity (Figure 1). A remarkable difference between them is the two insertions at −1897 to −1599 and −1157 to −389 in the promoter region of *DcPAL3* relative to that of *DcPAL4* (Figure 1). The shared structural features of the nucleotide sequences of the MITEs include imperfect terminal inverted repeats (TIRs), target site duplications (TSD), and internal AT-rich sequences (Figure 2). The two MITEs, which are 299 bp and 769 bp long, were designated MITE1 and MITE2, respectively. The nucleotide sequences of the imperfect TIRs of MITE1 are similar to those of members of the *Stowaway* family (Figure 3). The characteristic TSD sequence, TA, of members of the *Stowaway* family was observed at the insertion site of *DcPAL3* relative to the *DcPAL4* sequence (Figure 2), indicating that MITE1 belongs to the *Stowaway* family (Buerou et al. 1994). MITE2 has an unusual long imperfect TIR sequence, and the TSD sequence AAAAGAAAA was observed in *DcPAL3* relative to *DcPAL4* (Figure 2). The nucleotide sequence of the end of TIR, GGGATCTTTTTAAAAATACC of MITE2, was similar to that of the *MuDR* element, GaGATaaTTgccAttATAga (accession no. M76978, the upper and lower case letters indicate nucleotides identical to and different from the TIR sequence of MITE2, respectively). The *Mu* element occurs in a 9 bp TSD sequence at the insertion point (Hershberger et al. 1991), suggesting that MITE2 is a carrot *Mu*-related element. Southern analysis to the carrot genomic DNA using the DNA fragment of MITE2 as a probe showed smear patterns on every lane of the DNA digested by several restriction endonucleases, patterns similar to the profiles obtained using MITE1 as a probe (data not shown). These results suggest that the number of copies of MITE2 might be in the hundreds, as in MITE1, so that the sequence for MITE2 identified here might belong to a novel MITE family.

### *Gene expression and promoter activity of DcPAL3 and DcPAL4 genes*

We prepared a cDNA library from *PAL* transcript-enriched poly(A)$^+$ RNA fractions prepared from the anthocyanin-synthesizing cells cultured in the medium lacking 2,4-D by sucrose density-gradient centrifugation and isolated more than 30 independent clones corresponding to *PAL* cDNA (Ozeki et al. 1990), but found no cDNAs with sequences identical to that of the ORF of *DcPAL4* (data not shown). To confirm whether the *DcPAL4* gene is expressed in cultured cells or plant tissues of carrot, the expression of *DcPAL3* and *DcPAL4* was detected by RT–PCR using *DcPAL3*- and *DcPAL4*-specific sequences as primers. mRNAs were prepared from cultured cells and from the leaves, stems, roots, and flowers of carrot plants. The expression of *DcPAL3* was observed in anthocyanin-synthesizing cultured cells and all plant tissues (Figure 4, left), but was not quantifiable because of saturated PCR amplification in this condition. The expression of *DcPAL4* was detected in neither cultured cells nor any tissues (Figure 4, right), indicating that the expression of *DcPAL4* is highly repressed or ceased in this condition.

Although the nucleotide sequences of the promoter regions of *DcPAL3* and *DcPAL4* are highly similar, except for the MITE1 and MITE2 insertions, there are certain other differences in them. We found six candidate sequences for *cis*-elements: G-box, GCC-box homolog, and boxes-P, -P′, A, and L, in the TATA-proximal region

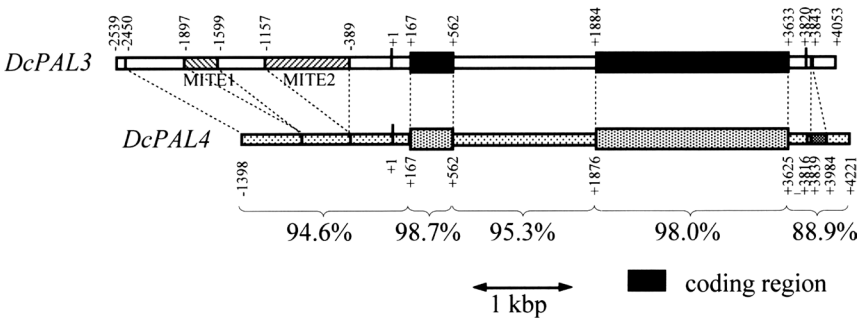


Figure 1. Comparison of the nucleotide sequences of the genomes of *DcPAL3* and *DcPAL4*. Thick and thin boxes in *DcPAL3* show coding and noncoding regions, respectively (Ozeki et al. 1990; Ozeki et al. 2003). The boxes shown in *DcPAL4* are the regions corresponding to *DcPAL3* and the similarities in the nucleotide sequences to *DcPAL3* are presented as percentages, excluding MITE1 and MITE2 in *DcPAL3*.

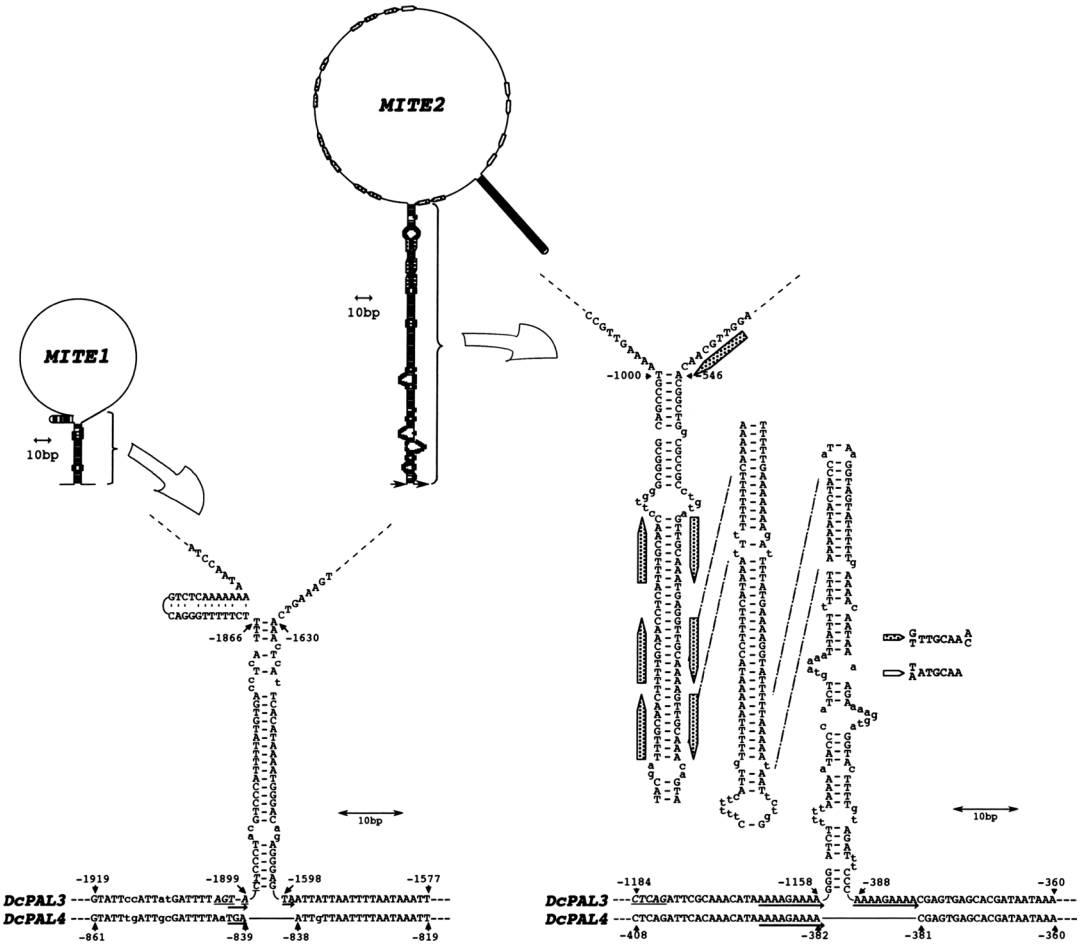


Figure 2. Stem structures of MITE1 and MITE2 and the nucleotide sequences of regions proximal to the insertion sites in *DcPAL3* relative to the *DcPAL4* sequence. The upper and lower case letters in the stems show the complimentary and uncomplimentary sequences, respectively, in the TIR regions. The nucleotide sequences of the regions proximal to the insertion sites in *DcPAL3* that are identical to and different from those in *DcPAL4* are shown in upper and lower case letters, respectively. Arrows indicate putative TSD sequences. The underlined sequence at the left border of MITE1 (−1902) and adjacent to the left border of MITE2 (−1184) indicates a *Sca*I and *Dde*I site, respectively.

| species | accession number | | size (bp) |
|---|---|---|---|
| *Daucus carota* MITE1 | | taCTCCCTACGTCCCATTTTATGTGACCTCATTTTCTTTTTG ----- ACATAAAATGGGGACAGAGGGAGTa | 299 |
| *Daucus carota* | X16131 | taCTCTTTCTATCTCATTTAATTCTATACATTTCTTTTTAAC ----- TACTACTCAGGGGACGGAGGGAGTa | 253 |
| *Daucus carota* | S47635 | taCTCCCTTCCGTAGCTTTTCAGTTATCACATTTCTATTTTT ----- CAACTAAAAAGGGACGGAGGGAGTa | 249 |
| *Petroselinum crispum* | X55736 | taCTCCCTCCGTCCCACCCAATTCTTTACATTGGGGATAGGG ----- AGAAATGGATGGGACGGAGGGAGTa | 243 |
| *Petroselinum crispum* | AF274564 | taCTCCCTCCGTCCCACCCAATTTTTACATTGGGGGATGGG ----- AGAAATGGATTGGACGGAGGGAGTa | 257 |
| *Petroselinum crispum* | U46217 | taCTCCCTCCGTCCCACCCAATTGTATACATTGGGGGACGGG ----- AGAAATGGATGGGACAGAGGGAGTa | 251 |
| *Petunia hybrida* | X14595 | taCTCCATCCGGTCCACTTTATTTGATGTTTTTCACTTTTCG ----- TCAAATAAAAATGGACCGGAGGAGTa | 257 |
| *Nicotiana tabacum* | X64519 | taCTCCCTCCGTTTCATATTAAATAAGATACCGACATATTTT ----- CAATAAAAAAATGAGCGAAGGAGTa | 375 |
| *Lycopersicon esculentum* | M83314 | taCTCCCTTCGTTTTTAAAAAAATACCTAGTTTGACTTGAAA ----- CATTGCTTTTTGAAACGGAGAGAGTa | 248 |
| *Medicago truncatula* | AC149806 | taCTCCATCCGTTTTCAAATACATGTCTAATTTTTGCAAAGT ----- CATGTATTTTGAAACGGAGGGAGTa | 256 |
| *Solanum tuberosum* | X03956 | taCTGTCTTGTTCCAATTTATCTCACACTTTTTGTTTTTCGA ----- CACATAAAAATGGGACAGAAGGAGTa | 255 |
| *Oryza sativa* | AP008212 | taCTTTCCTCCGTTTCACAATGTAAGTCATTCTAGCATTTTC ----- CTTACATTGTCAAACGGAGGGAGTa | 134 |
| *Zea mays* | AF215823 | taCTCCCTAAGTTCCAAATTAAAATCCATTTTAGCCTTTTAT ----- AAAACGTTGTGGGACGGAGGGAGTa | 101 |
| *Triticum aestivum* | X13580 | taCTCT.TCCGTTAAATTACTCGTCGCAGAAATGAATGTATC ----- CGAATAATTCGAAACGGAGGGAGTa | 104 |

Figure 3. Multiple sequence alignment of proximal TIR regions of *Stowaway* elements. Some representatives of the huge number of elements belonging to the *Stowaway* superfamily (Bureau and Wessler 1994) are listed in comparison with MITE1 identified here (top line of the sequences). Conserved nucleotides of MITE1 relative to the other elements are indicated by white letters on a black background. The 'ta' sequences are TSDs, which are excluded from the numbers of base pairs listed on the right side.

of the *DcPAL3* promoter. The transient expression experiments using deletion mutants of the *DcPAL3* promoter in carrot protoplasts indicated that the GCC-box homolog and box-L were required to give full activity to the *DcPAL3* promoter irrespective of the presence or absence of 2,4-D and that box-P′ might play the most important role in repressing the activity in response to the addition of 2,4-D to the protoplasts (Ozeki et al. 2003). We confirmed that the nucleotide sequences corresponding to all these *cis*-elements were conserved in the putative TATA-proximal region of the *DcPAL4* promoter (data not shown). However, it is possible that any lethal changes in the sequence of the promoter region of *DcPAL4* compared with that of the

unidentified *cis*-element(s) of *DcPAL3* in our previous experiments might cause the diminution or loss of its promoter activity. To verify this possibility, the promoter activities of *DcPAL3* and *DcPAL4* were examined using transient expression assays in carrot protoplasts. The promoter regions of *DcPAL3* and *DcPAL4* were translationally fused to the *luc* gene followed by the *NOS* terminator or the terminator region of *DcPAL3* or *DcPAL4*. The constructs were electroporated into carrot protoplasts, the protoplasts were cultured for 24 h, and the LUC activity was determined according to a previous report (Ozeki et al. 1993). Both the *DcPAL3* and *DcPAL4* promoters were active in this transient assay, indicating that the *DcPAL4* promoter could work well without loss of the activity compared with *DcPAL3* in the transient expression system. The activity of the *DcPAL3* promoter was slightly lower than that of *DcPAL4* (Figure 5), implying that the insertion of MITE1 and MITE2 reduces the promoter activity of *DcPAL3*, consistent with other reports that the insertion of transposable elements into promoter regions results in the loss or reduction of the promoter activity in transient expression analysis (Ozeki et al. 1997). Because the sequences of the terminator regions of *DcPAL3* and *DcPAL4* differ, possibly affecting gene activity, the *NOS* terminator was replaced by the terminator region of *DcPAL3* or *DcPAL4*. The terminator of *DcPAL4* worked well in the transient expression experiment, as did that of *DcPAL3* and the *NOS* terminator (Figure 5).

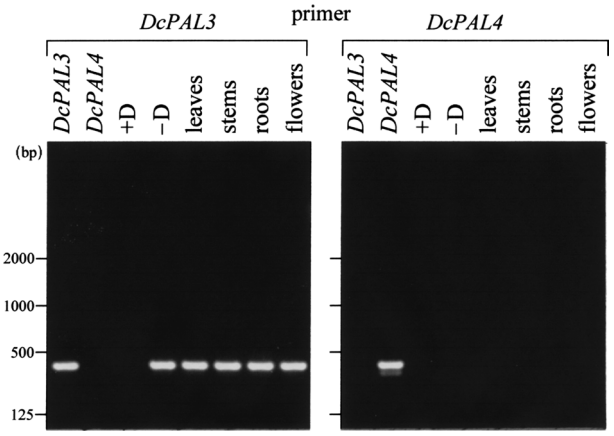


Figure 4. RT–PCR amplified products of first-strand cDNAs prepared from cells of carrot suspension cultures and the tissues of carrot plants used as templates, with either *DcPAL3*- (left) or *DcPAL4*- (right) specific primers. +D, cells cultured in medium containing 2,4-D under dedifferentiation condition; −D, cells cultured without 2,4-D, inducing anthocyanin synthesis; leaves, stems, roots, and flowers were harvested from carrot plants grown in a green house. '*DcPAL3*' and '*DcPAL4*' in the first and second lanes of each panel indicate the control reactions with plasmids encoding DNA fragments containing the second exon of *DcPAL3* or *DcPAL4*, respectively, to ensure the specificity of each primer set in the PCR reaction.

### *Effect of the MITE1 and MITE2 on transgene activity introduced into stable transgenic tobacco cells*

Like other DNA transposable elements, such as *En*/*Spm*,

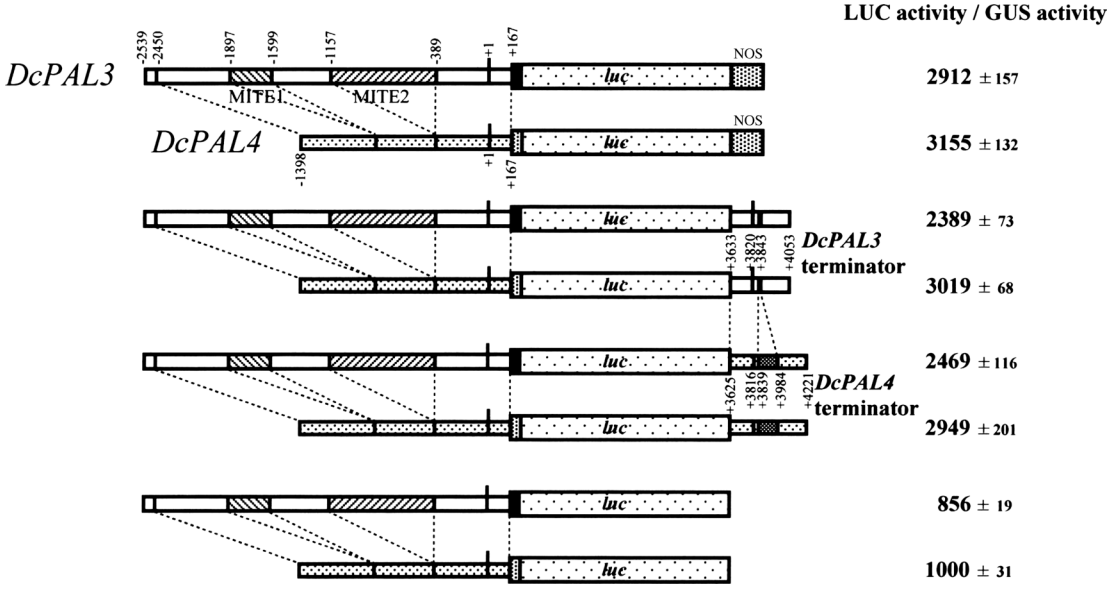


Figure 5. Transient expression of *DcPAL3* and *DcPAL4* promoter activities in carrot protoplasts. *DcPAL3* and *DcPAL4* promoter regions with part of the first exon were translationally fused to the *luc* gene as the reporter gene, followed by the *NOS* terminator (the first and second constructs shown, respectively), either the 3′ noncoding region of *DcPAL3* (the third and fourth constructs) or that of *DcPAL4* (the fifth and sixth constructs), and without any terminator (the seventh and eighth constructs). These constructs were introduced into carrot protoplasts using electroporation, together with the 35S–*GUS* construct (Ozeki et al. 1993). The LUC and GUS activities were measured in the extracts prepared from the protoplasts after culture for 24 h (Ozeki et al. 1993). To normalize for electroporation efficiency, the LUC activity was divided by the GUS activity. Mean values and standard errors (SE) were derived from three independent determinations.

*Ac*/*Ds*, and *Mu*, the insertion of MITEs possibly depresses or destroys the activity of the recipient gene (Nakazaki et al. 2003). However, in this case, *DcPAL3*, with two MITEs in the promoter region, is expressed, whereas the expression of *DcPAL4*, with no MITEs, is undetectable, even though the *DcPAL4* promoter activity was demonstrably active in transient expression. It is possible that the discrepancy between expression of *DcPAL3* and 4 *in vivo* might be caused by the presence or absence of MITE1 and 2 between *DcPAL3* and *4*. MITE1 and 2 might affect or modulate the expression of *DcPAL3* in the genome under the differentiated condition, but not the transient expression in protoplasts.

To identify the roles of the MITEs in the expression of the genes in the genome, MITE1 and/or MITE2 were inserted into a Ti-plasmid typically used for gene transfer into plants (Figure 6A). These constructs were introduced into *A. tumefaciens*, then transferred into tobacco BY-2 cells. Different numbers of cells were spread onto plates containing 100 mM or 300 mM kanamycin as the selective agent to reflect NPTII activity. The numbers of regenerated calli containing introduced MITE1/2 were higher than the numbers of controls without MITEs on plates containing 100 mM kanamycin (Figure 6B). MITE2 increased the numbers of regenerated calli relatively more weakly than did MITE1/2, but MITE1 did not increase the number of calli relative to the control, which had no MITEs. A concentration of 300 mM kanamycin (Figure 6C) is unusually high for a selection pressure exerted on transformed tobacco cells, which must express much more NPTII activity to survive than on 100 mM kanamycin plates. On 300 mM kanamycin, the number of regenerated calli with introduced MITE1/2 was much higher than the control calli without MITEs.

Some of these transformed and regenerated calli containing MITE1/2 and 35S were randomly picked and independently transferred to fresh medium to grow into clonally independent calli, from which protein extracts were prepared and the GUS activity in the extracts measured (Figure 7). Many previous reports have shown that the expression of the enzyme activities of foreign proteins derived from transgenes varies greatly among independent transformants, resulting from differences in their insertion positions in the genome, called the "position effect" (van Leeuwen et al. 2001). This fact was confirmed in the GUS activity of independent clonal calli, both with and without MITE1/2, although the mean GUS activity was higher in calli containing MITE1/2 than that in the control calli (Figure 7).

Because MITE1 and MITE2 were positioned upstream from the 35S promoter driving *GUS* (Figure 6A), it was possible that the MITEs acted as enhancer elements, upregulating the 35S–*GUS* activity in the transformants. However, the position of the MITEs was downstream

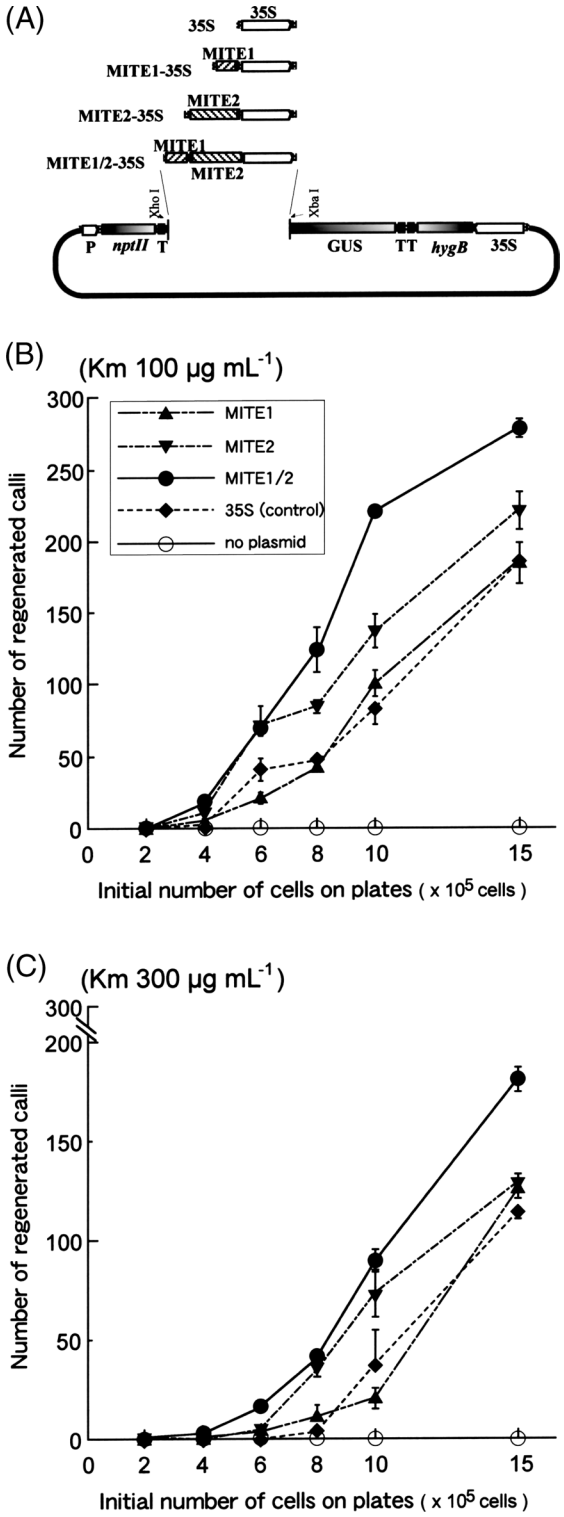


Figure 6. (A) Structure of the plasmids containing MITE1 and/or MITE2 upstream from the 35S–*GUS* gene, together with *nptII* and *hygB* as antibiotic-resistance marker genes. P and T, nopaline synthase (*NOS*) promoter and terminator, respectively. (B) and (C), The constructs shown in (A) were introduced into *A. tumefaciens*, followed by their transfection into tobacco BY-2 cells. Several series of different numbers of cells were spread onto plates of solid MS medium containing 100 $\mu$g ml$^{-1}$ (B) and 300 $\mu$g ml$^{-1}$ (C) kanamycin together with 500 $\mu$g ml$^{-1}$ cefotaxime. After one month in culture, the numbers of regenerated transformant calli on the plates were counted. Bars indicate SE (n=3).

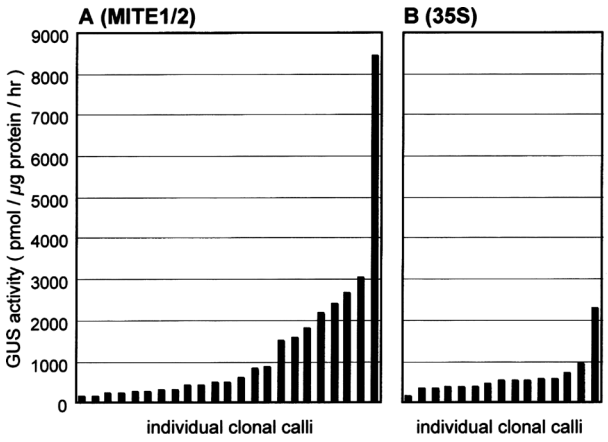


Figure 7. GUS activity in individual calli carrying the transgenes with and without MITE1/2 (A and B, respectively). The small regenerated calli transformed with MITE1/2–35S or 35S (see Figure 6) on plates containing 100 $\mu$g ml$^{-1}$ kanamycin were randomly picked and transferred to fresh MS medium containing 100 $\mu$g ml$^{-1}$ kanamycin to be grown for a further month. The GUS activities in crude extracts prepared from individual calli were then measured. The GUS activities in individual calli were aligned in ascending order, and are shown as bars in the figure.

from the terminator of the *nptII* gene, far from its promoter. It is assumed that the MITE1/2 and 35S constructs were introduced into the tobacco BY-2 cell genome randomly, with some transgenes introduced into active sites in the genome and others into cryptic sites. The quantities of transcripts and thus translated proteins might be higher from transgenes inserted into active sites, but lower from those inserted into cryptic sites. The range of these quantities, estimated as the range of the enzyme activity in individual transformants, should be distributed successively from lowest to highest, as is the GUS activity shown here (Figure 7). The amounts of NPTII protein are also expected to differ successively in individual transformed cells from lowest to highest. In the presence of the selection pressure exerted by kanamaycin, only transformed cells that can produce enough active NPTII protein to overcome the threshold level of the selection pressure will survive. The number of regenerated transformed cells might reflect how many cells produce active NPTII protein above this threshold level. If the level of active NPTII protein in individual cells correlates with the expression of the *nptII* gene, the greater numbers of regenerated calli containing MITE1/2 relative to those containing the 35S control might be attributable to greater numbers of cells expressing the *nptII* gene strongly. This suggests that MITE1/2 enhances the adjacent gene, here *npt*II, resulting in the production of enough NPTII protein to overcome the selection pressure. Our preliminary experiments with tobacco leaf discs and carrot seedlings transformed with the MITE1/2–35S or 35S constructs indicated that more regenerated shoots of tobacco and carrot contained the MITE1/2–35S construct than contained the 35S construct on plates with kanamaycin or hygromycin as the selective agents for tobacco or carrot, respectively (data not shown). The larger number of shoots regenerated from carrot seedlings transformed with the MITE1/2–35S construct relative to the number transformed with the 35S control construct implies that MITE1/2 maintains the activity of the *hygB* gene, which is located distant from MITE1/2 (Figure 6A). These results suggest that MITE1/2 allows the enhancing effect on the genes located at a vicinal region.

Here, our data indicate that such an enhancing effect of MITE1/2 on the activity of the inserted genes to the genome occurred in the stable transformants (Figures 6 and 7), but not in the transferred genes in the protoplasts of the transient expression system (Figure 5). This suggests that MITE1/2 may not act as transcription enhancer elements, as does the tobacco mosaic virus Ω sequence, which strongly enhances the expression of the introduced gene in both stable transformants and protoplasts of the transient system (Mitsuhara et al. 1996). Our results suggest that MITE1/2 increases the expression of both the transgenes in the genome and the endogenous gene, i.e., *DcPAL3*. Future studies are needed to elucidate the role of MITE1/2 in the expression of the endogenous *DcPAL3* gene.

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