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Rapid isolation of promoter sequences by TAIL-PCR: the 5′-flanking regions of *Pal* and *Pgi* genes from yams (*Dioscorea*)

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Abstract Using a modified TAIL-PCR technique, the 5′-flanking regions of the phenylalanine ammonia lyase (*Pal*) genes of a yam species, *Dioscorea bulbifera*, and the phosphoglucose isomerase (*Pgi*) gene of *D. tokoro* were successfully isolated. Two novel modifications of the TAIL-PCR procedure introduced here, namely (1) the use of a battery of random 10-mers (RAPD primers) as short arbitrary primers, and (2) the use of a total of five nested, gene-specific primers, allow the rapid isolation of the 5′-flanking region of any gene from organisms with large genomes. Isolated 5′-flanking regions were fused to the *gus* gene, and tested for transient expression in tobacco BY2 cells. All the isolated 5′-flanking regions were shown to drive reporter gene expression. Three *Pal* promoters responded to salicylic acid, presumably as a result of the binding of a MYB transcriptional activator to the multiple MREs (Myb Recognition Elements) present in these regions.

Key words TAIL-PCR · Promoter · *Dioscorea* · *PAL* gene · *PGI* gene

Introduction

The isolation of promoter and enhancer sequences is a crucial step in the study of the regulation of gene expression. Flanking regions of genes, containing these elements, have been conventionally isolated by screening genomic libraries using cDNAs as probes. However, the construction and screening of genomic libraries involves time-consuming procedures. As alternatives, PCR-based methods have increasingly been applied for this purpose. Inverse PCR (Ochman et al. 1988), and ligation-mediated PCR (Rosenthal and Jones 1990; Devon et al. 1995; Siebert et al. 1995; Balavoine 1996; Zhang and Chiang 1996) are the techniques most frequently used for the isolation of flanking regions of genes. These methods rely on the presence of restriction sites in the region to be isolated, so that the fragments can be self-ligated to form circular molecules (inverse PCR) or ligated to a DNA cassette (ligation-mediated PCR), prior to PCR. As information on restriction sites is usually not available in advance, there is no guarantee that digestion with a particular restriction enzyme will be successful, thereby necessitating trials with several different enzymes. Furthermore, in the case of ligation-mediated PCR, there exists the inherent problem of undesirable amplification of PCR products that are flanked by the DNA cassette sequence at both ends, at the expense of target sequence amplification. Several commercially available kits for the isolation of regions flanking a known DNA sequence try to minimize this problem by modifying the cassette structure (e.g. Siebert et al. 1995), but complete prevention of PCR amplification of non-target sequences is difficult. PCR products of the target sequence may be separated from non-target sequences by using biotinylated gene-specific primers and streptavidin-coated magnetic beads (Rosenthal and Jones 1990). However, this procedure requires biotin-labeling and capture/separation of the primers, which entails further costs, takes more time and reduces yields of target sequences. Therefore, simpler and more reliable techniques for promoter isolation are urgently required.

The TAIL-PCR (thermal asymmetric interlaced PCR) method developed by Liu and Whittier (1995) is such a simple, but nevertheless efficient, technique for genomic walking which does not require any restriction or ligation steps. PCR is carried out with long sequence-specific primers in combination with short degenerate

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Dedicated to Professor Dr. George G. Laties (UCLA, Los Angeles, Calif.) on the occasion of his 80th birthday

primers of arbitrary sequence. An elaborate thermal cycling program composed of "supercycles", each consisting of one low-stringency cycle and two high-stringency cycles, allows only sequence-specific fragments to be exponentially amplified. This method has been successfully used to isolate insert-end segments of P1 and YAC clones (Liu and Whittier 1995) and flanking regions of T-DNA inserts in *Arabidopsis* (Liu et al. 1995). But to the best of our knowledge, this method has never been employed for the isolation of flanking regions of any resident genes. Here we report the successful isolation of the 5′-flanking regions of *Pal* and *Pgi* genes of yams using a modified TAIL-PCR method. Two novel and essential modifications of the standard method were introduced for the systematic isolation of flanking regions of resident genes of organisms with large genome sizes: (1) use of a battery of random 10 mers originally developed for RAPD analysis (Williams et al. 1990) as the short arbitrary primers instead of three degenerate 16-mer primers as described in the original TAIL procedure (Liu and Whittier 1995), and (2) use of a total of five nested gene-specific primers instead of three. By using modification (1), we were able to exploit a whole battery of 10 mer primers from commercially available, low-cost primer sets designed for RAPD analysis (Williams et al. 1990). The use of a large number of 10 mers increases the probability of amplifying long target sequences. Modification (2) was necessary to amplify target sequences from the complex genomes of the genus *Dioscorea*, which are about five times larger (550 Mb; Arumuganathan and Earle 1991) than the *Arabidopsis* genome, for which the original TAIL-PCR technique was developed (Liu et al. 1995).

The *Pal* gene codes for phenylalanine ammonia lyase, the enzyme that catalyzes the conversion of phenylalanine to transcinnamic acid in the initial step of phenylpropanoid biosynthesis (Hahlbrock et al. 1976; Hahlbrock and Scheel 1989; Wanner et al. 1995). The *Pal* gene was one of the first plant defense genes to be identified, and was found to be induced by pathogens and environmental stresses (Kuhn et al. 1984; Edwards et al. 1985; Hahlbrock et al. 1995; Logeman et al. 1995). To obtain an elicitor-inducible promoter for the genetic engineering of yam crops, we have been trying to isolate the 5′-flanking region of this gene. The *Pgi* gene codes for phosphoglucose isomerase, a key enzyme in glycolysis, which is known to be constitutively expressed. Molecular population genetic studies of the coding region of the *Pgi* from *D. tokoro* (Terauchi et al. 1997) prompted us to isolate and characterize the 5′-flanking region of the gene.

Materials and methods

Plant materials, genomic DNA and cDNA

Total genomic DNA was extracted from individual *Dioscorea bulbifera* plants (cultivar *sativa*) and from *D. tokoro* using the standard CTAB (cetlytrimethylammonium bromide) method (Murray and Thompson 1980). DNAs were further purified by CsCl ultracentrifugation. Total RNA was isolated from *D. bulbifera* leaf tissue by a phenol/SDS method (Palmiter 1974), and cDNA was obtained from it by reverse transcription using Superscript II (Gibco-BRL).

Isolation of *Pal* coding region from *D. bulbifera*

Alignment of the amino acid sequences derived from phenylalanine ammonia lyase (*Pal*) genes from 11 plant species (*Arabidopsis thaliana*, alfalfa, avocado, *Camellia*, *Ipomoea*, parsley, *Pisum*, *Populus*, rice, *Trifolium*, tobacco and tomato, obtained from the Genbank database) identified highly conserved sequence regions. Based on the sequences of these regions, two degenerate primers were synthesized (Fig. 1): D0 (5′-CAYYTIGAAYGARGTIAAR-MRIATGGT-3′, the forward primer) and U3 (5′-GMRCTICCRTC-

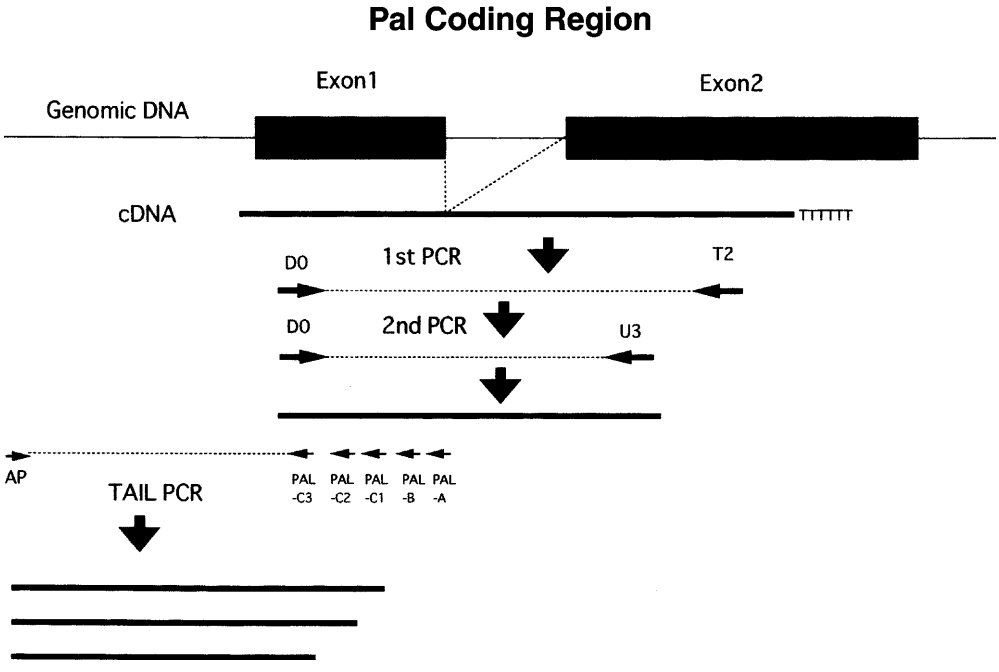

Fig. 1 Schematic outline of the procedures used to isolate *Pal* 5′-flanking regions from *Dioscorea bulbifera*. First, a partial coding region of *Pal* was amplified by two consecutive PCRs using the exon-specific primer pairs D0 and T2 (first PCR), and D0 and U3 (second PCR). On the basis of the sequence amplified, five gene-specific primers were synthesized and used in combination with an arbitrary primer (AP) for TAIL-PCR to obtain the 5′-flanking region

IARDATRTGYTCCAT-3′, the reverse primer). In addition, the primer T2 (5′-ACRTCYTGRTTRTGYTGYTC-3′, reverse primer) was synthesized according to Howles et al. (1994). PCR performed with the primer pair D0/T2 using *D. bulbifera* cDNA as template resulted in non-specific amplification. An aliquot of this PCR product was then used as the template for the secondary PCR with primer pair D0/U3. The resulting discrete PCR product of the expected size was cloned into pBluescript, and sequenced.

The TAIL-PCR procedure

In order to allow chromosome walking beyond the known *Pal* and *Pgi* sequences into the unknown 5′ flanking region, TAIL-PCR (Liu and Whittier 1995; Liu et al. 1995) was employed with two essential modifications: (1) the use of 10 mer random primers instead of degenerate 16 mers as the short primer, and (2) the use of a total of five rather than three gene-specific primers in nested positions to ensure selection of the correct target fragments.

On the basis of the cDNA sequence of a *Pal* gene from *D. bulbifera* and a genomic *Pgi* DNA sequence from *D. tokoro* (Terauchi et al. 1997), a total of five gene-specific primers in nested positions close to the 5′-end of the coding regions were designed and synthesized. The primers for *D. bulbifera Pal* were PAL-A (5′-CCCTGCTTGGTYCTCCKATGAG-3′), PAL-B (5′-CGCCAAAACCAGTAGTGACACC-3′), PAL-C1 (5′-TCTTCATCACCCAATCACTGCT-3′), PAL-C2 (5′-TGGTCTGGCCTCCTCTGAGAGC-3′) and PAL-C3 (5′-CTAAGCTGACAACAGGATTCCT-3′). The primers used for *D. tokoro Pgi* were PGI-A (5′-ACAGTGGCAGGAAGCACTCGTTGCCGGGAATAGTC-3′), PGI-B (5′-GCATGAGATCGCGGAGATGCGTCTTCTTGATTTCT-3′), PGI-C1-(5′-TTTGAGGGACGAATGGAGGAAGAG-3′), PGI-C2 (5′-GATACATTGAGGTCCTTCCACTGC-3′) and PGI-C3 (5′-TTCGCAGATAAGCGTGGACGTAGC-3′) (Fig. 2). These primers were designed such that the *Tm* calculated according to Mazers et al. (1991) would be higher than 62 °C for the primers used in the primary PCR (PAL-A and PGI-A) and the secondary PCR (PAL-B and PGI-B), and higher than 57 °C for the tertiary PCR (PAL-C1–C3 and PGI-C1–C3). Sixteen arbitrary 10 mer primers each for *Pal* and *Pgi* were chosen from the 10 mer primer sets available (Roth), and care was taken to ensure that they were not predicted by the Oligo program (National BioSciences) to form stable duplexes with either of the two gene-specific primers (A and B).

Three rounds of PCR (Table 1) were carried out on a Perkin Elmer 9600 thermal cycler using the product of the previous PCR as template for the next, and employing a common arbitrary primer and nested gene-specific primers in a consecutive manner. The annealing temperature for the low-stringency cycle was set to 29 °C, instead of 44 °C as in the original protocol (Liu and Whittier 1995). The primary PCR was carried out in a 20-µl volume containing 100 ng of genomic DNA, 0.2 µM gene-specific primer (Primer A), 2.0 µM 10 mer primer, 200 µM of each dNTP, 0.2 U Taq polymerase (Gibco-BRL) and 1× Taq polymerase buffer supplied with the enzyme. The secondary PCR was carried out with Primer B in combination with the same arbitrary primer as used in the primary PCR. The reaction solution was the same as for the primary PCR, except that 1 µl of a 1/50 dilution of the primary PCR product was used as template. For the tertiary PCR, three

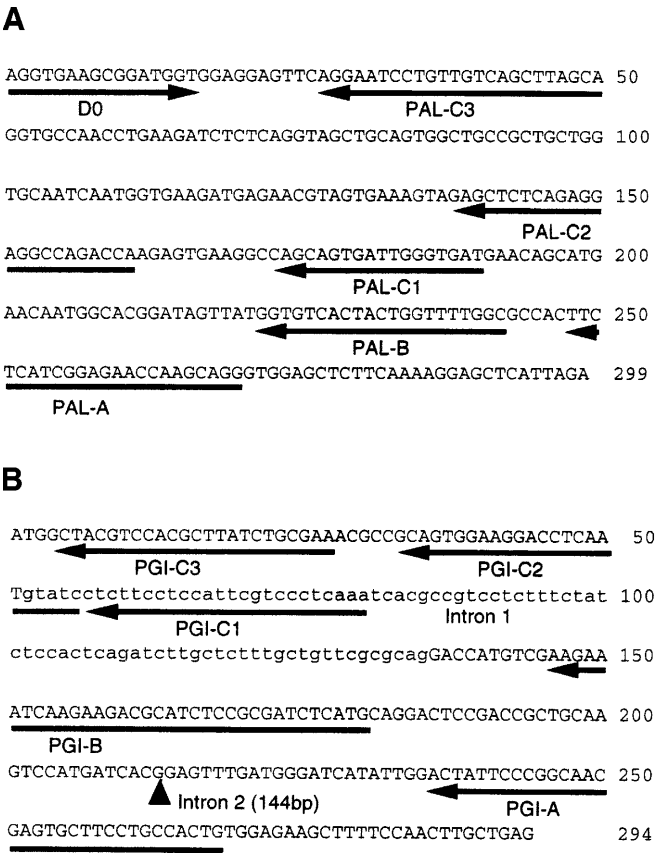

Fig. 2A, B Localization of the five gene-specific primers for the *Pal* gene of *D. bulbifera* (**A**) and the *Pgi* gene of *D. tokoro* (**B**)

Table 1 Reaction parameters for the TAIL-PCR used to amplify the 5′- flanking regions of the *Pal* genes of *Dioscorea bulbifera* and the *Pgi* gene of *D. tokoro*

Reaction (primer combination)	Program no.	Number of cycles	Cycle (supercycle) parameters
Primary PCR			
(AP/Primer A)	1	1	93 °C, 1 min; 95 °C, 1 min
	2	5	94 °C, 30 s; 62 °C, 1 min; 72 °C, 2.5 min
	3	1	94 °C, 30 s; 25 °C, 3 min; ramping to 72 °C over 3 min; then 72 °C, 2.5 min
	4	15	94 °C, 10 s; 68 °C, 1 min; 72 °C, 2.5 min; 94 °C, 10 s; 68 °C, 1 min; 72 °C, 2.5 min; 94 °C, 10 s; 29 °C, 1 min; 72 °C, 2.5 min
	5	1	72 °C, 5 min
Secondary PCR			
(AP/Primer B)	6	12	94 °C, 10 s; 64 °C, 1 min; 72 °C, 2.5 min; 94 °C, 10 s; 64 °C, 1 min; 72 °C, 2.5 min; 94 °C, 10 s; 29 °C, 1 min; 72 °C, 2.5 min
	5	1	72 °C, 5 min
Tertiary PCR			
(AP/Primers C1–C3)	7	20	94 °C, 15 s; 29 °C, 30 s; 72 °C, 2 min
	5	1	72 °C, 5 min

gene-specific primers (C1, C2 and C3) were separately used with the common arbitrary primer. The reaction solution for the tertiary PCR was the same as for the primary PCR except that 1 μl of a 1/10 dilution of the secondary PCR product was used as template, and the concentration of the arbitrary primer was 0.2 μM instead of 2.0 μM.

The products of the tertiary PCR (three PCRs for each arbitrary primer, corresponding to the three gene-specific primers C1 to C3) were separated in adjacent lanes on agarose gels to determine whether discrete PCR products from the three gene-specific primers show size differences corresponding to the relative positions of the nested primers. In the original protocol developed for *Arabidopsis* (Liu et al. 1995), PCR products of the secondary PCR (obtained with primer B) and tertiary PCR (obtained with primer C1) were separated in two adjacent lanes by agarose gel electrophoresis to detect the expected size difference. However, this procedure was not applicable for *Dioscorea*, as electrophoresis of the secondary amplicons usually resulted in smeared patterns. Use of three (C1, C2 and C3) instead of two primers for the tertiary PCR was also important to allow us to discriminate the true step-wise size differences corresponding to the different primer locations from spurious amplifications.

Cloning and sequencing of the tertiary PCR products

PCR products were excised from the agarose gel and reamplified. After polishing the ends with the Klenow fragment (NEB) and T4 polynucleotide kinase (NEB) in the presence of dNTPs and ATP, they were cloned into the *Sma*I site of pBluescript. DNA sequencing was performed on an ABI 373A automated sequencer.

Transient expression assay using cultured tobacco BY2 cells

Cloned 5′-flanking regions of *Pal* (PAL-AP140, PAL-AP122 and PAL-AP103) and *Pgi* (PGI-AP125) genes were PCR-amplified with primers carrying *Bam*HI (the distal end of the 5′-flanking regions) and *Xho*I (the proximal end) extensions at the 5′-ends. The products were ligated to a 2.6-kb *Xho*I-*Hin*dIII fragment containing the *gus* (*β*-glucuronidase) gene and the CaMV 35S polyadenylation signal derived from pRT101-gus (Töpfer et al. 1993), and cloned into the *Bam*HI-*Hin*dIII sites of pBluescript SK− (Stratagene), resulting in the plasmids pRG108 (PAL-AP140), pRG109 (PAL-AP122), pRG110 (PAL-AP103) and pRG103 (PGI2; see Fig. 5). Each of these GUS constructs was mixed in a 1:1 ratio with plasmid pRT101-LUC (a kind gift of Dr. C. Kirchner; Töpfer et al. 1993), harboring a firefly luciferase gene under control of the CaMV-35S promoter and 35S polyadenylation signal, and introduced into BY2 cells (*Nicotiana tabacum* cv. Bright Yellow; Ikeda et al. 1976) by particle bombardment (Biorad) following the manufacturer's instructions. Tobacco BY2 cells maintained in the BY-2 medium containing 2,4-D (Matsuoka and Nakamura 1991) were collected on a filter paper 4 days after subculturing, placed on a solid medium containing 0.2% gelan gum, and bombarded with the plasmids. After 24 h the cells were transferred to a solid BY-2 medium containing either (1) no added ingredients, (2) a culture filtrate of *Botrytis cinerea*, (3) N-acetylchitohexaose (0.02 mg/ml; Yamada et al. 1993), or (4) salicylic acid (SA; 20 μM), and cultured for another 24 h. Then cells were harvested and lysed in LC-*β* PicaGene cell lysis buffer (Wako Chemicals) by sonication. The lysate was centrifuged, and the supernatant immediately assayed for luciferase and *β*-glucuronidase activity using the PicaGene

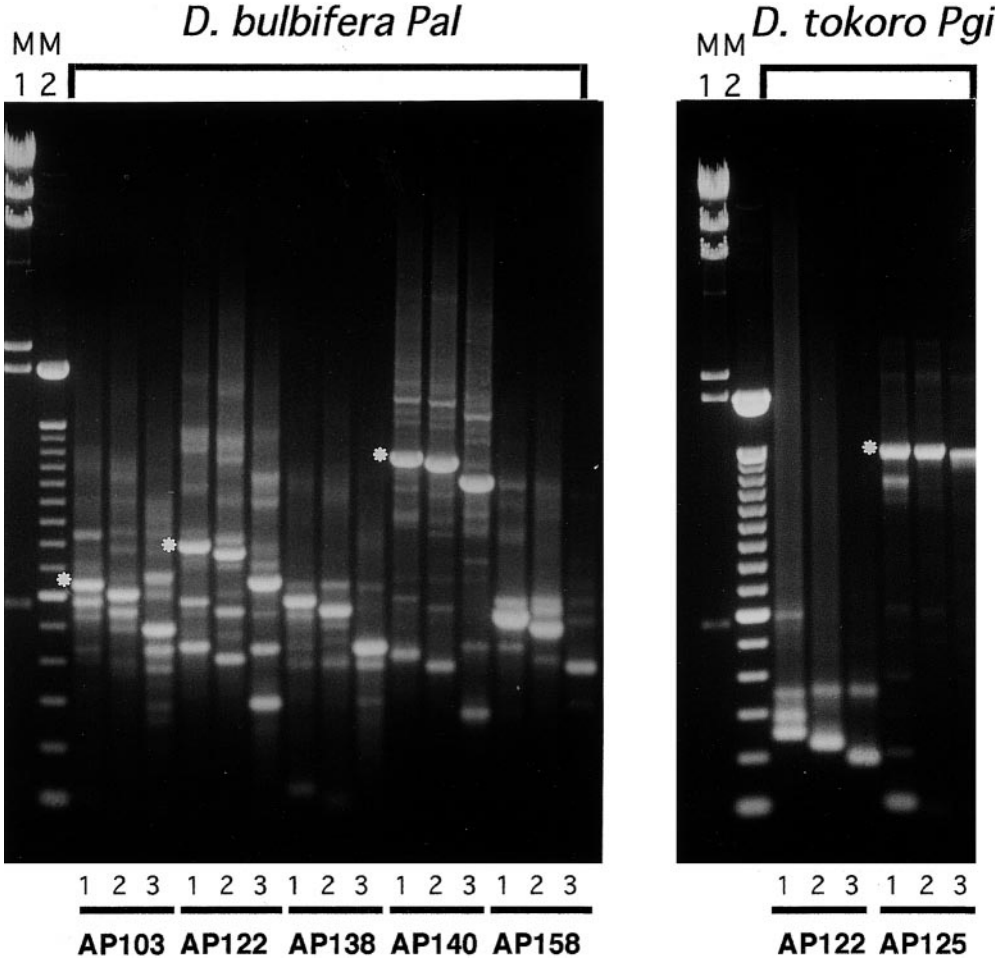

Fig. 3 Agarose gel electrophoresis of the tertiary PCR products of *D. bulbifera Pal* (left) and *D. tokoro Pgi* (right). M1 and M2 are molecular weight markers [*Hin*dIII digest of *λ*-DNA, and 100-bp ladder (Gibco-BRL), respectively]. DNA fragments marked with *asterisks* were excised from the gel and sequenced. The sequences of the arbitrary 10 mer primers were: 5′-GGTGCTCCGT-3′ (AP103), 5′-CGATGAGCCC-3′ (AP122), 5′-CTATCGCCGC-3′ (AP138), 5′-CGCAGACCTC-3′ (AG140), 5′-GTGTGCCCCA-3′ (AP158) and 5′-ACGGTGC-CTG-3′ (AP125)

Luciferase detection kit (Wako Chemicals) and the Gus-Light GUS detection kit (Tropix), respectively, and an ATTO luminometer. Promoter activity was expressed as the GUS value divided by the LUC value, the latter serving as a normalizing factor to reduce the interexperimental fluctuation caused by differences in cell viability and/or efficiency of plasmid delivery by particle bombardment.

Results and discussion

Isolation of the 5′-flanking regions of *Pal* genes from *D. bulbifera*

Among the 16 arbitrary primers tested in combination with a set of *Pal* gene-specific primers (PAL-C1, PAL-C2 and PAl-C3), ten primers resulted in amplification of discrete PCR products. Electrophoretic patterns obtained for five arbitrary primers are shown in Fig. 3. Successful walking can easily be confirmed by the stepwise change in the sizes of PCR products that correspond to the relative positions of the three nested, *Pal*-specific, primers (PAL-C1, PAL-C2 and PAL-C3). Further confirmation came from the observation that DNA sequences of PCR products obtained by PAL-C1 primer overlapped perfectly with the 5′-end sequence of a cDNA (data not shown). The sizes of PCR products ranged from 400 to 1300 bp (when PAL-C1 was used for walking).

Aligned DNA sequences of three PCR products (generated by the arbitrary 10 mers AP140, AP122 and AP103) are given in Fig. 4. DNA sequences were deposited in DDBJ, EMBL and Genbank under Accession Nos. AB016713–AB016715. The longest walk into the 5′-flanking region was obtained with AP140 (1014 bp upstream of the start codon), followed by AP122 (510 bp) and AP103 (352 bp). Although the region close to the coding sequences is highly conserved, there are extensive differences between the products obtained with

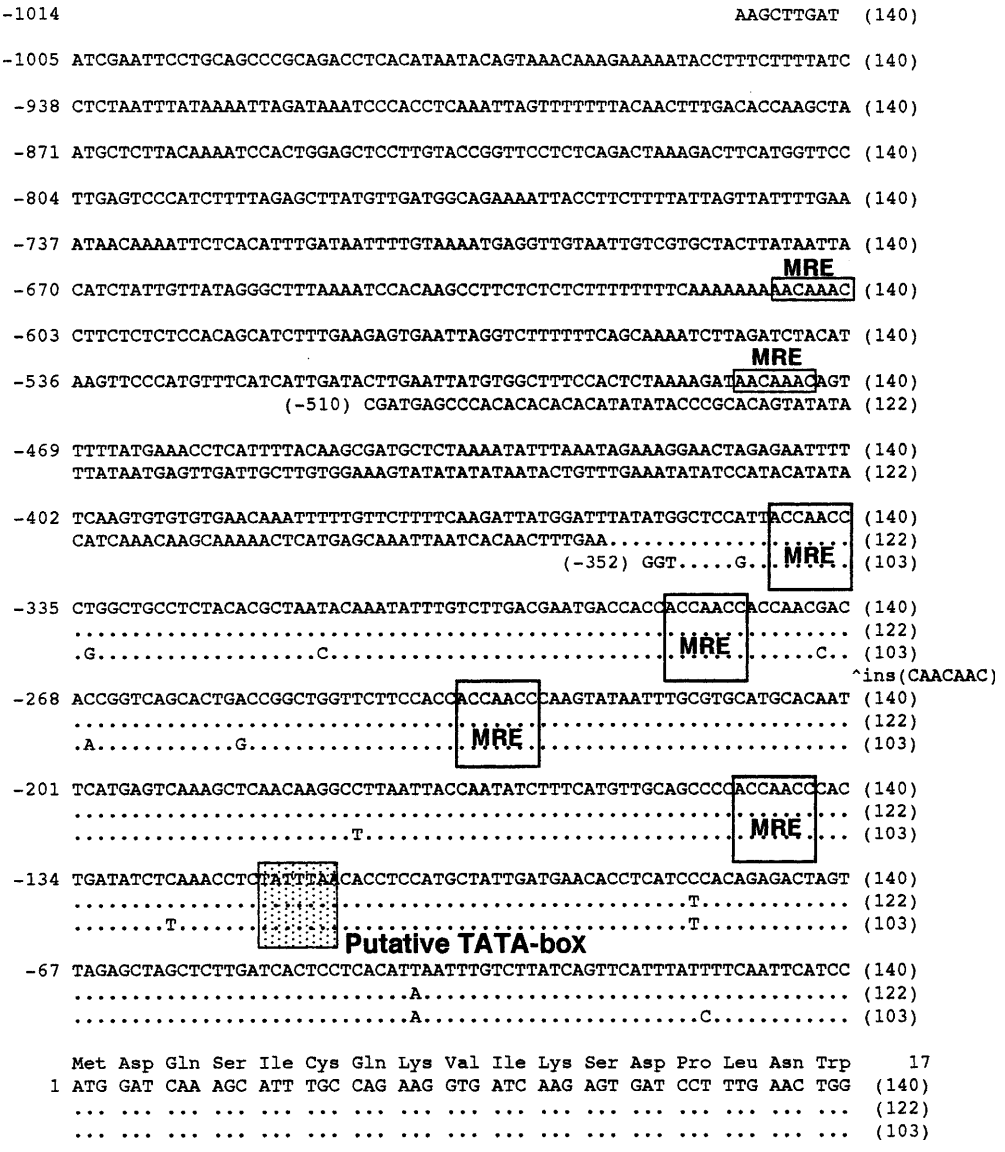

Fig. 4 Aligned DNA sequences of three TAIL-PCR products (amplified with the arbitrary primers AP140, AP122 and AP103) obtained from the 5′-flanking regions of *Pal* genes of *D. bulbifera*. The putative TATA box (sequence 5′-TATTTAA-3′) and MYB recognition elements (MRE consensus sequence: 5′-ACCAACC-3′) are *boxed*

the APs in regions further upstream (a 7-bp insertion in the AP103 sequence at position – 168 from the start codon; no sequence homology between AP140 and AP122 sequences upstream of position – 355). These observations indicate that we have isolated the 5′-flanking regions of different *Pal* loci. Indeed *Pal* genes are known to form a multigene family (Logeman et al. 1995; Wanner et al. 1995). In the regions isolated, a putative TATA box and several MREs [MYB recognition elements; 5′-G(G/T)T(A/T)G(G/T)T-3′; type II MYB consensus sequence; Yang and Klessig 1996] could be identified. MREs were previously identified in boxes P and L of parsley *Pal* genes, and obviously are address sites for the MYB-like protein BPF-1 (de Costa e Silva et al. 1993; Feldbrügge et al. 1997).

Isolation of the 5′-flanking region of the *Pgi* gene from *D. tokoro*

After testing 16 arbitrary 10 mers, five primers were found to result in the desired products. Two examples are shown in Fig. 3. The sizes of the PCR products obtained ranged from 200 to 1500 bp (when PGI-C1 was used for walking). The DNA sequence of the longest PCR product (obtained with arbitrary primer AP125) has been deposited in DDBJ, EMBL and Genbank under the Accession No. AB016716.

Transient expression of the GUS gene driven by the isolated 5′-flanking regions

Isolated 5′-flanking regions of *Pal* and *Pgi* genes were fused to the *gus* gene, and their activity was tested by transient transformation after delivery into tobacco BY2 cells by particle bombardment (Fig. 5). Three plasmids bearing *Pal* 5′-flanking regions (pRG108 for PAL-AP140, pRG109 for PAL-AP122 and pRG110 for PAL-AP103) and a plasmid carrying the *Pgi* 5′-flanking region (pRG103 for PGI-AP125) were found to drive expression of the *gus* gene (Fig. 5). This indicates that the isolated 5′ flanking regions contain the minimal promoter elements necessary for the transcription of the genes located downstream.

Responses of the promoters to external stimuli were tested by culturing the transformed tobacco BY2 cells on media containing various substances. As shown in Fig. 5, the presence of SA in the medium significantly increased the level of expression of *gus* driven by all three *Pal* 5′-flanking region constructs (pRG108, pRG109 and pRG110), relative to the expression level of the control (water), whereas culture filtrate of *Botrytis* and *N*-acetylchitohexamer had no effect.

Yang and Klessig (1996) have shown that the *myb* oncogene homolog from tobacco (*myb1*) is rapidly induced (within 15 min) by externally applied SA, and that Myb1 protein specifically binds to a Myb-binding consensus sequence, 5′-G(G/T)T(A/T)G(G/T)T-3′,

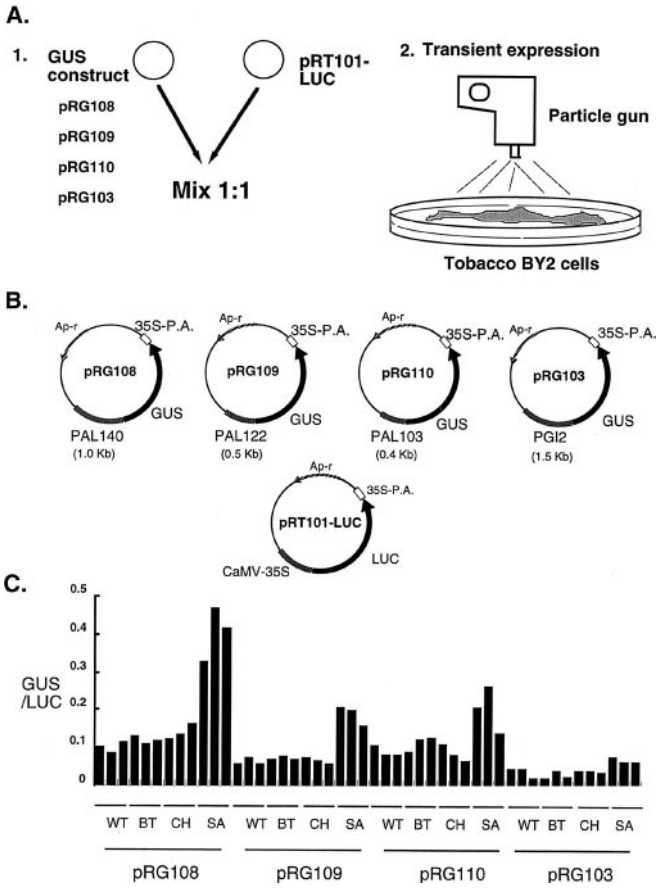

Fig. 5 A Schematic outline of the strategy for transient expression analysis using tobacco BY2 cells **B** Structure of the transient expression vectors carrying *Pal* (pRG108, pRG109 and pRG110) and *Pgi* (pRG103) 5′-flanking regions. **C** Transient expression of *gus* genes after introduction of plasmids into tobacco BY2 cells by particle bombardment. Some 24 h after plasmid introduction, cells were placed on medium supplemented either with water (WT), *Botrytis cinerea* culture filtrate (BT), *N*-acetochitohexamer (CH) or salicylic acid (SA). Experiments were performed in triplicate. The ordinate represents the ratio between the GUS and LUC measurements

found in the promoter region of the tobacco *PR-1a* gene, the transcription of which is induced several hours after *myb1* induction. They suggest that the tobacco *myb1* gene encodes a signaling component that acts downstream of SA, probably participating in transcriptional activation of PR genes that contribute to plant disease resistance. In view of the presence of multiple MREs in the 5′-flanking regions of *D. bulbifera Pal* genes, we assume that upon the treatment of transformed tobacco BY2 cells with SA, *myb1* was induced and Myb1 subsequently bound to MRE of the *Pal* promoter regions of the reporter plasmids, thereby activating the expression of *gus*.

Versatility of the TAIL-PCR method for the isolation of promoters

Using the TAIL-PCR technique with two modifications, we have successfully and rapidly isolated the 5′-flanking

regions of three *Pal* genes from *D. bulbifera* and the *Pgi* gene from *D. tokoro*. Transient expression studies showed that all the isolated sequences are transcriptionally functional. In the case of the *Pal* gene, 5′-flanking regions of multiple loci could be recovered. This demonstrates that we can isolate the promoter regions of most of the members of a particular gene family by systematically testing a large number of 10 mers of arbitrary sequence, in combination with consensus degenerate gene-specific primers. This versatile method recommends itself for the isolation of regulatory elements of genes from any organism.

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