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Tissue Specific Expression of Tomato Phenylalanine Ammonia-lyase Gene in Transgenic Tobacco Plants

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형질전환 담배에서 토마토 PAL유전자의 조직 특이적 발현

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Tomato phenylalanine ammonia-lyase 5 (tPAL5) was identified that alternate initiation sites were utilized differentially in response to environmental stimuli (Lee et al, 1992b). In this study, we tried to look into tissue- or cell- specific expression pattern of tPAL5 gene by fusing with β-glucuronidase (GUS) gene in transgenic tobacco plants. In transgenic plants, root and stem extracts contained 8~12 fold higher levels of GUS activity than petiole or leaf tissue while the highest levels of induction was observed from leaf tissue by mechanical wounding (5~11 fold). In *trans*-sections of stems and petioles, GUS activity was restricted to phloem cells(outer region) of developing vascular bundle and mainly at apical tip region in the root tissues. The levels of GUS activity was drastically reduced (10~12 fold reduction) when the 5'-upstream region of tPAL5 gene (-1151bp from ATG codon) was deleted up to -665. The levels of GUS expression, however, raised up by 6~8 fold when deleted up to -455. Therefore, we conclude that there are positive *cis*-elements at the region -1151 to -1008 and at -455 to -195 while the negative *cis*-element is at -1008 to -455.

Key words: expression patern, promoter analysis, *cis*-element

Phenylalanine ammonia-lyase (PAL) is one of the best studied enzymes involved in the plant secondary metabolisms synthesizing natural products such as flavonoid pigments, antibacterial phenolics, UV protestants, cell wall associated phenolics, and several forms of ester compound which are all derived from the phenylpropanoid pathway (Hahlbrock and Grisebach, 1979). Such products are synthesized in response to normal developmental cues, pathogen attack, and mechanical stresses (Lamb et al., 1989).

In this sense, genes involved in phenylpropanoid pathway have been an intensive research target for the regulation upon treatment with various environmental stimuli (Liang et al., 1989a, Lois et al.: 1989). The chalcone synthase (CHS) gene which catalyzes the first step in the biosynthesis of flavonoids from phenylpropanoid is also induced by fungal elicitor preparation, illumination, and mechanical wounding (Ryder et al., 1987).

In tomato, we have found that there are PAL5 genes in a small gene family (Lee et al., 1992b) and differentially regulated upon treatment with light, microbial infection, and mechanical wounding (Lee et al., 1994). During our studies on the responding of fungal infection on resistant and susceptible lines of tomato, we have found that the resistant line produced more PAL transcript than that of the susceptible.

In this study, we report the expression pattern of the

tomato PAL5 gene in transgenic tobacco by fusing with reporter gene (β-glucuronidase) and the effects of mutation on the promoter of the tPAL5 gene.

MATERIALS AND METHODS

Plasmid construction

A clone containing the upstream region including the first exon and intron of the tPAL5 gene was obtained from a λ charon 4A tomato genomic DNA library. Each deleted upstream region from the translation codon (ATG) of the PAL5 gene was generated by PCR amplification using each primer located at the numbered position in Figure 1. Two primers were synthesized to contain two restriction enzymes sites : *Hind* Ⅲ at 5'-end and *Bam*HI at 3'-end. The *Hind* Ⅲ-*Bam*HI fragment containing each deleted region was cloned into the same sites of GUS expression vector pBI101 to give PAL5-GUS gene fusions, pBIPAL1 to pBIPAL5.

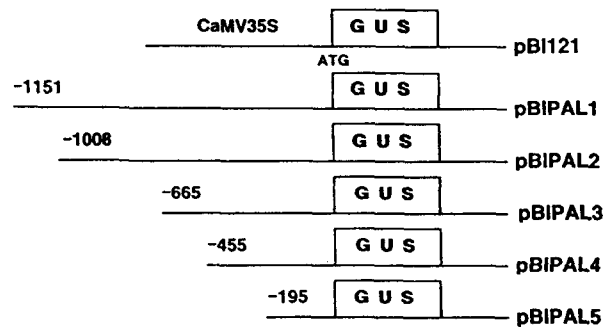

Figure 1. Structures of tPAL5-GUS gene fusions. Each structure was deleted fragments of tPAL5 substituted with CaMV35S promoter of plant binary vector pBI121. These were externally deleted mutants from 5'-terminus of native tPAL5 promoter.

Plant transformation

The binary vectors containing each deleted PAL5 upstream sequence and GUS gene were transferred from *E. coli* (DH5 α) into *Agrobacterium tumefaciens* LBA4404 by the direct transformation method. *Agrobacterium*-mediated *Nicotiana tabacum* var. Xanthi leaf disc transformation and regeneration transgenic plants were performed as previously described (An *et al*. 1985: An et al., 1988). Putative transformants showing kanamycin resistance were confirmed by observing the ability of germination on the media containing 500 mg/L. kanamycin. Transformants were grown at 25 ± 1°C under 16/8 h cycle.

GUS assay

Samples (5 mm×5 mm) were cut from leaf, stem, and petiole and then macerated with pestle in the extraction buffer (50 mM $NaPO_4$, 10 mM EDTA, 0.1%(w/v) sodium sarcosinate, 0.1%(v/v) Triton X-100, pH 7.0). Then they were vortexed with 0.5 g/L of glass bead for 1~2 min. Clear solution for GUS enzyme assay was obtained by centrifugation. From tissue extracts of transgenic plants, GUS was assayed as described by Liang *et. al* (1989b). 4-Methylumbelliferyl glucuronide (MUG) was used as a substrate for GUS enzyme. GUS activity was expressed as nmoles of product per min per g of tissue.

For histochemical localization of GUS activity *in situ*, chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-gluc) was used for staining. Each tissue section was cut and fixed in solution containing 0.3% formaldehyde, 10 mM MES (pH 5.6), and 0.3 M mannitol for 2 h. Then tissue was reacted with in solution containing 2 mM X-gluc in 50 mM Na_2PO_4 (pH 7.0) at 37°C for 14 h, bleached with 50%(v/v) and 100%(v/v) methanol solution and sectioned by hand with razor blade for microscopy. For observing the wound induced, the stem tissue was cut from the transgenic plants, epidermic cell was removed with razor blade and left on the wet paper tissue for 4 h.

RESULTS AND DISCUSSION

Cloning of deleted tPAL5 promoter regions

The sequence of the tomato PAL5 gene has been described by Lee *et al.* (1992a), and the putative *cis*-elements on the promoter region were discussed by comparing with other known *cis*-elements of PAL genes isolated from bean (Cramer et al., 1989), parsley (Lois et al., 1989). The full-length upstream region (-1151 bp) from the translation start site was inserted into *Hind* Ⅲ/*Bam*H I site of the pBI101 vector. Series of deleted promoter fragments were generated by PCR amplification with synthetic oligomer taken from at indicated positions and common oligomer at the translation start site to create *Bam*H I site to be translationally fused with the β-glucuronidase gene (Figure 1). The result of substituted each deleted fragment was shown in Figure 2.

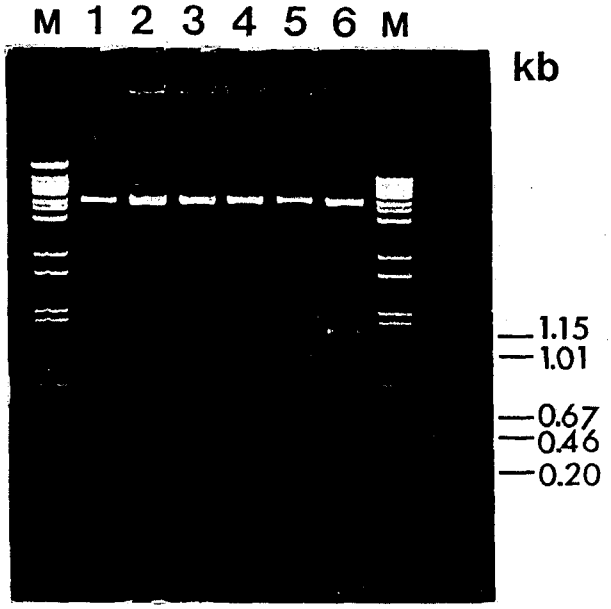

Figure 2. Electrophoretic patterns of externally deleted mutants which were subcloned into *Bam*HⅠ/*Hin*dⅢ site of pTZ19U. Lane M, lambda DNA/*Bst*EII digest as size marker: lane 1, pTZ19U: lane 2-6, series of deleted fragments of tPAL5 promoter.

Analysis of transgenic plants

In order to look into the functional properties of tPAL5 promoter, we have deleted promoter region as indicated in Figure 1 and each construct was transformed into tobacco plants. Transgenic plants were regenerated and seeds were harvested to confirm indirectly the integration of tPAL5-GUS fusion gene in the chromosome of tobacco plants by testing the ability of germination on the media containing kanamycin (500 mg/L). In general, the ratio of resistant to susceptible seeds was observed as 3 : 1 from each transgenic plants.

tPAL5-GUS expression of transgenic tobacco plants

Assay of extractable GUS activity was used to monitor the activity of the tPAL5 promoter in different organs during development and in response to wounding stress with those transgenic plants. When the full length of the tPAL5 promoter (-1151) was fused to the GUS gene, only low levels of GUS activity was observed in leaf extracts, whereas root and stem extracts contained 8 to 12 fold higher levels of GUS activity (Table 1). In general, the distribution of GUS activity in different organs of transgenic plants containing the tPAL5-GUS gene fusion closely resembles the pattern of accumulation of endogenous tPAL5 transcripts in tomato (Lee et al., 1994). The tPAL5 transcripts also accumulate at levels several fold higher than normal in tomato in response to wounding or dark-to-light transitions (Lee et al., 1994), and therefore, we examined the effects of mechanical wounding on the expression of the tPAL5-GUS gene fusion in transgenic tobacco. In fact, wounding of leaf tissue induced GUS activity 5 to 11 fold, whereas in root and stem, only 3 to 5 fold induction was observed (Table 1). This results also closely resemble the pattern of transcriptional level observed from endogenous tPAL5 gene's response to wounding in tomato by S1 nucleate mapping technique (Lee et al., 1994). Such an induction pattern of endogenous PAL transcription level was also observed from several other plant species including parsley (Lois and Hahlbrock, 1992).

Table 1. Tissue-specific and wound-induced expression of tPAL5-GUS construct.

Organ	Condition	No. of Analyzed Plants	GUS activity (nmole MU/g/min)
Leaf	*UW	5	109 ± 55
	**W	7	1090 ± 155
Petiole	UW	5	73 ± 21
	W	7	511 ± 95
Stem	UW	5	876 ± 87
	W	7	2628 ± 240
Root	UW	5	1202 ± 105
	W	7	6010 ± 550

*, unwounded; **, wounded

Putative *cis*-elements on the tPAL5 promoter region

Multiple functional *cis*-elements were identified to be located on the tPAL5 promoter region. The full length (-1151 bp) of promoter showed maximum levels of GUS expression but deletion up to -665 caused drastic decrease of GUS expression (almost to basal level). Interestingly, GUS activity was raised up again when deleted up to -455 (about 6 to 8 fold). Very low levels of GUS activity was observed by deletion up to -195. These results suggested that there are multiple *cis*-element on the tPAL5 promoter to regulate the expression pattern in the developing vascular tissue (Table 2). The results indicated that the -1151bp to -1008 region and -455 to -195 region of the tPAL gene contain the sequence essential for the tissue specific expression and there are suppressing elements at between -1008 and -455 region.

Series of deleted tPAL5 promoter showed that positive elements are located at the -1151 to -1008 and -455 to -195

Table 2. GUS activity of tPAL5 promoter in stem of transgenic tobacco plants.

Structure	No. of Analyzed Plants	GUS activity (nmole MU/g/min)
pBIPAL1	15	875 ± 120
pBIPAL2	17	67 ± 7
pBIPAL3	21	42 ± 5
pBIPAL4	15	611 ± 82
pBIPAL5	16	110 ± 32

and a negative element is located at -1008 to -455. These results might be related with alternate transcription initiation sites reported in previous report (Lee et al., 1994). It has been reported that the tPAL5 gene utilizes alternate transcription initiation sites in response to different environmental stimuli including high light illumination, wounding, and *Verticillium* infection. While the transcript initiated at site 2 behaves as constitutive in expression, showing no conspicuous changes to different stresses, the second transcription initiated at site 1 is dramatically influenced by either wounding, light or fungal infection.

In this study, we propose that the two initiation sites may be controlled by their own set of *cis*-element and transcription factor since multiple *cis*-elements were identified to be related to each initiation sites. While the transcription initiated at site 2 seems to be regulated by a *cis*-element located at -1151 to -1008, the transcription initiation at site 1 is regulated by a *cis*-element located at -455 to -195. The negative *cis*-element located at -1008 to -455 is activated when the region -1151 to -1008 is removed.

Histochemical analysis of tPAL5-GUS expression

The cell and tissue specificity of tPAL5-GUS expression was examined by histochemical analysis of the spatial pattern of GUS activity *in situ*. In *trans*-sections of stems, GUS activity was confined to prephloem cells, and no activity was observed in other tissues such as the epidermis, cortex, xylem, or pith (Figure 3A). Such pattern was not changed through developing stage to matured plant. In roots, pronounced GUS activity was mainly observed at the apical tip primarily in a band of tissue corresponding to the region of cell proliferation immediately behind the meristem when stained with X-glus as a substrate on the root. GUS activity was also observed further from the root, but, in these more mature regions, it was restricted to the vascular tissue (Figure 3B). In leaves, GUS activity was also observed mainly in the veins containing phloem and xylem cells (Figure 3C). In petioles, GUS activity was also restricted to the prephloem cells of developing vascular tissue as shown in Figure 3D when cross sectioned. An interesting result in this study is that the full length promoter (-1151 bp) of tPAL5 gene can activate the reporter gene (GUS) only at the outer region of vascular cambium (prephloem region) of stem. This result was highly reproducible from series of transformants.

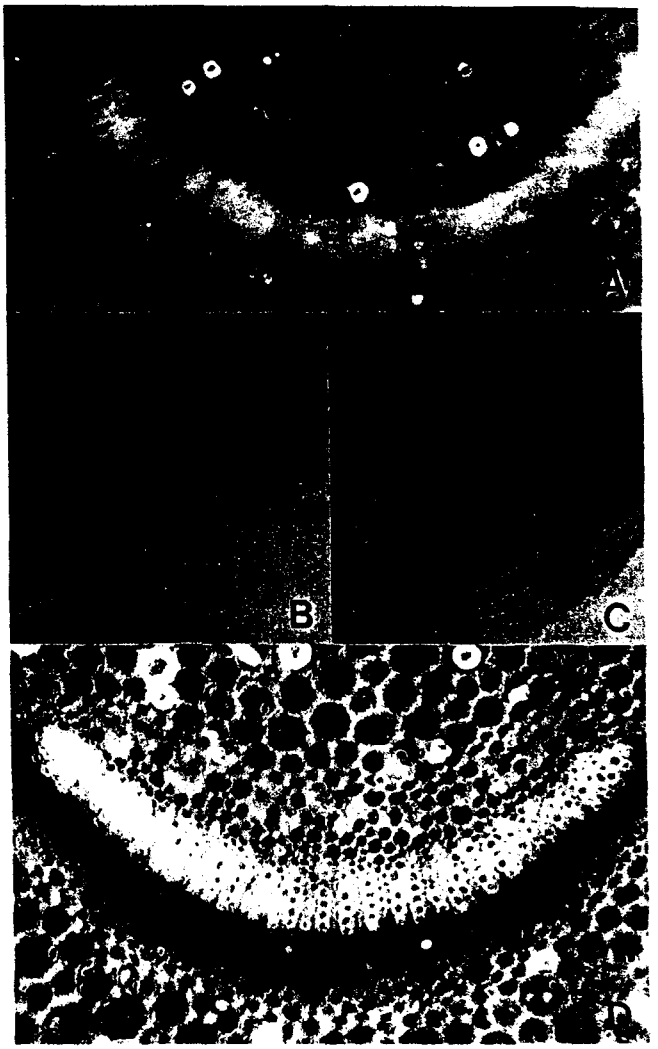

Figure 3. Histochemical analysis of GUS activity in transgenic tobacco tissues. Stem and petiole tissues were cross sectioned and root and leaf tissue were squashed after *in situ* staining for GUS activity. (A) stem, (B) root, (C) leaf (D) petiole.

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