Kasetsart J. (Nat. Sci.) 36 : 361 - 369 (2002)

Function of the Promoter of *PSPAL2*, a Pea Defensive Gene Encoding Phenylalanine Ammonia-lyase

Permpong Sriprasertsak[1], Anchalee Raweerotwiboon[1], Tetsuji Yamada[2] and Thira Sutrabutra[3]

ABSTRACT

The phenylalanine ammonia-lyase is a key enzyme in phenylpropanoid synthesis, a pathway for the biosynthesis of a wide range of natural products which play key roles in plant development and protection against environmental stresses including the structural polymer lignin, flavonoids (anthocyanin pigments and UV protectants), isoflavonoids and phytoalexins. In this study, *PSPAL2*, a member of pea *PAL* gene family was determined and structurally characterized. The structure of *PSPAL2* was divided into two exons by the single intron of 90 bp. In *PSPAL2*, some putative *cis*-regulatory elements of box I, box II, and box IV conserved among the promoter of several genes involved in the phenylpropanoid pathway and the retrotransposon-like sequences were found in the 5′-upstream region of *PSPAL2* promoter.

To discriminate the function of *PSPAL2* promoter, the expression of pea *PSPAL2* retrotransposon-like sequence in the region between –406 and –2196 and the three types of sequentially deleted chimeric promoter constructs designated as *PSPAL2-FLd1*, *PSPAL2-FLd2* and *PSPAL2-FLd3* in transgenic tobacco during developmental growth and upon fungal ingression were demonstrated. The histochemical GUS expression in young seedlings and mature plants were found in tissue and specific organs (roots, stems, leaves, flower organs and anthers). Moreover, the levels of GUS activities in tissues of transgenic plants depending on the 5′-upstream region of *PSPAL2* promoter were also determined. Extremely low GUS expression was observed in healthy or undisturbed mature leaves. However, the *PSPAL2* promoter activated in the leaves of transgenic tobacco plants after transferring to the greenhouse was induced upon fungal ingression, especially when the leaves were inoculated with *P. capsici* and incubated at 22-24°C for 48 hr. Marked expression was detected at the HR area surrounding the inoculation site of the transformant of *PSPAL2-FL*. Extremely low GUS expression was observed in the transformant of *PSPAL2-FLd3*. The results demonstrated that the region from –966 to –2196 of *PSPAL2* promoter played a crucial role in the regulation of induction of GUS activities in the mature leaves of transgenic tobacco plants. It was thus clear that the 5′-upstream region between +110 to –594 was insufficient to establish the full capacity of defense gene response under stress even though this region contained important box sequences such as box I, box II and box IV.

Key words: promoter, pea, defensive gene, plenylalanine ammonia-lyase

1 Central Laboratory and Greenhouse Complex, Kasetsart University Research and Development Institute, Kasetsart University, Kamphaeng Saen, Nakhon Pathom 73140, Thailand.

2 Laboratory of Plant Pathology and Applied Genetic Engineering, Faculty of Agriculture, Okayama University, Okayama 700, Japan.

3 Department of Plant Pathology, Faculty of Agriculture, Kasetsart University, Bangkok 10900, Thailand.

INTRODUCTION

Phenylalanine ammonia-lyase (PAL,EC 4.3.1.5) is one of the key enzymes that are essential for defense reactions in plants. PAL catalyzes the first step of the general phenylpropanoid metabolism, the deamination of L-phenylalanine to produce cinnamic acid and regulates subsequent reactions leading to the production of a wide variety of natural products. These essential roles of isoflavonoids in plant development and defense responses against various environmental stresses, including pathogen ingression, and the structural and regulatory function of phenylalanine ammonia-lyase (*PAL*) genes, have been extensively studied in various higher plants such as alfalfa (Gowri *et al*., 1991), *Arabidopsis* (Mauch-Mani and Slusarenko, 1996., Ohl *et al*., 1990 and Wanner *et al*., 1995), carrot (Takeda *et al*., 1997), French bean (Sablowski *et al*., 1995 and Shafflebottom *et al*., 1993), *Lithospermum erythrorhizon* (Yazaki *et al*., 1997), parsley (Lois *et al*., 1989), pea (Yamada *et al*., 1992), pine (Whetten *et. al*., 1992), poplar (Subramanian *et al*., 1993), rice (Minami *et al*., 1989 and Zhu *et al*., 1995), sweet potato (Tanaka *et al*., 1989), tobacco (Fukazawa-Akada *et al*., 1996), and tomato (Lee *et al*., 1992).

In pea, genes encoding phenylalanine ammonia-lyase (*PSPAL*) from a small multigene family comprised of at least three members (Kawamata *et al*., 1997). Two members of a *PSPAL* family, designated as *PSPAL1* and *PSPAL2*, were cloned and characterized (Yamada *et al*., 1994). Both *PSPAL1* and *PSPAL2* were induced by fungal elicitors but suppressed by fungal suppressors produced by a pea pathogen, *Mycosphaerella pinodes* (Berk. Et Blox.) Stone, a fungus pathogenic on pea (Yamada *et al*., 1992)

Recently, the temporal and spatial expression pattern of the *PSPAL1* promoter was characterized in response to pathogen ingreses and wounding in transgenic tobacco plants (Kawamata *et al*., 1997). The promoter dissection of *PSPAL2* has been performed in a transient transforming assay by electroporation of pea protoplasts, then determining their responses to environmental stimuli such as fungal elicitors, supprescin or UV light irradiation (Yamada *et al*., 1992). Functional analysis of 5′-nested deletions showed that an enhancer-like element is located in the TATA-distal region from – 2196 to –406, in which the consensus sequence motifs known as box II and box IV (Lois *et al.*, 1989 and Takeda *et al.*, 1997) were presented in close proximity.

The objective of the present study was to elucidate the function of the promoter of *PSPAL*, a pea defensive gene encoding phenylalanine ammonia-lyase.

MATERIALS AND METHODS

1. Construction of chimeric genes

The pea *PSPAL2* full length promoter (*PSPAL2-FL*, -2196 to +110) and three deleted chimeric promoters designated as *PSPAL2-FLd1* (-1486 to +110), *PSPAL2-FLd2* (-966 to +110) and *PSPAL2-FLd3* (-594 to +110) had been constructed into CAT reporter gene for analyzing the transient expression in electroporated protoplasts (Yamada *et al*., 1994). To investigate the expression of *PSPAL2* promoters in transgenic tobacco plants, the *PSPAL2-FL* promoter and the three selected deletion constructed promoters in the CAT reporter gene were amplified by PCR using specific primers and subcloned into pBluescriptII KS (+) at *Hin*d III and *Bam* HI sites, then ligated with the GUS reporter gene in pBI101.2 (Figure 1). The *PSPAL2 – GUS* chimeric constructed promoters were purified and transformed into *Agrobacterium tumefaciens* LBA4404 using the freeze-thaw method (Holster *et al*., 1978)

2. Preparation of sterile tobacco plants

Nicotiana tabaccum was used in the transformation experiments. Seeds were surface-sterilized in 5% hypochlorite for 10 min, followed

by soaking in 70% ethanol for 10 min, and rinsed in sterilized water. Sterilized seed were germinated in a sterile petri dish containing the MS medium (Murashige and Skoog, 1962)

3. Leaf disk transformation

Leaf disk of the sterilized tobacco was co-cultured in MS medium for 15-30 min with 1-5 × 10^8 cell/ml of *A. tumefaciens* LBA4404 (McCormick *et al*., 1986) carrying the specified chimeric genes. After drying on a sterilized Whatman 3 MM filter paper to remove excess bacteria, inoculated leaves were placed abaxial surface down on MS medium containing 1.0 mg/l of NAA and 1.0 mg/l of BA. The inoculated leaves were incubated at 22 - 24°C for 2 days, and then, transferred onto MS-selective medium containing the same concentration of NAA, BA, kanamycin (100 mg/l) and claforan (500 mg/l) until shoots formed. After 2-3 weeks of incubation, adventitious shoots were transferred to a new MS medium until roots were formed (Gelvin *et al*., 1990). All plant materials were incubated at 25-28°C under 16-hr light (150 m E/m^2/s), 8-hr dark conditions. Young seedlings were transplanted into soil and incubated in a greenhouse.

4. GUS histochemical assay and PCR analysis

GUS histochemical assay, mature leaves were fixed by soaking in 1% formaldehyde in 50 mM sodiumphosphate buffer (pH 7.0) for 10 min and rinsed three times with 50 mM sodiumphosphate buffer (pH 7.0). Then, they were incubated in X-Gluc solution (1.0 mM 5-bromo-4-chloro-3-indolyl-b-D-glucuronide, in 50 mM sodiumphosphate buffer (pH 7.0), 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, 10 mM EDTA) at 37°C for overnight as described by Jefferson *et al*. (1987). Staining and fixing of X-Gluc solution into tissues was facilitated by vacuum infiltration. Staining reactions were stopped by transferring the tissues into 70% ethanol. GUS-PCR analysis of the genomic DNA extraction for GUS-PCR was performed to confirm the integration of *PSPAL2-GUS* fusion in to the genome of transgenic tobacco plants as described by Hosaka (1994). GUS-PCR was amplified using GUS-specific primers [primer I (upstream) 20 mer : 5′-TAC GTA TCA CCG TTT GTG TG-3′; primer II (down stream) 20 mer : 5′-GTA ATA ACG GTT CAG GCA CA-3′]. DNA manipulation was performed according to the standard methods described by Sambrook *et al*. (1989) or as specified by the manufacturer's protocols.

RESULTS AND DISCUSSION

1. GUS expression in transgenic tobacco plants during developmental growth

Basal GUS expression of *PSPAL2-FL* promoter was observed in tissues of roots, stems and leaves during developmental growth of young seedlings before transplanting to soil. The histochemical GUS expression in young seedlings and mature plants seemed to be conserved in tissue and specific organs as observed in *PSPAL1* (Kawamata *et al*., 1997) and bean *PAL2* (Shufflebottom *et al*., 1993 and Hatton *et al*., 1995). *PSPAL2* promoter showed strong GUS expression in xylem, phloem elements of the vascular and endodermal tissues of lateral roots (Figure 2), stems (Figure 3) and vascular tissues in the veins of leaves, leaf tips, and petrioles (Figure 4). Strong GUS activity was also found in flower organs, especially in the pigment parts of petals, sepal tips, gland cells of trichomes (Figure 5) and anthers (Figure 6). However, the GUS expression could not be observed in root hairs as in *PSPAL1*. Moreover, the level of GUS activities in the organs of transgenic plants significantly declined in corresponding to the deleted 5′-upstream chimeric *PSPAL2* promoters from –2196 to –594, especially in roots, vascular tissues in the veins of leaves and stems as shown in Figure 7.

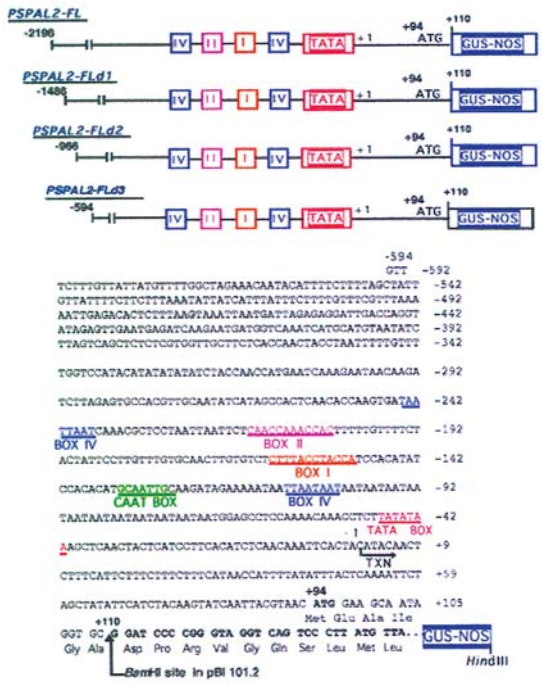

Figure 1 Schematic representation of *PSPAL2* promoter-*GUS-NOS* fusion. Full length (*PSPAL2-FL*) promoter-sequence and the three deleted chimeric constructed promoter (*PSPAL-FLd1*, *PSPAL-FLd2* and *PSPAL-FLd3*) being fused to GUS in pBI101 (Toyobo Inc, Kyoto, Japan) at *Hin*dIII and *Bam*HI sites. The nucleotide sequences of all chimeric constructed promoter, the positions where *GUS-NOS* cassette being connected, a putative translation initiation codon and transcription start site (TXN) being indicated. Putative TATA box, CAAT box and characteristic sequences motifs such as box I, II and IV in 5/-upstream region relative to the transcriptional start site being denoted by colors with those for *PSPAL2* beneath them. The numbers on top denoing the nucleotide position from the transcriptional start point. *GUS* and *NOS* : b-glucuronidase gene in pBI 101.2 and *Agrobacterium tumefaciens* nopaline synthase gene terminator sequence.

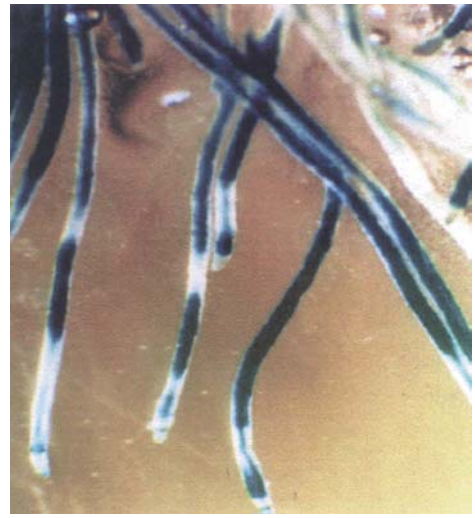

Figure 2 Histochemical localization of GUS activity in young roots of transgenic tobacco plants containing promoter of *PSPAL2-FL-GUS-NOS* construct.

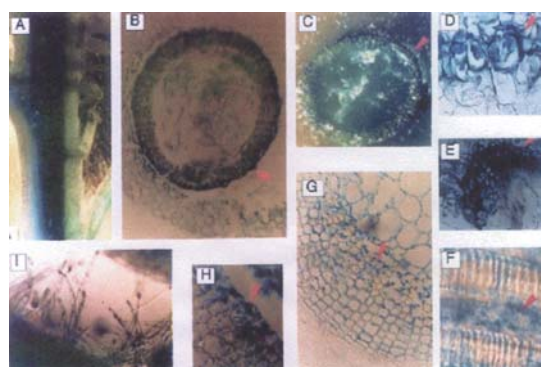

Figure 3 Histochemical localization of GUS activity in young stems both transverse and cross-sections of transgenic tobacco plants containing promoter of *PSPAL2-FL-GUS -NOS* construct.
A : vascular tissue of stem; B,C : cross-section of stem exhibiting high levels of GUS activity localized in the xylem rays (arrow) and in the internal and external phloem tissue; D,E,G : closer view to the cross-section of stem; F,H: transverse-section of stem; I : stem trichomes

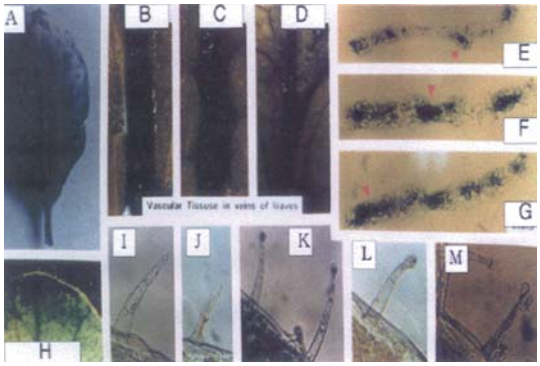

Figure 4 Histochemical localization of GUS activity in young leaves of transgenic tobacco plants containing promoter of *PSPAL2-FL-GUS-NOS* construct.
A : whole leaf; B-D : vascular in veins of leaves; E-G : transverse-section in leaf veins H : leaf tip; I-M : leaf trichomes

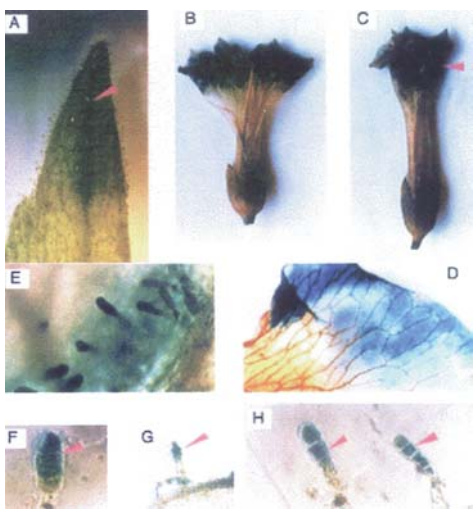

Figure 5 Histochemical localization of GUS activity in flower organ of transgenic tobacco plants containing promoter of *PSPAL2-FL-GUS-NOS* construct.
A: sepal tip; B,C: whole flower with high levels of GUS activity localized in the petal; D: a portion of a petal, showing GUS activity in the pigmented rim; E-H: gland cells of trichomes

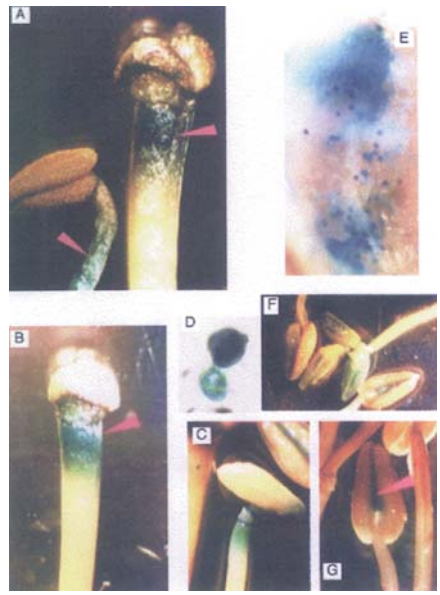

Figure 6 Histochemical localization of GUS activity anther of transgenic tobacco plants containing promoter of *PSPAL2-FL-GUS-NOS* promoter construct.
A-C :filaments; D-E :pollens and anther wall ; F-G : anthers

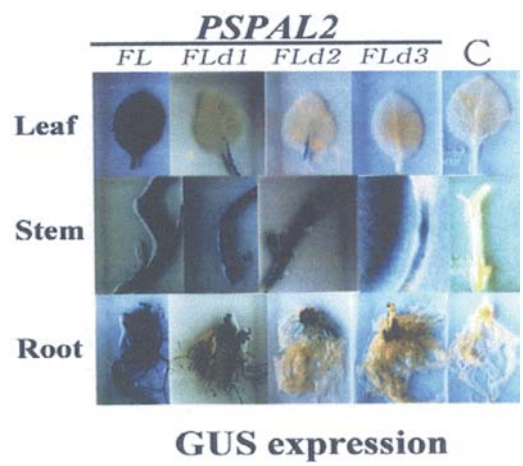

Figure 7 GUS expression of the pea *PSPAL2* full length promoter (*PSPAL2-FL*, -2196 to +110) and three deleted chimeric promoters designated as *PSPAL2-FLd1* (-1486 to +110), *PSPAL2-FLd2* (-966 to +110) and *PSPAL2-FLd3* (-594 to +110) in leaves, stems and roots of transgenic tobacco seedling during developmental growth.

2. GUS expression in transgenic tobacco plants upon fungal ingression and injuries

Histochemical GUS expression

The leaves of transgenic tobacco plants carrying *PSPAL2-FL* were inoculated with *Phytophthora nicotianae*, a tobacco pathogenic fungi (P) or with *P. capsici*, a nonpathogen (NP). The results showed that histochemically detected GUS expression in transgenic tobacco plants was highest 48 hr after inoculation with *P. capsici* (Figure 8B) and incubated at 22-24°C. Then GUS expression gradually faded away at the hypersensitive response (HR) area around the inoculation site after 72 hr of incubation (Figure 8C), where a plant defense system had presumably been established for blocking fungal invasion, in a manner similar to the expression of *PSPAL1* promoter (Kawamata *et al*., 1997.) The pattern of GUS expression after inoculation with a pathogen was not so clear as that observed in the necrotic area after inoculation with a nonpathogen and the expression never faded until the whole leaf was colonized.

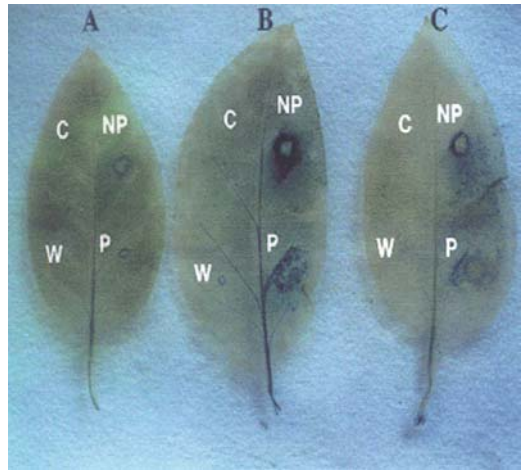

Figure 8 Histochemical GUS expression of *PSPAL2-FL* promoter upon wounding (W) and fungal ingression with pathogen (P, *P. nicotianae*) and non pathogen (NP,*P. capsici*) in transgenic tobacco leaves. Control treatment is shown as C.
A: 24 hr after inoculation or wounding
B: 48 hr after inoculation or wounding
C: 72 hr after inoculation or wounding

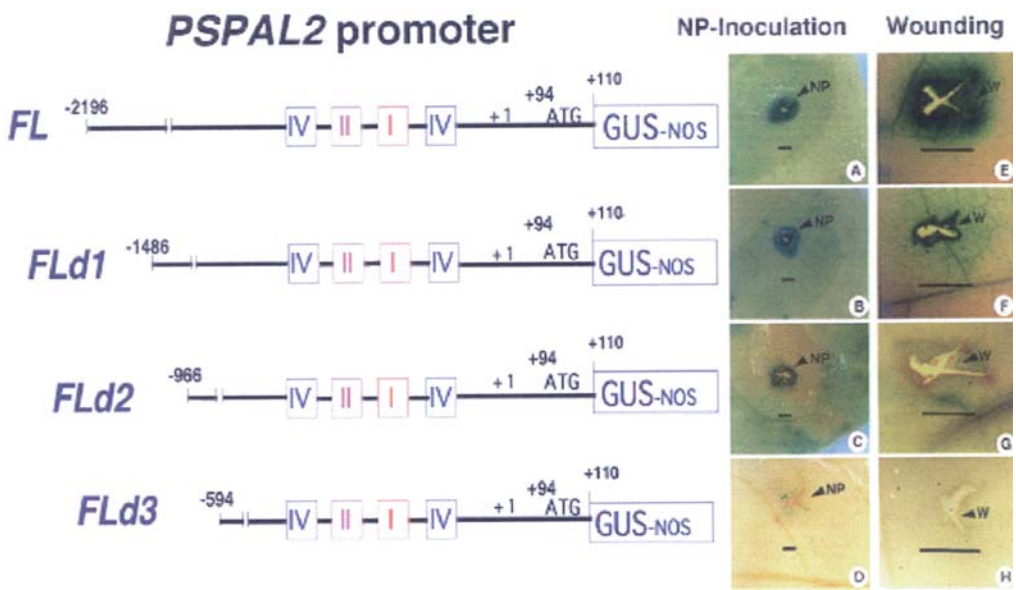

Figure 9 Expression of full (*PSPAL2-FL* : A,E) and deleted *PSPAL2* promoters (*PSPAL2-FLd1* : B,F) *PSPAL2-FLd2* : C,G; *PSPAL2-FLd3* : D,H) at 48 hr after wounding (W) and inoculation with a nonpathogen (NP,*P. capsici*) in transgenic tobacco leaves. Bars equal 1 mm.

Expression of deleted *PSPAL2* promoters upon fungal infection

To discriminate the expression of the deleted *PSPAL2* promoters upon fungal infection, mature leaves of transgenic tobacco plants carrying the *PSPAL2* chimeric constructed promoters were inoculated with *P. capsici*. This nonpathogenic fungus induced a very large, clear GUS expression zone around the site of the hypersensitive response (HR), especially in the transformants of *PSPAL2-FL* (Figure 9A) and *PSPAL-FLd1* (Figure 9B) at 48 hr after the inoculation. However, the responses to fungi infection were not high in the transformants of *PSPAL2-Fld2* (Figure 9C); the expression zone was restricted to the area around the inoculation site. GUS expression in the transformant of *PSPAL-FLd3* did not clearly appear (Figure 9D). The results showed that the pea *PSPAL2* promoter expression in transgenic tobacco was strongly affected by the sequences in 5/-upstream region as previously shown with the transient CAT expression in electroporated pea protoplasts (Yamada *et al*., 1994). The functional analysis of 5/-nested deletions of *PSPAL2* promoter in electroporated protoplasts showed that an enhancer-like element located at the TATA-distal region from –2196 to –406, and this promoter was activated by fungal elicitor from *M. pinodes* and partially suppressed by the suppressor from the same fungus (Yamada *et al*., 1994). Interestingly, the GUS expression of the constructs upon fungal ingression in transgenic tobacco leaves demonstrated the induction of positive defense responses at the sites of infection at different levels depending on the additional sequences of the 5/-upstream region. Because a very low level of the GUS expression was observed in the transformants of *PSPAL2-Fld3* compared to *PSPAL2-FL*, the lower level of GUS expression is not due to a position effect of the integration of the chimeric promoters. This phenomenon might be explained by the lack of some active elements needed for the regulation of the *PSPAL2* promoter. These elements were likely to span from –966 to –2196. Moreover, the GUS expression was observed in mature transgenic tobacco leaves being injured by sterile razor blade. Intense blue colorations were observed at wounding sites (W) and restricted adjacent areas. The GUS expression of the deleted *PSPAL2-FL* promoters after wounding also declined from a high level to an extremely low one as the deletion was extended, in a manner similar to the expression after fungal ingression (Figure 9E-H).

The results demonstrated that the region from –966 to –2196 of *PSPAL2* promoter played a crucial role in the regulation of induction of GUS activities in the mature leaves of transgenic tobacco plants. It was thus clear that the 5/-upstream region between +110 to –594 was insufficient to establish the full capacity of defense gene response under stress even though this region contained important box sequences such as box I, box II and box IV. The additional sequences from –594 to –2196 that include the region of retrotransposon-like sequence are obviously necessary for the functional expression of the gene not only during developmental growth but also in response to fungal ingress and injuries.

LITERATURE CITED

Fukazawa-Akada, T., S. Kung, and J.C. Watson. 1996. Phenylalanine ammonia-lyase gene structure, expression, and evolution in Nicotiana. Plant Mol. Biol. 30 : 711-722.

Gelvin, S.B., R.A. Schilperoort, and D.P.S. Verma. 1990. Plant Molecular Biology Manual. Kluwer Academic Publishers, NY.

Gowri, G., N.L. Paiva, and R.A. Dixon. 1991. Stress responses in alfalfa (*Medicago sativa*). 12. Sequence analysis of phenylalanine ammonia-lyase (PAL) cDNA clones and appearance of PAL transcripts in elicitor treated cell cultures and developing plants. Plant Mol. Biol. 17 : 415-426.

Hatton, D., R. Sablowski, M.H. Yung, C. Smith, W. Schuch, and M. Bevan. 1995. Two classes of *cis* sequences contribute to tissue-specific

expression of a *PAL2* promoter in transgenic tobacco. Plant J. 7 : 859-876.

Holsters, M., D. de Waele, A. Depicker, E. Messens, N. van Montagu, and J. Schell. 1978. Transfection and transformation of *Agrobacterium tumefaciens.* Mol. Gen. Genet. 163 : 181-187.

Hosaka, K. 1994. Current RAPD Technology. Academic booklet, Kobe University, Kobe.

Jefferson, R A., T.A. Kavanagh, and M.W. Bevan. 1987. GUS fusion: β-glucuronidase as a sensitive and versatile gene marker in higher plants. EMBO J. 6 : 3901-3907.

Kawamata, S., K. Shimoharai, Y. Imura, M. Ozaki, Y. Ichinose, T. Shiraishi, H. Kunoh, and T. Yamada. 1997. Temporal and spatial pattern of expression of the pea phenylalanine ammonia-lyase gene1 promoter in transgenic tobacco. Plant Cell Physiol. 38 : 792-803.

Lee, S.W., J. Robb, and R.N. Nazar. 1992. Truncated phenylalanine ammonia-lyase expression in tomato (*Lycopersicon esculentum*). J. Biol. Chem. 267 : 11824-11830.

Lois, R., A. Dictrich, K. Hahlbrock, and W. Schulz. 1989. A phenylalanine ammonia-lyase gene from pasley : structure, regulation and identification of elicitor and light responsive *cis*-acting elements. EMBO J. 8 : 1641-1648.

McCormick, S., J. Niedermeyer, and J. Fry. 1986. Leaf disc transformation of cultivated tomato (*L .esculentum*) using *Agrobacterium tumefaciens.* Plnat Microbe Interact. 6 : 453-466.

Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15 : 473-497.

Mauch-Mani, B. and A. Slusarenko. 1996. Production of salicylic acid precursors in a major function of phenylalanine ammonia-lyase in the resistance of *Arabidopsis* to *Peronospora parasitica*. Plant Cell 8 : 203-21.

Minami, E., Y. Ozeki, M. Matsuoka, N. Koizuka, and Y. Tanaka. 1989. Structure and some characterization of the gene for phenylalanine ammonia-lyase from rice plants. Eur. J. Biochem. 185 : 19-25.

Ohl, S., S.A Hedrick, J. Chory, and C.J. Lamb. 1990. Functional properties of a phenylalanine ammonia-lyase promoter from *Arabidopsis*. Plant Cell 2 : 837-848.

Sablowski, R.W.M., D.C. Baulcombe, and M. Bevan. 1995. Expression of a flower-specific Myb protein in leaf cells using a viral vector causes ectopic activation of a target promoter. 92 : 6901-6905.

Sambrook, T., E.F. Fritsch, and J.Manitis. 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Habor, NY.

Shufflebottom, D., K. Edwards, W. Schuch, and M. Bevan. 1993. Transcription of two members of a gene family encoding phenylalanine ammonia-lyase leads to remarkably different cell specificities and induction patterns. Plant J. 3 : 635-645.

Sriprasertsak, P. 2000. Plant defense responses and control of gene expression: Structure and function of the promoter of *PSPAL2*, a pea defensive gene encoding phenylalanine ammonia-lyase. Ph.D. Dissertation, Okayama University, Japan.

Subramaniam R., S. Reinold, E.K. Molitor, and C.J. Douglas. 1993. Structure, inheritance, and expression of hybrid poplar (*Populus trichocarpa* x *Populus deltoides*) phenylalanine ammonia-lyase genes. Plant Physiol. 102 : 71-83.

Takeda, J., Y. Ozeki, and K. Yoshida. 1997. Action spectrum for induction of promoter activity of phenylalanine ammonia-lyase gene by UV in carrot suspension cells. Phytochem. Photobiol. 66 : 464-470.

Tanaka, Y., M. Matsuoka, N. Yamamoto, Y. Ohashi, Y. Kano-Murakami, and Y. Ozeki. 1989. Structure and characterization of a cDNA for phenylalanine ammonia-lyase from cut-injured

roots of sweet potato. Plant Physiol. 90 : 1403-1407.

Wanner, L.A., G. Li, D. Ware, I.E. Somssich, and K.R. Davis. 1995. The phenylalanine ammonia-lyase gene family in *Arabidopsis thaliana*. Plant Mol. Biol. 27 : 327-338.

Whetten, R.W. and R.R. Sederoff. 1992. Phenylalanine ammonia-lyase from loblolly pine : purification of the enzyme and isolation of complementary DNA clones. Plant Physiol. 98 : 380-386.

Yamada, T., P. Sriprasertsak, H. Kato, T. Hashimoto, H. Shimizu, and T.Shiraishi. 1994. Functional analysis of the promoters of phenylalanine ammonia-lyase genes in pea. Plant Cell Physiol. 35 : 917-926.

Yamada, Y., P. Sriprasertsak, H. Kato, T. Hashimoto, H., Shimizu, and T. Shiraishi. 1994. Functional analysis of the promoters of phenylalanine ammonia-lyase genes in pea. Plant Cell Physiol. 35 : 917-926.

Yamada, Y., Y. Tanaka, P. Sriprasertsak, H. Kato, T. Hashimoto, S. Kawamata, Y. Ichinose, H. Kato, T. Shiraishi, and H. Oku. 1992. Phenylalanine ammonia-lyase genes from *Pisum sativum* : structure, organ-specific expression and regulation by fungal elicitor and suppressor. Plant Cell Physiol. 33 : 715-725.

Yazaki, K., M. Kataoka, G. Honda, K. Severin, and L. Heide. 1997. cDNA cloning and gene expression of phenylalanine ammonia-lyase in *Lithospermum erythrorhizon Biosci.* Biotech. Biochem. 61 : 1995-2003.

Zhu, Q., T. Dabi, A. Beeche, R. Yamamoto, M.A. Lawton, and C.J. Lamb. 1995. Cloning and properties of a rice gene encoding phenylalanine ammonia-lyase. Plant Mol. Biol. 29 : 535-550.

Received date : 05/08/02
Accepted date : 28/10/02