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Cyclized Induction of Phenylalanine Ammonia-Lyase Gene Expression in *Rhizoctonia solani*-Infected Stems of Tomato

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Abstract

Soil-borne fungal pathogens such as *Verticillium* and *Rhizoctonia* can colonize in the stem tissue of plant through root and lead to wilting symptoms of plant by blocking water transportation. During the colonization of *Rhizoctonia solani* in the vascular tissue of tomato stems, particularly, phenylalanine ammonia- lyase (PAL) gene induction pattern was cyclized showing peak induction at two different time points (10 and 80 h) after fungal spores inoculation *in vivo*. In leaves or roots, however, no such cycling pattern was observed. The first induction peak may be due to an initial sporulation events leading to a second induction peak by a proliferation of fungal spores to the upper stems or other tissues from an initial spore trapping sites. Tomato PAL gene was also dramatically induced by wounding, light illumination and mercury chloride treatment but was not cyclized. Mercury chloride showed the earliest induction with all tissues even at half an hour after treatment.

Key words: *Rhizoctonia*, phenylalanine ammonia-lyase (PAL), tomato

Introduction

Phenylalanine ammonia-lyase gene is encoded by a small gene family and represents the first enzyme in the complex phenylpropanoid pathway which produces many different types of phenolic compounds by diverse branched pathways such as suberin, lignin, flavonoid, etc. (Hahlbrock and Sched 1989; Baker et al. 1989; Dalkin et al. 1990; Beno-Moualem and Prusky 2000). Therefore, much attention has been focused on the regulation mechanism during the plant developmental stages as well as various environmental stress treatments. For instances, all three genes (PAL 1, 2, 3) are induced by mechanical wounding of bean hypocotyls, but fungal infection activates only bean PAL 1 and PAL 2, but not the PAL 3 (Liang et al. 1989). In particular, enormous amount of reports has been published to elucidate the molecular plant-fungal interaction mechanism for manipulating resistant line against fungal pathogen (Lamb et al. 1989).

Heinz et al. (1998) examined the correlation between the fungal spore colonization pattern and tomato PAL 5 gene expression pattern during *Verticillium albo-atrum* infection from root tissues through wounds or via the apical meristem. They reported that both fungal colonization and PAL 5 gene expression were found to be fully systemic from the earliest sporulation events and to occur in cycles, the defense gene cycle following the colonization cycle. In stem tissues, tylose formation and rishitin production (Tjamos and Smith 1975) were also examined to be cycled upon infection of fungal pathogen, actually coincide closely with peaks of tomato PAL 5 gene expression cycle (Heinz et al. 1998). In addition, earlier studies by authors identified alternative transcription initiation sites of tomato PAL 5 gene respond differentially to various environmental stimuli (Lee et al. 1993; 1994) and also reported that transgenic potatoes containing the promoter region of tomato PAL 5 gene fused to *shiva-1* gene, encoding a small synthetic antibacterial peptide, enhanced the reliability of induced resistance to soft rot disease in potato when com- pared to a constitutive promoter, CaMV 35S (Yi et al. 2004).

In this study, the cyclization of tomato PAL gene

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expression upon treatment with another tomato fungal pathogen, *Rhizoctonia solani* was observed. Tissue and stress-specific expression pattern of tomato PAL 5 gene were also examined after treatment with various environmental stresses.

Materials and Methods

Plant material and experimentation

Tomato hypocotyls (var. Sekwang) were produced from seeds that were surface-sterilized with 1% (v/v) sodium hypochloride (3 min) and washed in sterile distilled water. Seeds were placed on moist blotting paper and germinated at room temperature in complete darkness for 8 days. For tissue-specific analysis, whole plants were grown for 4 weeks in pots in the green house and tissue samples were separated into leaves, stems and roots. For wounding induction, hypocotyl segments (5 mm) and tissues were cut with sharp scissors and collected samples for RNA at indicated time points. For light induction, etiolated hypocotyls and whole plants were exposed to continuous light (L58 Osram Lumlux : 500 μmol/m^2 s^{-1}) until indicated time points. Each tissue was treated with 100 μM $HgCl_2$ in a petri dish until indicated time points. For *R. solani* AG-4 infection, the pathogen was procured from the Division of Plant Pathology, NIAST (National Institute of Agricultural Science and Technology) and subcultured in potato dextrose broth at 30℃ for 1 week. The mycelial mats, on the surface of the medium, were then homogenized (4,000 rpm, 2 min) in sterile water and inoculated into the plant at the ratio of one-eighth piece of the mat. After inoculation, the plants were incubated at the 30℃ and the moisture was kept at 60% of the maximum water-holding capacity (Yeo 1997). All harvested hypocotyls and plant tissues were immediately frozen in liquid nitrogen and stored at -80℃ until required.

Total RNA extraction and northern blot hybridization

The total RNA was extracted from each samples and subjected for northern blot hybridization analyses as previously described by Lee et al. (1992a). Total RNA (20 μg per lane) was separated in 1% agarose-formaldehyde gels and transferred to Hybond-N membrane (Amersham) by irradiation with ultraviolet light (UV Stratalinker, Stratagene). A conserved region of a tomato PAL gene fragment (580 bp) was used as a probe and labeled with ^{32}P by random oligonucleotide-primed synthesis (Lee et al. 1992a). Prehybridizations were performed at 42℃ in 50% (v/v) formamide, 6X SSC, 5X Denhardt's solution, 0.2% (w/v) SDS and 100 μg/ml denatured salmon sperm DNA. Hybridizations were carried out in the same solution for 24h at 42℃. The filter was washed twice in 2X SSC, 0.1% SDS at room temperature, and twice in 0.1X SSC, 0.1% SDS at 65℃, and exposed to Kodak X-Omat SX film.

RNase protection analyses

The plasmids with gene-specific probe were constructed by subcloning a 1.4kb (tPAL 1), 1.6kb (tPAL 4) and 0.7kb (tPAL 5) into the vector pBluescript-SK$^+$ (Stratagene) which had been cleaved with *SmaI* for ligating to PCR fragments, respectively. The each insert was amplified by using PCR with gene-specific primers and M13/pUC forward primer on the template strands containing a fragment of 5'-flanking and first exon region. Each primer was designed at the 1st exon of each gene: tPAL 1, 5'-CATTTAAATGACTCCCTC-TTAAAGAATCAGCAGCC-3'; tPAL 4, 5'-TTGATGGTGCC-ATTGGATCAAG-3'; tPAL 5, 5'-TCCATTACCCAATCACT-3'. The nucleotide sequence of tPAL 1 and tPAL 4 gene was reported by Yeo et al. (1999) and tPAL 5 gene was reported by Lee et al. (1992b). For *in vitro* transcription and RNase protection procedure was followed as described in the manual provided in the kit (Promega).

Results and Discussion

Lee et al. (1992a) have reported that tomato PAL gene was not induced by *Verticillium albo-atrum* of susceptible plant. In this study, attempts were made to extend investigation on more stresses, including mercury chloride treatment, physical wounding, light illumination and infection with another destructive pathogen, *R. solani*.

In general, the transcriptional level of PAL gene in hypocotyls was induced at 1.0 h after excision wounding and gradually decreased while it was continuously increased up to 12 h by light illumination although the levels were up and down at some time points. Much earlier and stronger induction was observed with mercury chloride treatment i.e. half an hour after treatment while *R. solani* infection showed the highest induction level at 20 h after treatment and declined gradually (Figure 1).

In leaves of 4-week old plant, the light illumination caused the highest induction peak at 3 h and high levels were lasted up to 12 h after treatment while mercury chloride treatment and fungal infection did not induce the transcriptional levels as much as in hypocotyls (Figure 2). At 1 h after excision wounding, similar level of peak induction was observed and gradually decreased. Similar induction

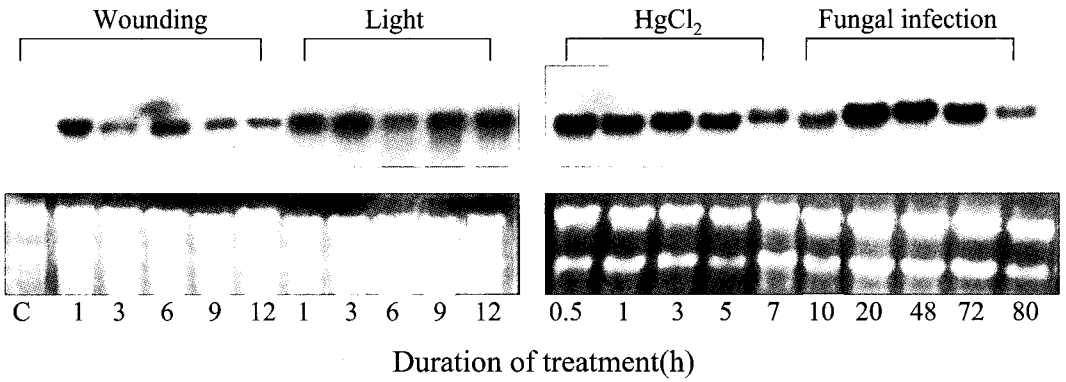

Figure 1. Northern-blot analysis of PAL transcript levels in hypocotyls of tomato by environmental cues. Tomato hypocotyl tissues were wounded, exposed to white light following 8 days of dark adaptation, transferred to a 100-μM $HgCl_2$ solution and inoculated with *R. solani* and harvested at different time points after treatment. Twenty micrograms of total RNA per lane was fractionated on a 1.0% formamide denaturing gel, blotted and hybridized with the conserved region of tPAL 5 as probe. C, Control plant harvested immediately after treatment. The equivalence of total RNA loaded in each lane was demonstrated by EtBr staining of rRNA.

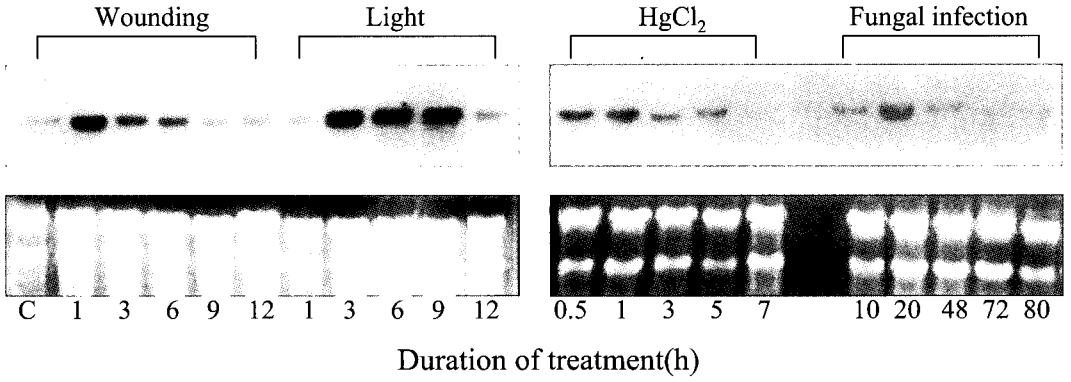

Figure 2. Northern-blot analysis of PAL transcript levels in leaves of tomato by environmental cues. Tomato leaves were wounded, exposed to white light following 8-days of dark adaptation, transferred to a 100-μM $HgCl_2$ solution or inoculated with *R. solani* and harvested at different time points after treatment. Twenty micrograms of total RNA per lanes were fractionated on a 1.0% formamide denaturing gel, blotted and hybridized with the conserved region of tPAL 5 as probe. C, Control plant harvested immediately after treatment. The equivalence of total RNA loaded in each lane was demonstrated by EtBr staining of rRNA.

pattern was also observed by mercury chloride treatment at half an hour after treatment but not as much as in hypocotyls. In leaves, the lowest induction pattern was observed by the fungal infection as compared to all other tissues observed in this work. The results indicate that leaves are not much involved in the colonization of fungal spores but sensitive to light illumination and wounding.

In stems, almost no induction was detected by excision wounding as well as light illumination while *R. solani* infection caused two different peak induction at 10 h and 80 h after treatment (Figure 3). In mercury chloride treatment, the induction level was increased gradually up to 7 h. Many fungal pathogen such as *R. solani* and *V. albo-atrum* colonize at the vascular tissue of stem from the root in soil and result in wilting symptom by blocking the transportation system of water or nutrients. During the infection process of fungal pathogen, the late induction of PAL gene may be the result of second infection cycle from the first infection site. Heinz et al. (1998) also observed that the colonization of fungal pathogen and defense related gene expression pattern in tomato stem showed cyclization during an *in vivo* infection through root tissues.

In root tissues, in general, much higher basal level was observed from the sample given no stresses at all and highly induced by excision wounding (at 1 h) (Figure 4). Mercury chloride and fungal infection showed the highest induction at 1 h and 48 h after treatment, respectively, where no induction was observed by light illumination (Figure 4). This result may suggest that more active root elongation was performing at the time of sampling and

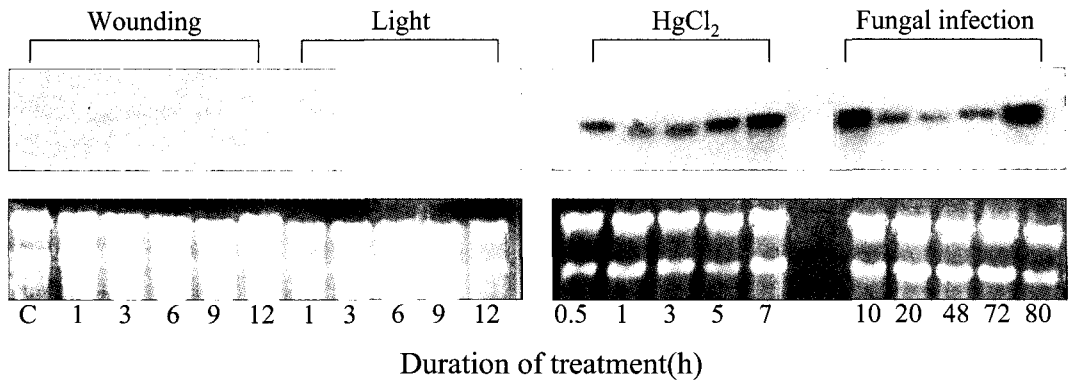

Figure 3. Northern-blot analysis of PAL transcript levels in stems of tomato by environmental cues. Tomato stem tissues were wounded, exposed to white light following 8-days of dark adaptation, transferred to a 100-μM $HgCl_2$ solution or inoculated with *R. solani* and harvested at different time points after treatment. Twenty micrograms of total RNA per lanes were fractionated on a 1.0% formamide denaturing gel, blotted and hybridized with the conserved region of tPAL 5 as probe. C, Control plant harvested immediately after treatment. The equivalence of RNA loaded in each lane was demonstrated by EtBr staining of rRNA.

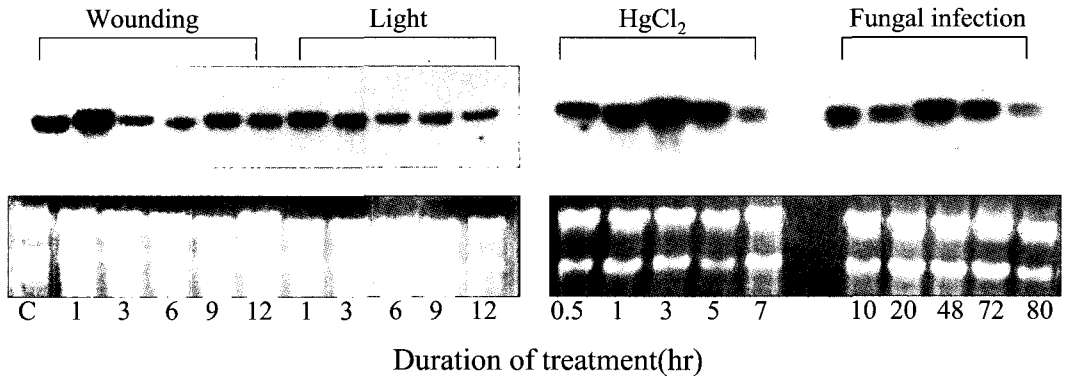

Figure 4. Northern-blot analysis of PAL transcript levels in roots of tomato by environmental cues. Tomato root tissues were wounded, exposed to white light following 8-days of dark adaptation, transferred to a 100-μM $HgCl_2$ solution or inoculated with *R. solani* and harvested at different time points after treatment. Twenty micrograms of total RNA per lanes were fractionated on a 1.0% formamide denaturing gel, blotted and hybridized with the conserved region of tPAL 5 as probe. C, Control plant harvested immediately after treatment. The equivalence of RNA loaded in each lane was demonstrated by EtBr staining of rRNA.

continuous growth requires lignin synthesis in membrane.

In addition, Lee et al. (1993; 1994) reported the differential response pattern of alternate initiation sites of tomato tPAL 5 gene to wounding, light illumination and *V. albo-atrum* infection. In this study, comparison was made between the gene specific expression pattern of tPAL 1, tPAL 4 and tPAL 5 gene using RNase protection analysis technique as described in materials and methods. The 5'-upstream regulatory regions of tPAL 1 and tPAL 4 gene of tomato and their transcription initiation sites were already determined by primer extension techniques (Yeo 1997). RNase protection analyses showed that these two genes were not induced at all by any stresses included in this study (data not shown) but only tPAL 5 gene was induced with alternate initiation sites to various environmental stimuli.

In hypocotyls, a fast and strong induction of longer transcript of tPAL 5 gene was observed from the basal level after stress treatments and stayed in high levels. The short transcript, however, showed a peak induction at 6 h (wounding), 3 h (mercury chloride), 10 and 48 h (fungal infection), respectively (Figure 5A). In leaves of 4 weeks old plants, light illumination induced later than excision wounding and both longer and shorter transcripts were induced at 3 h after light illumination. However, induction by mercury chloride as well as *R. solani* infection was lower compared to other tissues (Figure 5B), similar to the total PAL gene expression pattern (Figure 2). In stem of mature plants, wounding, light illumination and mercury chloride treatment also induced the transcript levels at 1 to 3 h after treatments (Figure 5C). In particular, both longer and shorter transcripts were highly induced by light illumination. In case of *R. solani* infection in stem tissues, the cyclic induction pattern was also observed

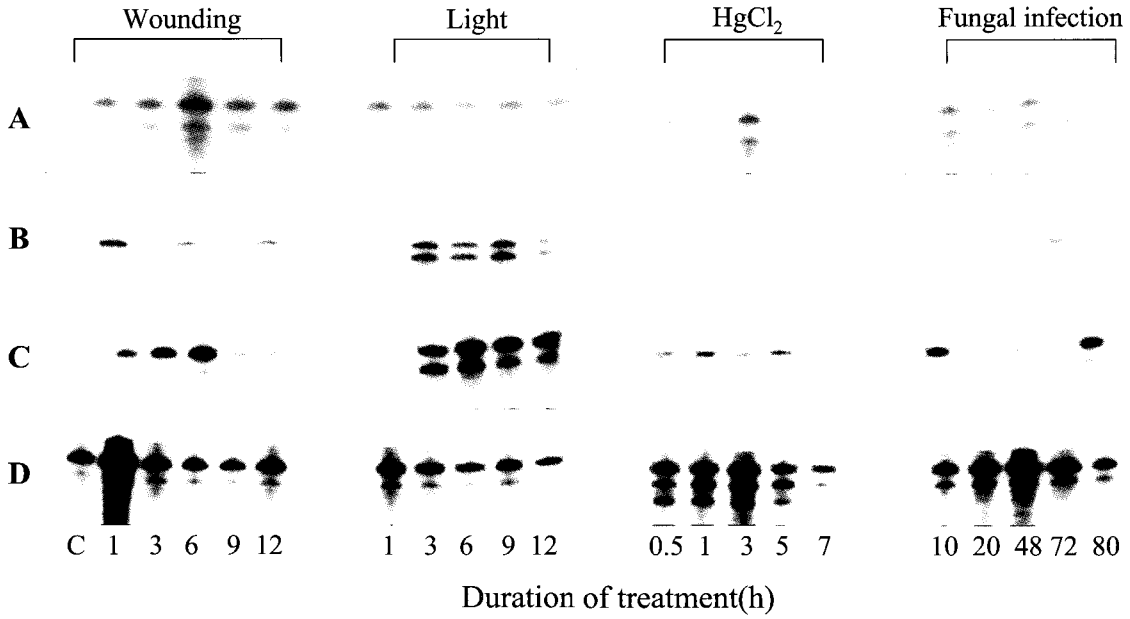

Figure 5. RNase protection analysis of tPAL 5 transcript by environmental cues. A radiolabel RNA probe was hybridized to 5 μg of total RNA isolated at given time after stress treatment from hypocotyls (A), leaves (B), stems (C) and roots (D), digested with RNase mixture and separated on a 5% sequencing gel. C, Control plant harvested immediately after treatment. The autoradiograph was exposed for 2 days.

at 10 h and 80 h (Figure 5C) as examined in hypocotyl tissues (Figure 5A). However, the induction was more delayed compare to hypocotyl tissues. This result suggests that the infection procedure of fungal pathogen is faster in hypocotyl stage than in mature growth stage of plants. As total PAL gene expression study is similar, higher amounts of tPAL 5 transcripts were observed in the root tissues of mature plants without any treatments, and higher induction of tPAL 5 gene in root was measured by all stresses shown in this study indicating a peak at certain time points after infection and then gradually decreased (Figure 5D).

In summary, it was found that PAL gene is induced by fungal pathogen in a cyclic manner in the hypocotyls and stem tissues, in the initial colonizing tissue from the root in soil.

References

Baker CJ, O' Neill NR and Tomerlin JR (1989) Accumulation of phenolic compounds in incompatible clone/race interactions of *Medicago sativa* and *Colletotrichium trifolii*. Physiol and Mol Plant Pathol 35: 231-241

Beno-Moualem D, Prusky D (2000) Early events during quiescent infection development by *Colletotrichum gloeosporioides* in unripe avocado fruits. Phytopathology 90: 553-559

Dalkin K, Edwards R, Edington B and Dixon RA (1990) Stress responses in alfalfa (*Medicago sativa* L.) I. Induction of phenylpropanoid biosynthesis and hydrolytic enzymes in elicitor-treated cell suspension cultures. Plant Physiol 92: 440-446

Hahlbrock, K and Sched D (1989) Physiology and molecular biology of phenylpropanoid metabolism. Ann Rev Plant Physiol Plant Mol Biol 40: 347-369

Heinz R, Lee SW, Saparno A, Nazar RN, and Robb J (1998) Cyclical systemic colonization in *Verticillium*-infected tomato. Physiol and Mol Plant Pathol 52: 385-396

Lamb CJ, Lawton MA, Dron M and Dixon RA (1989) Signals and transduction mechanisms for activation of plant defenses against microbial attack. Cell 56: 215-224

Lee SW, Nazar NR, Powell DA and Robb J (1992a) Reduced PAL gene suppression in *Verticillium*-infected resistant tomatoes. Plant Mol Biol 18: 345-352

Lee SW, Robb J and Nazar, NR (1992b) Trucated phenylalanine ammonia-lyase expression in tomato (*Lycopersicon esculentum*). J Biol Chem 267: 11824-11830

Lee SW, Eun MY and Ross RN (1993) Transcription initiation sites of tomato phenylalanine ammonia-lyase 5 gene and their usage. Mol Cells 3: 295-300

Lee SW, Heinz R, Robb J and Ross RN (1994) Differential utilization of alternate initiation sites in a plant defense gene responding to environmental stimuli. Eur J Biochem 226: 109-114

Liang X, Dron M, Cramer CL, Dixon RA and Lamb CJ (1989) Differential regulation of phenylalanine ammonia-lyase genes during plant development and environmental cues. J Biol Chem 264: 14486-14492

Tjamos EC and Smith IM (1975) The expression of resistance

to *Verticillium* wilt. Physiol Plant Pathology 6: 215-225

Yeo YS (1997) Organization and expression of a gene family encoding phenylalanine ammonia-lyase in tomato. Ph. D Thesis, Univ. of Chungnam pp 1-94

Yeo YS, Weh WH, Lee SW, Bae SC, Ryu JC and Chang YD (1999) Complete nucleotide sequence analysis and structural comparison of 3 members of tomato phenylalanine ammonia-lyase gene. Korean J Plant Tissue Culture 26: 41-47

Yi JY, Seo HW, Yang MS, Robb EJ, Nazar RN and Lee SW (2004) Plant defense gene promoter enhances the reliability of shiva-1 gene-induced resistance to soft rot disease in potato. Planta. in press.