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Phenylalanine Ammonia-Lyase Gene (*NtPAL4*) Induced by Abiotic Stresses in Tobacco (*Nicotiana tabacum*)

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Abstract - Phenylalanine ammonia-lyase (PAL), a key enzyme of the phenylpropanoid biosynthesis pathway, is activated by a number of developmental and environmental cues. The coding region of the *NtPAL4* gene was 2,154 bp in length, and its deduced protein was composed of 717 amino acids. Sequence analysis of *NtPAL4* cDNA from tobacco (*Nicotiana tabacum* L.) revealed high structural similarity to *PAL* genes of other plant species. The *NtPAL4* gene exists as a single copy in the tobacco plant, and its transcripts were strongly expressed in flowers and leaves. *NtPAL4* expression was significantly induced in response to NaCl, mannitol, and cold treatments, but it was not induced by abscisic acid (ABA). *NtPAL4* expression decreased gradually after treatment with ABA and H_2O_2; however, *NtPAL4* transcripts accumulated after treatment with methyl viologen (MV). Our results suggest that the *NtPAL4* gene may function in response to abiotic stresses.

Key words - Abiotic stress, *Nicotiana tabacum*, Oxidative stress, Phenylalanine ammonia-lyase, Reactive oxygen species

Introduction

In plants the phenylpropanoid metabolic pathway produces a variety of compounds, such as flavonoids, lignins, and anthocyanins. Phenylpropanoid metabolites serve valuable functions, such as mechanical support, pigmentation, production of signaling molecules in plants, and protection against abiotic and biotic stresses (Douglas, 1996). Phenylpropanoids are synthesized during the normal course of plant development. Phenylpropanoid metabolism is activated in response to a number of developmental and environmental cues, and phenylpropanoids are synthesized under various stress conditions such as UV irradiation, mechanical wounding, or pathogen attack (Lawton and Lamb, 1987).

Plants are subject to various abiotic stresses (e.g. high salinity, drought, high and low temperature, and exposure to heavy metals that damage cellular metabolism) that lead to the generation of reactive oxygen species (ROS) and the inhibition of photosynthesis (Hasegawa *et al*., 2000). Plant stresses are triggered by the specific signal transduction of genes involved in defense response and the production of certain secondary metabolites (Yang *et al*., 1997).

Phenylalanine ammonia-lyase (PAL) is a key enzyme of phenylpropanoid biosynthesis pathway, which leads to the synthesis of a variety of compounds including lignins, hydroxycinnamates, isoflavonoids, flavonoids, and anthocyanins (Brődenfeldt and Mohr, 1988). In plants *PAL* genes constitute a small gene family and are found in many plant species: bean, parsley, rice, potato, and Arabidopsis (Wanner *et al*., 1995). Tobacco PALs are encoded by a small family of four unclustered genes (Pellegrini *et al*., 1994; Richert *et al*., 2009).

PAL activity is extraordinarily sensitive to the plant physiological state and is induced by various stresses and plant signal molecules (Brődenfeldt and Mohr, 1988). Arabidopsis *PAL1* and citrus *PAL* can be induced by cold and chilling injury (Leyva *et al*., 1995; Lafuente *et al*., 2003). In *Lactuca sativa*, PAL activity increased following heat shock and methyl jasmonate (MeJA) treatments (Campos-Vargas *et al*., 2005; Kim *et al*., 2007). In tomato, *SlPAL5* transcripts were significantly induced by cold, salt and drought stresses (Guo and Wang, 2009). PAL showed pathogen-induced response in parsley (de Costa e Silva *et al*., 1993), and salicylic acid (SA) can induce PAL activity in grape berries

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(Chen *et al*., 2006).

The *NtPAL4* from *N. tabacum* was isolated and expressed under various environmental stresses. Our results show that abiotic stresses stimulate the transcripts of *NtPAL4* and suggest that *NtPAL4* plays an important role in multiple signaling pathways activated by abiotic and oxidative stresses.

Materials and Methods

Plant material and treatment

Tobacco (*Nicotiana tabacum* L.) seeds were cultured in MS medium containing 3% sucrose and 0.8% agar (Murashige and Skoog, 1962). The germinated plants were transferred to pots and kept in a growth chamber at 24°C for six weeks. For cold treatment, the leaves were placed in distilled water and kept in a 4°C cold chamber under dim light for 24 h. The leaves were incubated for various lengths of time in 200 mM NaCl, 200 mM mannitol, 100 μM ABA, 10 mM H_2O_2, 50 μM methyl viologen (MV). All the tissue cultures and potted plants were cultured at 24°C under a 16 h day/8 h dark photoperiod in a growth culture room.

Multiple amino acid sequence alignment

NtPAL4 cDNA has been isolated from *N. tabacum* and a BLASTP search was conducted against proteins in the NCBI database for matching proteins (Lee *et al*., 2004). Multiple sequence alignments of PAL proteins from various plants were performed by the Clustal W programme (http://us.expasy.org/tools).

Tissue-specific expression pattern analysis of *NtPAL4*

Total RNA was isolated from young and old tobacco leaves, flowers, stems, and roots using TRIzol-reagent® (Invitrogen, USA) according to the manufacturer's instructions. The first-strand cDNA was synthesized using Moloney murine leukemia virus reverse transcriptase (M-MLV RT, Invitrogen, USA).

Forward (5'-CATTGCCACATTCAGCAACA-3') and reverse (5'-GGGTGGTGCTTCAACTTGTG-3') primers were designed to amplify the specific fragment of the *NtPAL4* gene. The PCR reaction was carried out as follows: initial 5 min of denaturation at 94°C; followed by 30 cycles at 94°C for 1 min, 55°C for 1 min, 72°C for 1 min; and a final 7 min at 72°C. The reaction products were separated by electrophoresis on 1% agarose gels and visualized after staining with ethidium bromide.

Southern blot analysis

Genomic DNA was isolated from mature tobacco leaves, and samples (20 μg) were digested with *Eco*RI and *Xba*I. Digested genomic DNA was separated by electrophoresis on a 1% agarose gel, denatured, and blotted onto a nylon membrane (Amersham Pharmacia, Uppsala). Membranes were hybridized with the *NtPAL4* cDNA labeled with [α-^{32}P] dCTP as the probe. The cDNA fragments were obtained in the course of RT-PCR analysis. Hybridization was performed overnight at 65ºC in 5% dextran sulfate, 0.25 M disodium phosphate (pH 7.2), 7% (w/v) SDS, and 1 mM EDTA. After hybridization, the membranes were washed twice with 2×SSC and 0.1% SDS for 10 min each at room temperature and twice with 0.1×SSC and 0.1% SDS for 5 min each at 65ºC. The blots were dried and exposed onto X-ray film at -80ºC for 1 week.

Northern blot analysis

Equal quantities of total RNA were loaded on 1% agarose gels containing 7.4% formaldehyde. After electrophoresis and visualization under UV light, the RNA was transferred to nylon membranes (Hybond N^+, Amersham) and hybridization was performed using a *NtPAL4* cDNA probe labeled with [α-^{32}P] dCTP. After hybridization, the blots were washed twice with 2× SSC and 0.1% SDS for 10 min each at room temperature and twice with 0.1× SSC and 0.1% SDS for 5 min each at 65ºC. The blots were dried and placed on X-ray film at -80ºC for one week.

Results

Sequence analysis of *NtPAL4*

A full-length *NtPAL4* cDNA is 2,154 bp in length (accession No. EU883670) and codes for protein containing 717 amino acids. The deduced amino acid sequences of NtPAL4 had a predicted molecular weight of 77.951 KDa and a theoretical pI (isoelectric point) of 6.32, respectively.

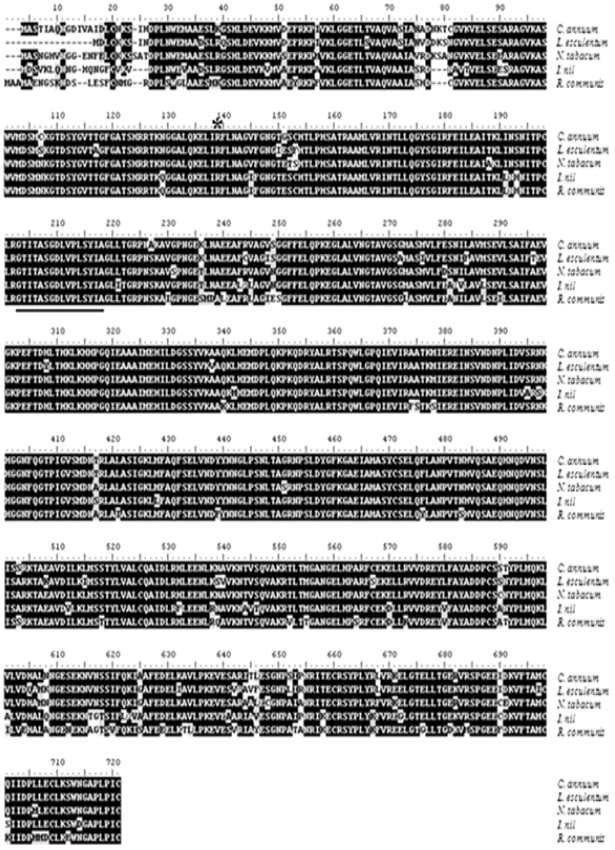

Fig. 1. Multiple alignment of *NtPAL4* with other PAL proteins. Comparison of the derived amino acid sequences of *NtPAL4* with other PAL proteins. Residues shaded in black are identical between the different proteins. Conserved sequence of active site is underlined; Arg residue (Arg^{139}) is labeled by asterisk. *Capsicum annuum* (ACF17667); *Lycopersicon esculentum* (AAA34179); *Nicotiana tabacum* (EU883670); *Ipomoea nil* (AAG49585); *Ricinus communis* (XP002519521).

Fig. 1 showed a multiple alignment of the deduced amino acid sequence of NtPAL4 and PAL proteins from other plants. The first 20 amino acids of the N-terminal regions are very different, while the other downstream residues are highly conserved in all PAL proteins. These PAL proteins share a conserved active site sequence: G-[STG]-[LIVM]-[STG]-[AC]-S-G-[DH]-L-x-P-L-[SA]-x-[SAV]. A codon for an Arg residue (Arg^{139}) is found in *NtPAL4* cDNA (Fig. 1). The deduced NtPAL protein showed high identities to other PAL proteins from several plant species. NtPAL4 was 92% identical to *C. annuum* (accession No. ACF17667), 91% to *L. esculentum* (accession No. AAA34179), and 88% to *I. nil* (accession No. AAG49585).

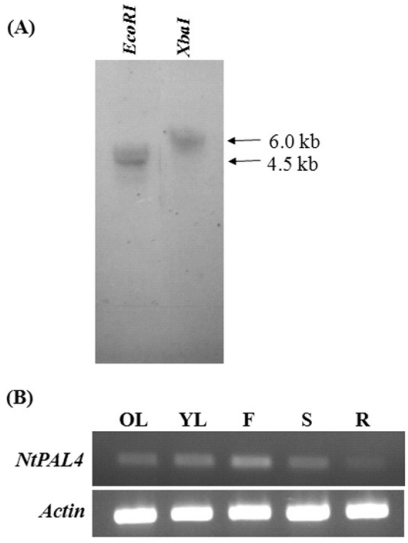

Fig. 2. Gel blot analysis of *NtPAL4*. (A) Southern blot analysis. Genomic DNA was digested with *Eco*RI and *Xba*I, loaded on an agarose gel, and hybridized with the ^{32}P-labeled probe corresponding to the full-length *NtPAL4* cDNA. (B) Tissue RNA expression of the *NtPAL4* gene. *NtPAL4* RNA (20 μg) levels were monitored in mature leaves (OL), young leaves (YL), flowers (F), roots (R) and stems (S) from tobacco plant.

Southern blotting analysis and tissue-specific expression pattern of *NtPAL4*

To assess the copy number of the *NtPAL4* gene in tobacco, genomic DNA was digested with *Eco*RI and *Xba*I. In Southern blot analysis using a *NtPAL4* full-length cDNA as the probe, the genomic DNA blot produced a single band in genomic DNA digested with both *Eco*RI and *Xba*I (Fig. 2A). These findings indicate that the *NtPAL4* gene is present as a single copy in the tobacco genome.

The expression pattern of the *NtPAL4* gene in various tissues was determined by RT-PCR (Fig. 2B). The *NtPAL4* transcript was strongly expressed in flowers; it also was expressed in mature and young tobacco leaves. A comparatively weak signal was observed in stems, but a very weak signal was detected in roots.

Expression of *NtPAL4* mRNA in response to various abiotic and oxidative stresses

To determine the regulation of the *NtPAL4* expression in response to abiotic stress, the expression of this gene was investigated by Northern blotting (Fig. 3). Distilled water

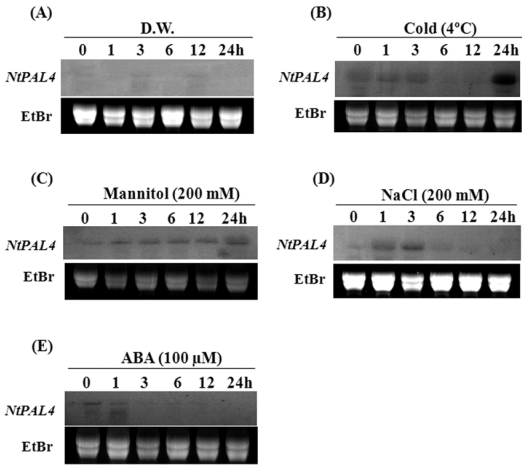

Fig. 3. Expression of the *NtPAL4* gene in tobacco leaf tissues that were exposed to various abiotic stresses. (A) D.W. treatment was used as control. Cold treatment (B; 4°C), mannitol (C; 200 mM), NaCl (D; 200 mM), and ABA (E; 100 μM) were used as the abiotic stresses. Total RNA (20 μg) from leaf samples at various time points after treatment was loaded into each lane, and *NtPAL4* cDNA was used as a probe. To ensure equal loading of RNA, a duplicate gel was stained with ethidium bromide as an RNA loading control.

treatment was used as a control (Fig. 3A). In response to cold treatment (Fig. 3B), the amount of the *NtPAL4* transcript slightly increased until 3 h, and it reached a maximum 24 h after treatment. In mannitol-treated tobacco leaves (Fig. 3C) the *NtPAL4* transcripts were induced after 1 h and maintained at a comparatively high level until 24 h. In tobacco leaves treated with NaCl, the *NtPAL4* transcripts were induced within 1 h and reached a maximum by 3 h, before the their amount gradually decreased (Fig. 3D). The *NtPAL4* transcript level decreased after exposure to ABA for 3 h (Fig. 3E).

Treatment with 10 mM H_2O_2 caused the *NtPAL4* transcript level to decrease after 1 h (Fig. 4A). PAL transcripts were significantly induced 1 h after MV treatment, and the amount declined at 3 h (Fig. 4B). These results indicate that regulation of *NtPAL4* expression may play an important role in protection mechanism under various stress conditions.

Discussion

In the present study, full-length *NtPAL4* cDNA was isolated from *N. tobacum*, and it was shown that *NtPAL4*

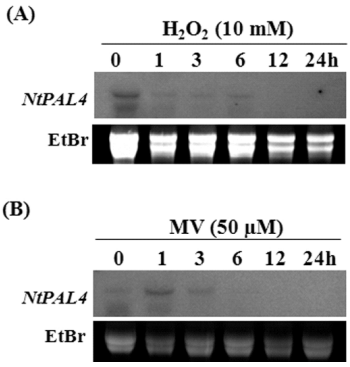

Fig. 4. Expression of the *NtPAL4* gene in tobacco plants treated with chemicals related to oxidative and osmotic stresses. Total RNA was extracted from leaves exposed to 10 mM H_2O_2 (A), 50 μM MV (B) at the indicated times after treatment.

expression was regulated in response to abiotic and oxidative stress conditions.

An active motif containing the sequence G-[STG]-[LIVM]-[STG]-[AC]-S-G-[DH]-L-x-P-L-[SA]-x-[SAV] has been observed in the *NtPAL4* gene. In various plants, PAL proteins share this active site, which is well conserved and can be used as a signature pattern of *PAL* genes (Lee *et al*., 2004). Thus, this active site may assist in the recognition and regulation of the *NtPAL4* gene. In most *PAL* genes, a single intron that splits an Arg codon is located at the same position (Pellegrini *et al*., 1994).

PAL1 and *PAL2* are well conserved; therefore, the Southern blot of these two genes showed multiple bands in samples of tobacco (Fukasawa-Akada *et al*., 1996). The tomato *SlPAL5* gene also produced multiple bands (Guo and Wang, 2009). In Arabidopsis, *PAL1*, *PAL2*, and *PAL3* showed multiple bands (Wanner *et al*., 1995). However, in our study, *NtPAL4* showed a single band suggesting that the *NtPAL4* is a single copy gene with low similarity compared to other *PAL* genes in tobacco.

NtPAL4 transcripts accumulated differently in various organs of the mature tobacco plant, and the developmental regulation of *NtPAL4* gene expression varies from one organ to another. High steady-state levels of the *PAL1* transcript have been found in the roots and flowers of tobacco plants (Fukasawa-Akada *et al*., 1996). In bean, the flower petals contain approximately ten times more PAL transcripts than roots and distinct PAL proteins exhibit markedly different

patterns of regulation of organ specificity during normal development (Liang *et al*., 1989).

Similar to the activity of the *PAL* gene in other plants, *NtPAL4* was readily induced by several abiotic stresses, such as NaCl, cold, and mannitol stresses. It was reported that *SlPAL5* was significantly induced by abiotic stresses, such as salt, cold, and drought in tomato (Guo and Wang, 2009). Furthermore, PAL activity can be induced by various stresses including ozone, pathogen invasion, the plant hormone ethylene, and plant signal molecules, including jasmonic acid (JA), SA, and MeJA (Campos-Vargas and Saltveit, 2002). Our data confirmed that the *NtPAL4* gene is related to responses to abiotic stresses. Although PAL is encoded by a family of several gene isoforms, distinct PAL polypeptides exhibit markedly different patterns of regulation of both organ specificity during normal development and of single organ response to diverse environmental stimuli (Liang *et al*., 1989). Some environmental stresses induce the accumulation of flavonoid compounds in the plant by activating their biosynthesis (Rivero *et al*., 2001). *Sorghum* varieties resistant to biotic and abiotic stresses had on average higher contents of flavonoid compounds than susceptible varieties (Dicko *et al*., 2005). These results suggested that stress induction of phenylpropanoid biosynthesis is involved in the increase of the transcript levels for various biosynthetic enzymes.

The *NtPAL4* transcript level decreased after ABA treatment (Fig. 3E). It was reported that ABA can mediate the expression of many stress-responsive genes in response to drought or saline stress (Zhu, 2002). The *NtPAL4* transcription was significantly induced after treatment with various abiotic stresses but not by ABA. These results suggest that the *NtPAL4* gene is related to NaCl, cold, and mannitol stress and may therefore belong to an ABA-independent regulation system. The mechanisms underlying the activation of the abiotic stress response by *NtPAL4* remain to be elucidated in detail.

The expression pattern of the *NtPAL4* gene in tobacco plants may be related to responses to oxidative stress (Fig. 4). ROS, such as superoxide anion radicals (O^{2-}), hydroxyl radicals (·OH), hydrogen peroxide (H_2O_2), and singlet oxygen (O_2^1), are toxic molecules capable of causing oxidative damage to proteins, DNA, and lipids (Apel and Hirt, 2004). Plant injury caused by environmental stress may be related to ROS-initiated oxidative damage at the cellular level. All organisms produced reactive oxygen species as by-products of normal metabolic processes (Foyer *et al*., 1994). MV generates O^{2-} in the chloroplast and is considered a type of oxidative stress (Babbs *et al*., 1989). MV treatment can significantly induce *NtPAL4* transcription in tobacco, suggesting that *NtPAL4* responded to some oxidative stress. These results showed that *NtPAL4* may play a regulatory role in abiotic stress and antioxidant signaling in tobacco.

In the present study, we investigated the expression of the *NtPAL4* gene during abiotic and oxidative stress by Northern blotting. *NtPAL4* was significantly induced by NaCl, cold, and mannitol stress but not by the plant hormone ABA. These observations suggest that the *NtPAL4* gene is related to abiotic stress response and may belong to an ABA-independent regulation system. Furthermore, *NtPAL4* transcripts accumulated under MV. The mechanisms underlying the activation of the abiotic response by *NtPAL4* remain to be fully elucidated.

Acknowledgements

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