SHORT COMMUNICATION

ANALYSIS OF A PHENYLALANINE AMMONIA-LYASE GENE SEQUENCE FROM *ARACHIS HYPOGAEA* L. AND ITS TRANSCRIPT ABUNDANCE IN INDUCED SYSTEMIC RESISTANCE AGAINST *SCLEROTIUM ROLFSII*

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SUMMARY

Phenylalanine ammonia-lyase (PAL) is an important plant enzyme in pathogen defense. The purpose of this work was to analyze the phylogenetic relationships of a partial *PAL* gene sequence from *Arachis hypogaea L.* (peanut) cv. Tegua (designated *AhPAL*), a *PAL* sequence from cultivar JL24 and *PAL* sequences from other legumes and non-legumes plants. In addition, changes in the *AhPAL* transcript abundance related to induced systemic resistance (ISR) were also evaluated. *AhPAL* sequence shows high level of similarity with *PAL* genes and proteins from several plant species, including legumes and non-legumes. Quantification of relative transcript abundance indicated that *AhPAL* could be involved in the *A. hypogaea* cv. Tegua response towards the phytopathogen *Sclerotium rolfsii.*

Key words: biotic stress, *Bacillus* sp., defense response, peanut, phenylalanine ammonia lyase.

Phenylalanine ammonia-lyase (PAL, EC 4.3.1.5) catalyzes the non-oxidative elimination of ammonia from L-phenylalanine to give *trans*-cinnamic acid, a substrate common to the biosynthesis of different phenylpropanoid products (Xu *et al*., 2008).

PAL genes have been identified in all higher plants tested (Hahlbrock and Scheel, 1989; Joos and Hahlbroock, 1992; Huang *et al*., 2010; Lepelley *et al*., 2012; Gao *et al*., 2012). PAL is an important enzyme not only for plant development but also for biotic and abiotic stress response. In some plants, *PAL* gene expression is believed to be activated by the jasmonic acid/ethylene signal pathway during induced disease resistance (Diallinas and Kanellis, 1994; Mitchell and Walters, 1995; Kato *et al*., 2000; Distefano *et al*., 2008).

Studies on the characteristics and roles of *PAL* genes in legumes are scarce. Specifically for *Arachis hypogeae* L. (peanut), there is no available information about the phylogeny of this gene and on the role of PAL enzyme, even though, a *PAL* gene sequence from cultivar JL24 has been recently published in GenBank (accession No. GU477587). This knowledge is, however, essential for a better understanding of the molecular processes involved in peanut resistance against pathogens.

PAL is a key enzyme in plant response to biotic stress, including the rhizobacteria-induced systemic resistance (ISR). This is a type of systemically enhanced resistance against a broad spectrum of pathogens that is triggered upon root colonization by selected strains of non-pathogenic bacteria (Kloepper *et al*., 1992). PAL expression is activated by the ethylene/jasmonate signaling pathway, which leads to ISR. Therefore, the expression of this gene and the enzyme activity could be considered as markers of this defense response (Distéfano *et al*., 2008; Shoresh *et al*. 2004).

Considering the importance of PAL in plant responses to biotic stresses, the objectives of this work were to obtain a partial-lenth genomic DNA sequence of a *PAL* gene from *A. hypogaea* cv. Tegua, to characterize its evolutionary relationships with other members of the *PAL* family and to determine its expression in ISR against the phytopathogen *Sclerotium rolfsii*

In order to obtain a *PAL* gene sequence from peanut plants, genomic DNA from young leaves was isolated using extraction NucleoSpin PlantII (Macherey-Nagel, Germany) kit, according to the manufacturer's instructions. PCR primers PAL_F (5'AAGCACCACCCTGGTCAAATTGAG-3') and PAL_R (5'-GACAAGCTCGGAGAATTGAGCAAAC-3') were designed based on conserved sequences of *PAL* genes from the legumes *Stylosanthes humilis* (L36822), *Pisum sativum* (D10003), *Phaseolus vulgaris* (M11939), *Trifolium subterraneum* (M91192), and *Glycine max* (X52953). The *PAL* gene sequence from A. *hypogaea* cv. JL24 had not been released yet when this work was started, so it was not included. PCR was performed in 10 μl reaction mixtures containing 1X PCR buffer, 2.5 mM $MgCl_2$, 200 μM of each nucleotide (Promega, USA), 1 μM of each primer, 1 U of Taq DNA polymerase (Promega, USA) and 1 μl of template DNA solution. Temperature profile was as follows: initial denaturation at 95°C for 3 min; 35 cycles of denaturation at 94°C for 1 min, annealing at 56°C for 1 min, extension at 72°C for 1 min, and a final exten-

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sion step at 72°C for 10 min. A fragment of approximately 370 bp was amplified, custom sequenced (Macrogen, Korea) and deposited in GenBank under the accession No. GU123139.

Homologous genes were identified using the algorithms BLASTN and BLASTX (Altschul *et al*., 1997). A high level of similarity was found with *PAL* genes and proteins from several plant species, including legumes and non-legumes, confirming that even among evolutionary distant taxa, *PAL* sequences are highly conserved (Butland *et al*., 1998; Kumar and Ellis, 2001). Among *PAL* sequences from different plants, the *Ah-PAL* gene fragment had a nucleotide sequence identity of 79% (99% query coverage) with *Vigna unguiculata* and of 93% with *Glycine max* (99% query coverage).

For phylogenetic analysis, *PAL* gene sequences from GenBank were aligned using Clustal W (Larkin *et al*., 2007). Phylogenetic analyses were conducted using BioEdit (Hall, 1999), MEGA version 4 (Tamura *et al*., 2007) and PhyML (Guindon and Gascuel, 2003) soft-

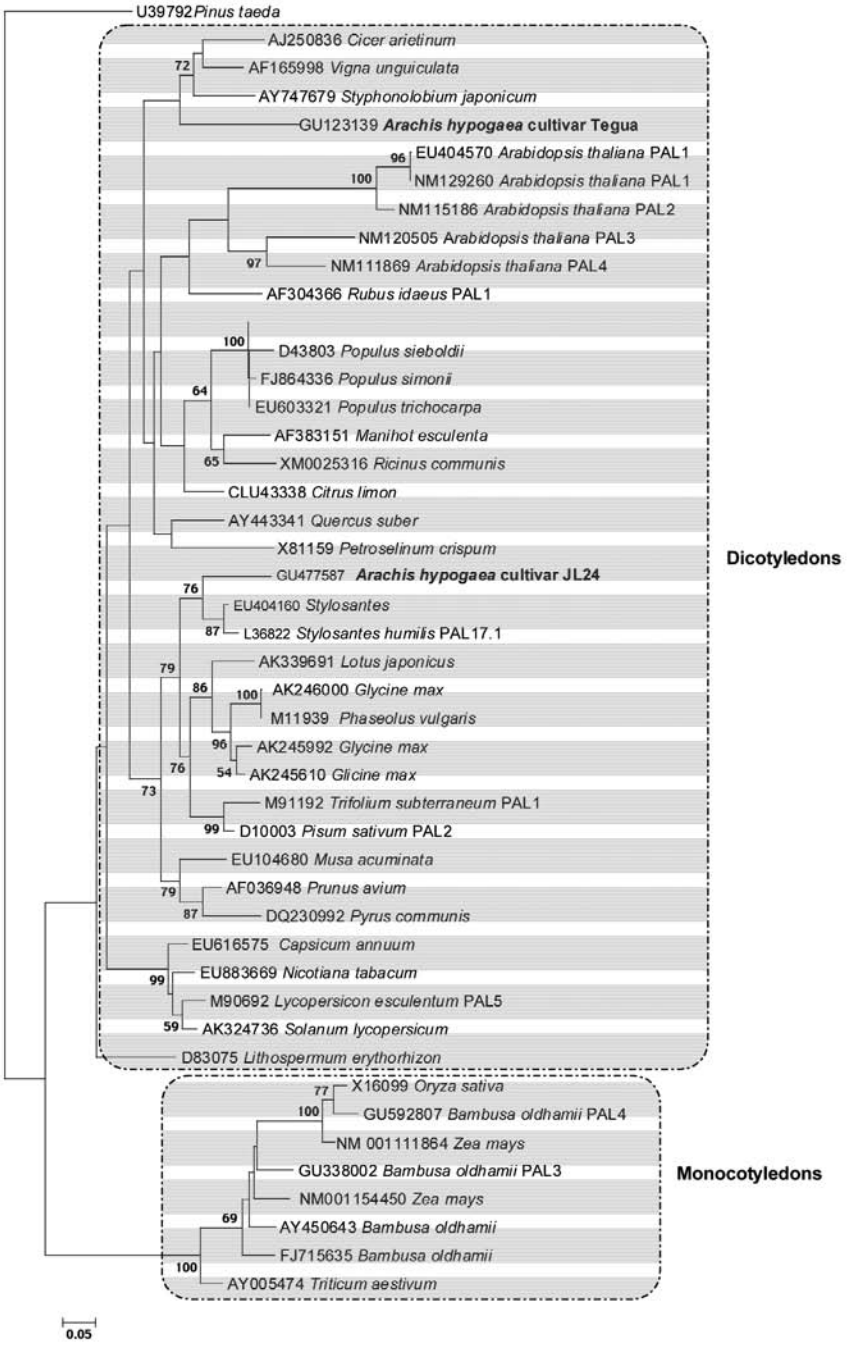

Fig. 1. Phylogenetic tree of plant PAL genes. The tree was inferred by ML method under HKY+G substitution model. Bootstrap values (over 50%) for 100 replicates are shown. *Pinus taeda* PAL gene was used to root the tree.

ware. HKY+G (Hasegawa *et al*., 1985) was selected as the best-fit nucleotide substitution model according to jModeltest (Posada, 2008).

The phylogenetic tree revealed that, as expected, *AhPAL* gene fragment clustered with sequences from dicotyledonous plants (Fig. 1). Moreover, this sequence and that of *A. hypogaea* cv. JL24 shared 80% nucleotide sequence identity and 88% amino acid sequence similarity. Interestingly, four of the six sequences that encode fragments of the active sites in *A. hypogaea* cv. JL24 *PAL* were also found in the *AhPAL* sequence. However, *PAL* sequence of cv. JL24 and *AhPAL* do not cluster together in the phylogenetic tree, possibly indicating that they encode different PAL isoforms, which might evolved by divergence after a gene duplication event. This event has been reported to be a main issue in the evolution process of *PAL* gene (Kumar and Ellis, 2001). Duplication events that occurred later in the evolution of angiosperms resulted in *PAL* genes, within the same species, that cluster together. On the other hand, it has been reported that paralogous copies of *PAL* genes within *P. vulgaris*, *Rubus idaeus* and *Coffea canephora* (Rubiaceae) genomes do not cluster together in phylogenetic trees (Kumar and Ellis, 2001; Lepelley *et al*., 2012). These paralogous genes encode unique PAL isoforms that respond to different stimuli.

In a previous work we had shown that rhizobacterium *Bacillus* sp. CHEP5 is able to reduce peanut disease severity caused by the phytopathogen *Sclerotium rolfsii* by inducing systemic resistance (Tonelli *et al*., 2011). In this study, to determine if *AhPAL* is involved in this protection, the amount of its transcript was measured in the leaves of plants whose roots had previously been inoculated with *Bacillus* sp. CHEP5, then challenged with *S. rolfsii*. In this assay, physical separation between the rhizobacterium and the fungus was guaranteed by using the root split method described by Fuchs *et al*. (1997).

Total RNA was isolated from leaves using the Extraction NucleoSpin RNA/Protein kit (Macherey-Nagel, Germany) and its purification was performed using the RNeasy Minikit (Qiagen, USA). RNA samples were extracted at 24 and 72 h after fungal challenge. cDNAs was obtained from 1 µg of DNase-treated total RNA from the different treatments in a 20 µl reaction containing 200 U of SuperScript III reverse transcriptase (Invitrogen, USA), according to the manufacturer's protocol.

Real Time-PCR was performed in 10 µl reaction mixtures containing 2X Brillant SYBR green QPCR master mix (BioRad, USA), 1:10 dilution of synthesized cDNA and 200 nM of the primers $RTPAL_F$ (5'-CGCTCTTC-GAACTTCGCCTCA-3') and $RTPAL_R$ (5'-ATG-GCCAGCCGGGTGTTATC-3'. The thermocycle parameters were as follows: initial polymerase activation 5 min at 95°C followed by 40 cycles of 30 sec at 95°C, 30 sec at 60°C and 45 sec at 72°C, finally one step of 1 min at 95°C. The specificity of the PCR amplification procedure was checked with a heat dissociation protocol (from 70 to 100°C) after the final cycle of the PCR. The results obtained from the different treatments were standardized to the Ubiquitin mRNA levels, which was amplified with the primers UBQ_F (5'-AAGCCCAA-GAAGATCAAGCAC-3') and UBQ_R (5'-GGTTAGC-CATGAAGGTTCCAG-3') (Luo *et al*., 2005). Real Time-PCR determinations were carried out with RNA extracted from two independent biological samples with the threshold cycle (Ct) determined in triplicate. The relative levels of transcription were calculated by using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

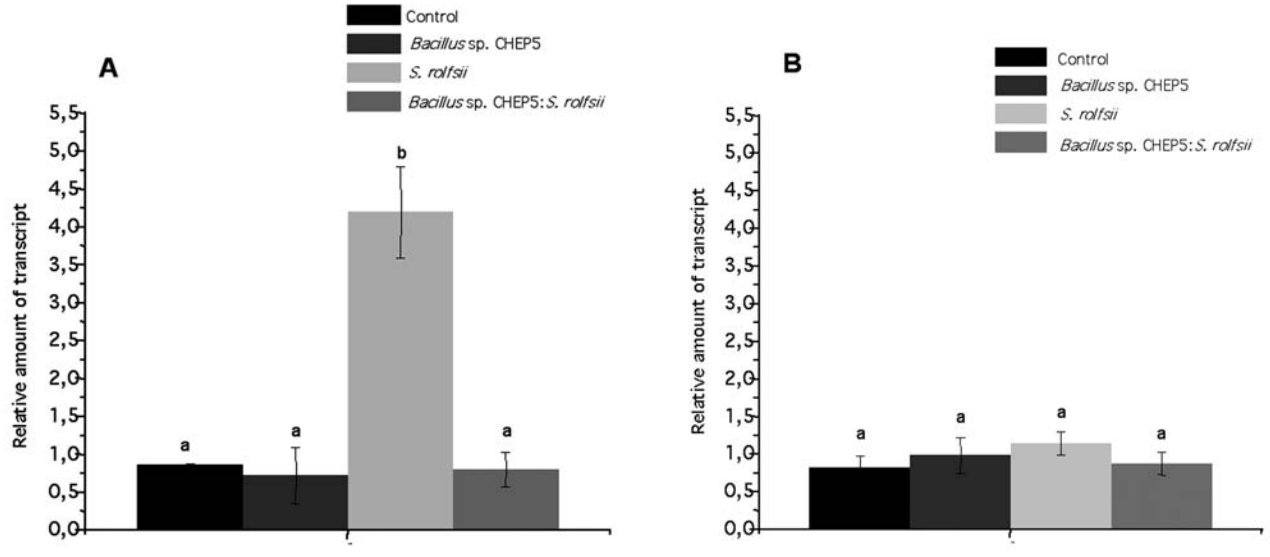

Fig. 2. Real Time-PCR analysis of *AhPAL* relative amount of transcript. A: 24 h post inoculation of *S. rolfsii.* B: 72 h post inoculation of *S. rolfsii*. Values are the means ± S.E. of two biological replicates and three analytical replications (n=3). Different letters indicate significant differences according to Duncan's test ($p < 0.05$).

AhPAL relative transcript abundance increased only 24 h after *S. rolfsii* challenge (Fig. 2), suggesting that it is related to the first stages of plant biotic stress response. No variation in *AhPAL* relative transcript abundance was detected following *Bacillus* sp. CHEP5 inoculation. The same results were obtained when the *PAL* gene sequence from cv. JL24 was analyzed (data not shown). However, in a previous work we found an increase in *PAL* activity in the leaves of plants inoculated with this biocontrol agent before *S. rolfsii* challenge, pointing out the ability of this rhizobacterium to prime systemically plant defense responses, besides reducing disease severity (Tonelli *et al*., 2011). Considering that in all studied plants, PAL proteins are encoded by a multi-gene family ranging from a few to a dozen or more copies (Rozen and Skaletsky, 2000; Kumar and Ellis, 2001; Cramer *et al.*, 1989), it is possible that the increase in the PAL activity that we previously reported was related with a different PAL isoform or that an increase in the *AhPAL* expression level occurred earlier than the time at which it was evaluated. Other explanations for the discrepancy between PAL activity and relative transcript abundance are that mRNA may be very unstable, making difficult its quantification, and that besides transcriptional gene control, post-transcriptional control has a key role in enzyme activity, including PAL regulation (Dixon and Paiva, 1995; Dixon *et al*., 2002, Paré *et al*., 2005; Naiumkina *et al*., 2010; Lepelley *et al*., 2012).

In conclusion, results from this study demonstrate that the increase in the relative *AhPAL* transcript abundance is related to the systemic response towards biotic stress induced by *S. rolfsii*. Furthermore, it was found that *AhPAL* shows high level of similarity with *PAL* genes and proteins from several plant species, including legumes and non-legumes.

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