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Molecular cloning and characterization of a phenylalanine ammonia-lyase gene (*LrPAL*) from *Lycoris radiata*

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Abstract *LrPAL* is a novel full-length cDNA isolated from *Lycoris radiata* by degenerate oligonucleotide primer PCR (DOP-PCR), 3′- and 5′-RACE approaches, harbours an open reading frame (ORF) encoding a 708 amino acid product. Sequence alignment showed that the deduced amino acid sequence of *LrPAL* shared more than 80% identity with other PAL sequences reported in *Arabidopsis thaliana* and other plants. RT-PCR revealed that *LrPAL* transcripts were higher in bud flowers and wilting flowers (5 days after blooming) than in blooming flowers. The transcript levels of *LrPAL* in leaves were significantly induced by methyl jasmonate (MJ) and nitric oxide (NO), and salicylic acid (SA). Similarly, HPLC analysis showed that galantamine (GAL) content was also higher in bud flowers and wilting flowers than in blooming flowers. The GAL content in leaves was significantly induced by MJ and NO, and inhibited by SA. This study enables us to further elucidate the role of *LrPAL* in the biosynthesis of GAL in *Lycoris radiata* at a molecular level.

Keywords Phenylalanine ammonia-lyase · Galantamine biosynthesis · *Lycoris radiata*

Introduction

Species of *Lycoris* produce amaryllidaceae alkaloids, which are structurally unique and have a wide range of medical functions. Galantamine (GAL), one of these alkaloids, is an AChE inhibitor, an important drug for the treatment of Alzheimer's disease newly approved by the food & drug administration in the USA and by the European registration bureau in Europe [1, 2]. The low yield of GAL from natural sources and its high value in chemotherapy enhance researches involving elucidating the biosynthetic pathway of GAL and other related alkaloids, the key enzymes and genes in these pathways.

Phenylpropanoid pathway is one of the important secondary metabolism pathways and it produces a large number of biologically important secondary metabolites such as caffeic acid, flavonoid compounds, lignin, coumarins, salicylic acid, phytoalexins, chlorogenic acids, stilbenes, and so on [3–7]. These compounds play important roles in the courses of plant development, mechanical support, and disease resistance [8–10]. Phenylalanine ammonia-lyase (PAL) is one of important enzymes in the phenylpropanoid pathway. Recent researches postulated that PAL may play an important role in biosynthesis of some important secondary metabolites, such as biosynthesis of rosmarinic acid in *S. miltiorrhiza* [11], flavonoid biosynthesis in *A. membranaceus* var [5]. For their important roles in higher plant,

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manipulation of phenylpropanoid pathway metabolites has long been a hotspot [8, 12].

Phenylalanine ammonia-lyase is one of the branch point enzymes between primary and secondary metabolism, and catalyzes the conversion of L-phenylalanine to *trans*-cinnamic acid, which is the first step and also a key step of phenylpropanoid pathway [13]. L-phenylalanine (L-phe) and L-tyrosine (L-tyr) are considered as primary precursors of galanthamine [14]. PAL is one of the most extensively studied enzymes in plants with reference to its crucial function in the biosynthesis of various secondary metabolites [15]. At present, PAL genes have been cloned from various angiosperm species, such as *Oryza sativa* [16], *Petroselinum crispum* [17], *Arabidopsis thaliana* [18, 19], raspberry [20], pine [21], *Salvia miltiorrhiza* [11], and *Bambusa oldhamii* [22]. However, few data have been reported about genes contributing to phenylpropanoid pathway in *Lycoris*. Moreover, PAL in *Lycoris* has not been cloned yet, and its role in amaryllidaceae alkaloid synthesis has not been elucidated. In this study, we have reported the cloning of a novel full-length cDNA (*LrPAL* gene) from *Lycoris radiata*, its expression pattern and GAL contents in flowers of various stages, and the leaves treated by MJ, SA, and NO. The possible function of the *LrPAL* was discussed based on the experimental results.

Materials and methods

Cloning vectors, plant material and treatments

Plasmid pMD18-T Easy vector (TaKaRa, Dalian, China) was used as vectors for cloning. *E. coli* DH5α (TaKaRa) was used as the host for plasmid amplification. Biennial *Lycoris radiata* plants with two-month old leaves growing in incubators were treated by 100 μM MJ, 500 μM SNP (sodium nitroprusside) and 250 μM SA respectively.

Generation of partial cDNA from *Lycoris radiata*

Degenerate oligonucleotide primers: (f1) 5′-TNCCNCTNT CNTACATHGCCGG-3′ and (r1) 5′-AARCAYCAYCCNG GNCARATH-3′, were designed based on highly homologous sites on both the amino acid and nucleotide levels in the plant PAL. cDNA fragment encoding phenylalanine ammonia-lyase from *L. radiata* was produced by RT-PCR, and DNA amplification was performed under the following conditions: 30 cycles of 94°C, 30 s; 58°C, 30 s; 72°C, 30 s. At the end of 30 cycles, the reaction mixtures were incubated for an additional 5 min at 72°C prior to cooling to 4°C. The amplified DNA products were run on 1% agarose gel; the target bands were purified with Gel Extraction Kit (HuaShun, Shanghai) according to the manufacturer's protocol. The purified products were then subcloned into the pMD18-T Easy vector (TaKaRa, Dalian, China) and sequenced (Invitrogen, P. R. China).

Cloning of the full-length *LrPAL* cDNA

The full-length cDNA of phenylalanine ammonia-lyase gene was isolated by 3′- and 5′-RACE methods. Two gene-specific primers (3R1 and 3R2) were designed from the sequence between primers f1 and r1, for. A 711-bp fragment PCR product was obtained by 3′ RACE (TaKaRa, 3'Full RACE Core set Ver.2.0), using the two primers (3R1 and 3R2). The cDNA fragment was then purified, subcloned and sequenced. By using this sequence, two gene-specific primers (5R1 and 5R2) were designed for 5' RACE (TaKaRa, 5'-Full RACE kit). A 1952-bp fragment was amplified, subcloned and sequenced. Full-length of 2124-bp DNA was then produced by RT-PCR using two gene-specific primers: 5′-GGCATATGGCATACGCCAACGGTA-3′ and 5′-GGC TCGAGGCATATAGGAAGAGGAGCACCAT-3′.

Nucleotide and protein sequence analysis

The full-length phenylalanine ammonia-lyase gene and deduced amino acid sequence were compared to those available at the GenBank and were aligned by the BLAST program.

Expression analysis of *LrPAL* by RT-PCR

Reverse transcription-polymerase chain reaction (RT-PCR) was used to semi-quantitatively determine the expression profile of the *LrPAL* genes in flowers at various stage and stress-treated leaves mentioned above. Reverse transcription was achieved by AMV using oligo dT primers, as per instructions (TaKaRa, Dalian, China). The PCR cycle was 30 cycles of 94°C, 30 s; 56°C, 30 s; 72°C, 60 s. The 252 bp *LrPAL* fragment was amplified by primers *PAL-F* (5′-GACATTAAGAACAGGATTGAGGA-3′) and *PAl-R* (5′-AGAAGCTGAGAATGAATCACAAC-3′). A 530 bp fragment of the constitutively expressed 18S RNA gene was amplified by RT-PCR using two primers (INS-F: 5′-CCCCAGGCTTTACACTTTATGCTTC-3′ and INS-R: 5′-AATGCTTTCGCAGTTGTTCGTCTTT-3′) for use as an internal standard.

Galanthamine extraction and analysis

Determination of GAL was accomplished based on the method of Colque et al. [23]. The residual grinded tissue for RNA extraction was dried in an oven at 45°C until constant weight. 50 mg of powder was macerated by 10 ml of methanol for 24 h at room temperature; at this time they

are sonicated thrice at regular intervals (30 min each). The methanolic extracts were centrifuged at 4,000 rpm for 20 min, and then filtered through a 0.22 μM pore filter (Millipore) before HPLC analysis. The quantitative amounts of GAL were determined by HPLC analysis as described by Li et al. [24]. The standards of GAL were purchased from Fujian Like Bio-pharmaceutical Technology Co., Ltd. (Batch No. 061210-2, purity ≥98.0%).

Results and discussion

Isolation and characterization of *Lycoris radiata* *PAL* cDNAs

A new PAL cDNA was obtained from *L. radiata* by DOP-PCR, 3′-and 5′-RACE methods. By using the degenerate oligonucleotide primers, several parts of cDNA sequences were obtained by PCR screening. One of the fragments was confirmed to be phenylalanine ammonia-lyase gene by DNA sequencing. Then, by using 3′- and 5′-RACE methods, a full-length cDNA of *LrPAL* was obtained. It contains a 2,124 bp open-reading frame that encodes 708 amino acids with a calculated molecular weight of 77,512.3 Da. A theoretical isoelectric point (pI) of 6.52 was predicted by DNAMAN analysis software (Lynnon Biosoft, Vaudreuil, Canada).

BLAST P in NCBI (http://www.ncbi.nlmnih.gov/BLAST) and multi-alignment analysis by DNAMAN indicated that LrPAL was with highly identity to PALs reported in other species, sharing a similarity of 83, 82, 81, and 80% identity with MbPAL (BAG70992), PcPAL (P45729), DcPAL (BAG31 930), and PaPAL (O64963) (fig. S1). Therefore, the gene cloned in this study was designated *LrPAL* and the sequence information was deposited in the GenBank database (accession number FJ603650).

Phylogenetic tree depicting the relationships among plant PALs

To study the evolutionary relationship among *PAL* genes, sequences of a set of *PAL* gene family members from other plant species, including *L. radiata*, were analyzed by using the neighbor-joining method. The amino acids sequence of PAL from *Ginkgo* was selected as the out-group, because *Ginkgo* is a species of gymnosperms, which is considered on the basis of both morphological characters and 18S RNA sequences [25]. It is also known that dicotyledon and monocotyledon PALs independently form each of subfamilies in the angiosperm-type PAL family [20, 26]. On the whole, phylogenetic analysis of plant PALs may suggest that their sequence diversity agrees with the genetic evolution of the spermatophyte, excepting MbPAL, LrPAL and AcPAL. MbPAL is clustered in group of dicotyledon PALs, LrPAL, and AcPAL were clustered in an independent group between dicotyledon and monocotyledon PALs (Fig. S2). The results demonstrated that MbPAL, LrPAL, and AcPAL were at inter-evolutional type in plant PAL family.

LrPAL expression and galantamine content in flowers of *Lycoris radiata*

As it is well known, PAL genes are generally organized in a small multigene family in plants, *PAL* paralogues differ in both their sequences and in the regulation of their expression [27, 28]. In *Nicotiana tobacum*, Fukasawa-Akada [29] postulated the presence of four putative *NtPAL* genes (*PAL1–4*). Cochrane et al.[19] described four *Arabidopsis thaliana* putative *AtPAL* genes. In *Lycoris* species, the exact number of PAL genes has not yet been established but our results indicated that more than one *PAL* gene was present in the *L. radiata* genome (data not shown). To ensure the specifity of *LrPAL* fragment, we designed the specific reverse RT-primer from 3′UTR region of *LrPAL* for semi-quantitative PCR.

Our early study indicates that galantamine content in flowers changes smartly at various stages. We are interested in *LrPAL* expression pattern during this period. RNA of bud flowers, wilting flowers (5 days after blooming), and blooming flowers was extracted for *LrPAL* expression analysis (Fig. 1a). The results showed that *LrPAL* was expressed most actively at pre-florescence and after florescence stages, moderate expressed at florescence stage.

Phenylalanine ammonia-lyase catalyses the first step of the phenylpropanoid pathway leading to the synthesis of a wide range of chemical compounds including flavonoids, coumarins, hydroxycinnamoyl esters, and lignin [15]. Recently the roles of PAL were investigated in traditional medicine or economic plants, such as *Salvia miltiorrhiza* [11], *Astragalus membranaceus* [5], coffee [6], and tea [7]. We are interested in whether LrPAL is a limiting factor for galantamine synthesis in *L. radiata*, the residual grinded tissues for RNA extraction were assessed for GAL content. The results showed that the content of GAL was 85, 67, 84 μg/g DW in bud flowers blooming flowers, and wilting flowers (5 days after blooming) respectively (Fig. 1b). The changing trend of expression and GAL content is consistent, indicating that PAL may be a limiting factor in GAL synthesis.

LrPAL expression and galantamine accumulation in response to various elicitors

Expression of PAL gene can be induced by various environmental factors such as light, infection, mechanical

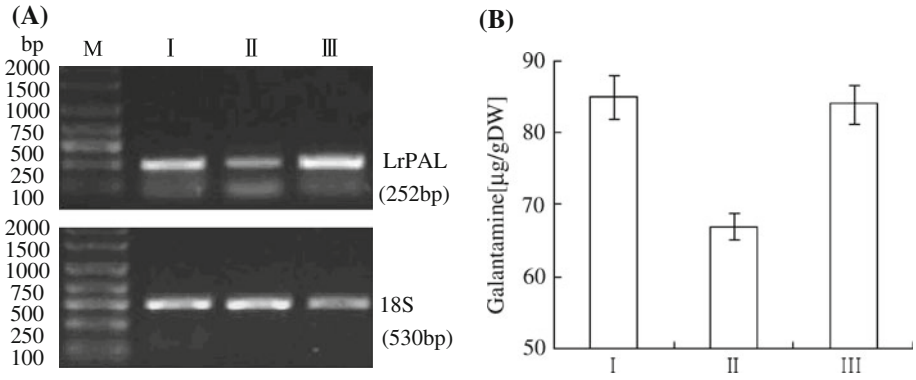

Fig. 1 *LrPAL1* semi-quantitative PCR analysis and GAL content of flowers during florescence. **a** Semi-quantitative PCR analysis: *I* bud flowers, *II* blooming flowers, *III* wilting flowers (5 days after blooming). **b** GAL accumulation in flowers at different stage. *M* DL2000 markers. Values represent the means of five replicates ± standard deviation

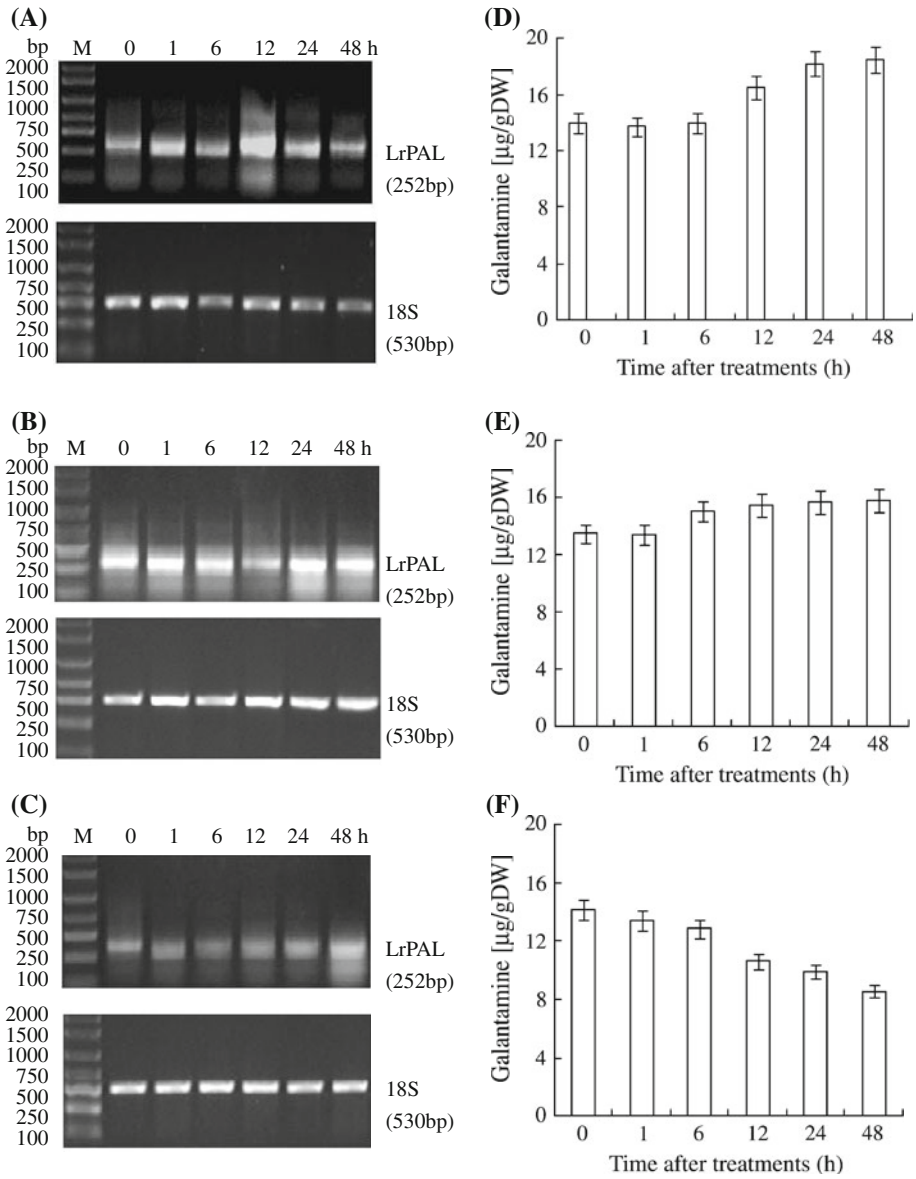

Fig. 2 Time course of *LrPAL* expression and GAL accumulation in leaves following elicitors treatment. *LrPAL* semi-quantitative PCR analysis of leaves treated by **a** 100 μM MJ, **b** NO (500 μM SNP), and **c** 250 μM SA; GAL content of leaves treated by **d** 100 μM MJ**,** *e* NO (500 μM SNP), and **f** 250 μM SA. *M* DL2000 markers. Values represent the means of five replicates ± standard deviation

wounding, low temperature and some signal substances [11, 30–32]. To characterize the transcription pattern of *LrPAL* in response to MJ, NO, and SA, the biennial *L. radiata* plants were treated for 1-, 6-, 12-, 24-, and 48 h by 100 μM MJ, 500 μM SNP, and 250 μM SA, respectively. The leaves total RNA was then extracted,

and used as template in the semi-quantitive RT-PCR analysis. As shown in Fig. 2, *LrPAL* transcripts was markedly increased in response to MJ and NO, and moderatively increased in response to SA. In leaves treated for 12 h by MJ, the *LrPAL* transcripts reached the highest level (Fig. 2a). In the experiment of treatment by NO, *LrPAL* transcripts were firstly up-regulated in 0–1 h, down-regulated in 1–12 h point, then reached the highest level at 24 h (Fig. 2b). In leaves treated for 48 h by SA, the *LrPAL* transcripts reached the highest level (Fig. 2c).

Recently, some studies have shown that MJ and NO can induce production of secondary metabolites such as galantamine [23], ginseng saponin [33] and artemisinin [34]. Mu et al. have reported that MJ and NO can significantly promote the accumulation of the total alkaloids and GAL accumulation, although SA promote the accumulation of the total alkaloids, but restrained the accumulation of GAL, in *Lycoris chinensis* [35]. In this study, we analyzed the correlation between GAL accumulation and *LrPAL* transcripts induced by these signal substances in *L. radiata*. The GAL content of the same *L. radiata* leaves used in the RT-PCR analysis, was examined by HPLC. The results showed that GAL increased after treated by NO and MJ, and was restrained by SA, similar to the results of Mu et al. [35]. In the experiment of treatment by MJ, GAL content began to up-regulate at 12 h, and reached the highest level at 48 h, which was 1.32-fold higher than the control (Fig. 2d). In the experiment of treatment by NO, GAL was 1.17-fold higher than the control at 48 h, (Fig. 2e). In the experiment of treatment by SA, the GAL content began to restrain at 12 h, and reached the lowest level at 48 h, which was 60% of the control (Fig. 2f).

These results suggest that MJ, NO and SA can induce *LrPAL* expression. Interestingly, the similar trend of GAL accumulation was not observed, after treated by SA. This indicates that although MJ, NO, and SA are capable of inducing the expression of *LrPAL*, but may have different effects over the expression of other genes of galantamine pathway. Similar situation was observed in the case of isoflavone metabolism in *Psoralea corylifolia* and *Medicago truncatula* [36, 37]. The characterization of other genes in amaryllidaceae alkaloids pathway should reveal the molecular events to account for the galantamine level in different tissues, development stages and their responses to different elicitors.

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