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# Phenylalanine ammonia lyase isolation and functional analysis of phenylpropanoid pathway under salinity stress in *Salvia* species

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**Abstract**

*Salvia mirzayanii* and *Salvia macrosiphon* are medicinal and aromatic plants belonging to the Lamiaceae family, which have many pharmaceutical properties. In the present study, the nucleic acid sequences of small-subunit ribosomal RNAs (*18s rRNA*) were isolated from *S. mirzayanii* and *S. macrosiphon* and then the genetic diversity and phylogenetic relationships of these two species with 29 related *Salvia* species were investigated. Phenylalanine ammonia lyase (*PAL1*) was isolated from these two *Salvia* species. The phylogenetic and multi-alignment analyses of amino acids sequences of PAL1 revealed that PAL1 from both *S. mirzayanii* (GenBank accession no. KP336898) and *S. macrosiphon* (GenBank accession no. KP336899) has high identity with the other *Salvia* PALs in the GenBank (71%-96%) and has a closer relationship with other angiosperm species. Promoter analysis demonstrated that *PAL* includes *cis*-acting elements such as ABRE, ARE, HSE, TCA-elements, TC-rich repeat and GA, CGTCA, TGACG, WUN-motif in the upstream region which respond to stress hormones and might show its vital role in biotic and abiotic stresses. To investigate the effects of salinity on *Salvia* plants, twelve-week old seedlings of *S. mirzayanii* and *S. macrosiphon* were irrigated with 100 mM NaCl salinity. Leaf tissue was harvested at 0, 6, 12, 24 and 48 h after end of treatments for subsequent analyses. The results showed that salt stress could induce *PAL1* expression (12 to 18 times) and subsequently increase PAL activity (42%-45%) and total phenolic accumulation (35%-43%) in the early hours after stress treatment. These results provide new information about molecular phylogeny of these two *Salvia* species and phenylpropanoid pathway under salt stress.

**Keywords:** Molecular systematic, *PAL1* expression, Promoter analysis, Salt stress.
**Abbreviations:** ABRE _ abscisic acid response element; ARE _ ABA-responsive element; HSE _ heat shock elements; MBS _ myb-binding site; MeJA _ methyl jasmonate; MSA-Like _ multiple system atrophy like; PAL _ phenylalanine ammonia lyase; WUN-motif _ wounding responsive element.

## Introduction

Phenylpropanoid compounds are precursors to a wide range of phenolic compounds with many functions in plants (Huang et al., 2010). These compounds have important roles in several different pathways such as plant defense against pathogens, protection from abiotic stresses, signal transduction and communication with other organisms, and also as regulatory molecules (Vogt, 2010; Kim and Hwang, 2014). Thus, the evolutionary emergence of the phenylpropanoid pathway in plants is an important adaptation that enables plant defense against abiotic and biotic stresses (Kim and Hwang, 2014). Besides their importance in plant biology, phenolic compounds also exhibit a wide range of physiological properties, such as anti-allergenic, anti-atherogenic, anti-inflammatory, anti- microbial, anti-thrombotic, cardioprotective, and vasodilatory effects (Balasundram et al., 2006). Therefore, as phenylpropanoid pathway is a gateway for production of many secondary metabolites including phenolic compounds under stress conditions and physiological processes, there is considerable interest in the regulation of phenylpropanoid biosynthesis, both as a model for understanding flux control in a complex biosynthetic pathway and for the identification of targets for biotechnological manipulation of product accumulation. Phenylalanine ammonia-lyase (PAL; EC 4.3.1.5) catalyzes the deamination of phenylalanine to provide cinnamic acid, which is the first step in the phenylpropanoid pathway and an important regulation point between primary and secondary metabolism (Huang et al., 2010). In addition, genes encoding subsequent enzymes of phenylpropanoid biosynthesis are often coordinately regulated with PAL and indeed PAL mRNA and enzyme levels are highly regulated spatially and temporally, associated with the tissue-specific accumulation of phenylpropanoid products (Bate et al., 1994). It has been shown that *de novo* synthesis of PAL isoforms is induced by biotic and abiotic factors and so, the differential expression of PAL isoforms leads to the synthesis and accumulation of phenolic compounds in different tissues (Jahnen and Hahlbrock, 1988). The genus *Salvia* L. (Lamiaceae) represents an enormous and broad-based assembly of nearly 1000 species, exhibiting a remarkable range of variation. It is distributed throughout the Old and New world, in subtropical and temperate areas (Zhang et al., 2012). In Iran, there are 58 *Salvia* species some of which are endemic to Iran (Sadeghi and Alizadeh, 2013). *S. mirzayanii* and *S. macrosiphon* are two herbaceous biennial or perennial, strongly aromatic and endemic plants growing in Iran, which belong to genus *Salvia* (Javidnia et al., 2002; Karami et al., 2012). Some studies have reported antimicrobial and immunomodulatory activities of *S. mirzayanii* and its activities in cell-mediated and humoral antibody-mediated responses (Amirghofran et al., 2010). *S. macrosiphon* is also generally known for its multiple pharmacological effects including its analgesic and

anti-inflammatory activity (Karami et al., 2012). Moreover, some studies have reported powerful antimicrobial properties (Vahdani et al., 2011) and strong antioxidant activity (Gohari et al., 2011) of the essential oils and extract of this plant. Although there are reports on the medicinal properties of these two species, literature review has not revealed any previous research on their molecular responses under stress conditions. Previously, salt stress was used as an abiotic elicitor to induce phenolic compounds biosynthesis in the medicinal plant, *Salvia mirzayanii* (Valifard et al., 2014). Results showed that salinity stress caused increase in total phenolic content and antioxidant activity of this valuable medicinal plant. Therefore, to complete our previous research on the effects of salt stress on phenolic compound of *Salvia* species, it was decided to investigate the functional analysis as well as the expression patterns of crucial gene, *PAL*, in production of phenolic compounds to better comprehend defense mechanisms toward salinity stress. In the present study, the phylogenetic status of these two *Salvia* species were analyzed for the first time among other *Salvia* species published in the GenBank database based on isolation of *18s rRNA* genes. In addition, the coding sequences of *Salvia PAL1*, which are typically encoded by small multi-gene families, have been partially isolated in two species and their expression profiles during salinity stress were monitored. Regarding the prominent role of promoter analysis in identifying the unknown function and underlying regulatory mechanism, promoter analysis carried out on *PAL* genes. The promoter of a drought-, high salinity-, and cold-inducible gene contains the major *cis*-acting elements, which are involved in stress-inducible gene response (Yamaguchi-Shinozaki et al., 2005). These results provide new information about phylogenetic position by molecular approach, *PAL* expression pattern, PAL activity, and phenolic compound production under salinity stress in these two *Salvia* species.

## Result and Discussion

### *Phylogenetic analysis based on 18S ribosomal RNA gene sequences*

In the present study to investigate the genetic diversity and phylogenetic relationships of *S. mirzayanii* and *S. macrosiphon* with related species, the 18S ribosomal RNA gene sequences of these two plants with 29 *Salvia* sequences for *18S rRNA* that had previously been published in GenBank database were compared. Fig. 3 shows that all *Salvia* species formed three major clades based on world distribution and morphological characters. Clade I consisted of 21 species in two subclades (subclade A and B) separately. With the introduction of new species or isolates, *S. mirzayanii* and *S. macrosiphon*, 9 species were identified in clade I, subclade B. Interestingly, several of the species in this subclade including *S. mirzayanii*, *S. macrosiphon*, *S. scalerea*, *S. aethiopis* and *S. verbascifolia* are all endemic plants in Iran. Clade II consisted of 9 species and several of them including *S. miltiorrhiza*, *S. flava*, *S. przewalskii*, *S. pauciflora*, *S. digitaloides* and *S. cynica* are from China (The Old World) (Zhang et al., 2012). Clade III comprised one species of *Salvia*, *S. apiana*, which has been reported from the Americas (New World) (Zhang et al., 2012). These results show that the position of *Salvia* species in three groups is not only based on morphological characters but also these species have the same geographical habitats. It is evident that *Salvia* has undergone marked species radiations in 3 regions of the world: Central and South America, Central Asia/Mediterranean, and Eastern Asia (Zhang et al., 2012).Our results using *18S rRNA* sequences of *S. mirzayanii* and *S. macrosiphon* also showed that these two species of *Salvia* which have been isolated for the first time by molecular approach have a close relationship with other *Salvia* species which are endemic to Iran. This finding is consistent with cladistics analysis of morphological characters of these two plants (Jamzad, 2012). Both of these species have similar morphological characteristics with other *Salvia* species in this group including, stamen positions, sterile and fertile parts of anthers in stamen, filament form, corolla length and shapes (Jamzad, 2012). The phylogenetic study based on traditional methods mainly described slight differences in morphological characteristics and analysis of compounds by high performance liquid chromatography (HPLC) fingerprints to distinguish *Salvia* species from likely species. The accuracy of authentication has limitations because of the amount of samples, stability of chemical constituents, variable sources, and chemical complexity. However, DNA can be extracted from fresh or dried organic tissue of the plant materials and is not restricted by the form of the samples (Zhang et al., 2012). Nucleic acid sequences from small-subunit ribosomal RNAs (*18S rRNA*) have proved useful for phylogenetic analysis in eukaryotes. Because of their ubiquity and evolutionary conservation, these molecules are useful for inferring distant phylogenetic relationship, providing a means of assessing relationships between organisms, which have limited informative homologous morphological or developmental traits (Turbeville et al., 1991).

### *Phylogenetic analysis, homology and conserved domains of Salvia PAL1*

According to the results of conserved domain analysis using the Pfam database (http://pfam.xfam.org/), the isolated region of two *Salvia* PALs contain the conserved domains of L-Aspartase-Like Super-family (IPR008948) and aromatic amino acid lyase (IPR001106) from residues 6-91. The enzyme L-aspartate ammonia lyase (aspartase) catalyzes the reversible deamination of the amino acid L-aspartic acid to produce fumaric acid and ammonium ion (Fibriansah et al., 2011). Aromatic amino acid ammonia-lyase catalyzes the deamination of L-His, L-Phe, and L-Tyr, yielding ammonia plus aryl acids bearing an α, β-unsaturated propenoic acid (Louie et al., 2006). These groups of enzymes contain a 4-methylidene-imidazole-5-one (MIO) cofactor formed by the spontaneous (autocatalytic) cyclization and dehydration of an internal Ala-Ser-Gly tripeptide segment. It has been suggested that the electrophilic MIO cofactors have the main role in catalyzing the elimination of the α-amino group and the stereo-specific abstraction of a β-proton from the L-amino acid substrate, representing highly conserved residues with the PAL protein families (Louie et al., 2006). The isolated regions of cDNA *PAL1* from *S. mirzayanii* and *S. macrosiphon* were translated to amino acids by vector NTI 10 software. The translated sequences of *PAL1* comprising 70 amino acids in two species were used for protein-protein Blast search in NCBI database and for multi-alignment by vector NTI 10 (Fig. 4A). Amino acid sequences of *S. mirzayanii* showed 93%, 88%, 81%, 63%, and 61% homology with the PAL amino acids from *S. macrosiphon*, *S. hypoleuca*, *S. virgata*, *Oryza sativa*, and *Z. mays* respectively. In addition, the amino acid sequences of *S. macrosiphon* showed 96%, 88%, 74%, 71%, and 68% identity with the PAL from *S. hypoleuca*, *S. virgata, S. officinalis, Panicum virgatum, and O. sativa* respectively. Because of the homo-

**Table 1.** Sequences of primer pairs used for isolation and quantitative RT–PCR.

| Gene | Primer | Sequence (5´-3´) | Amplicon size (bp) |
|---|---|---|---|
| *PAL1* (RT-PCR) | PAL-Fwd | TCTACAACAACGGATTGCCGTCC | 320 |
| | PAL-Rev | CACGGTGTTCTTCACCGCGTGC | |
| *18S rRNA* (Isolation) | 18S rRNA-Fwd | CGTAACAAGGTTTCCGTAGGTG | 721 |
| | 18S rRNA-Rev | TTATTGATATGCTTAAACTCAGCGG | |
| *18S rRNA* (RT–PCR) | 18S rRNA-Fwd | CGCCCAGGAAAACTAAACG | 110 |
| | 18S rRNA-Rev | GCAATTCACACCAAGTATCG | |


**Fig 1.** Seed collection sites of *S. mirzayanii* (yellow circle) and *S. macrosiphon* (orange circle) from Iran.

**Table 2.** The list of the significant several hormone and environment related cis-elements between promoters of *PAL* in three model organisms identified by the AC pairwise test ( p-value ≤ 0.1). *, significant.

| Element | Element No. | | | P-value | | | Function |
|---|---|---|---|---|---|---|---|
| | *Arabodopsis thaliana* | *Mimulus guttatus* | *Zea mays* | *A.thaliana vs M.guttatus* | *A.thaliana vs Z. mays* | *M.guttatus vs Z. mays* | |
| ABRE | 2 | 3 | 4 | 0.152 | 0.112* | 0.134* | involved in the abscisic acid responsiveness |
| TC-rich repeats | 3 | 1 | 1 | 0.164 | 0.14* | 0.234 | involved in defense and stress responsiveness |
| TCA-element | 2 | 2 | 1 | 0.202 | 0.202 | 0.165* | involved in salicylic acid responsiveness |
| MBS | 4 | 1 | 4 | 0.112* | 0.141* | 0.087* | involved in drought-inducibility |
| ARE | 3 | 2 | 0 | 0.184 | 0.073* | 0.104* | essential for the anaerobic induction |
| Box 4 | 5 | 14 | 0 | 0.004* | 0.019* | 0.000012* | involved in light responsiveness |
| O2-Site | 2 | 0 | 2 | 0.166 | 0.194 | 0.132* | involved in zein metabolism regulation |
| RY-Element | 1 | 0 | 0 | 0.302 | 0.27 | | involved in seed-specific regulation |
| Skn-1 motif | 4 | 3 | 7 | 0.16 | 0.074* | 0.068* | required for endosperm expression |
| HSE | 1 | 2 | 0 | 0.184 | 0.27 | 0.104* | involved in heat stress responsiveness |
| G-Box | 5 | 4 | 11 | 0.14* | 0.027* | 0.029* | involved in light responsiveness |
| GA-motife | 1 | 0 | 0 | 0.302 | 0.27 | | part of a light responsive element |
| MSA-Like | 1 | 0 | 0 | 0.302 | 0.27 | | involved in cell cycle regulation |
| CGTCA-motif | 2 | 0 | 3 | 0.166 | 0.155 | 0.071* | involved in the MeJA-responsiveness |
| TGACG-motif | 2 | 0 | 3 | 0.166 | 0.155 | 0.071* | involved in the MeJA-responsiveness |
| WUN-motif | 1 | 0 | 0 | 0.302 | 0.27 | | wound-responsive element |

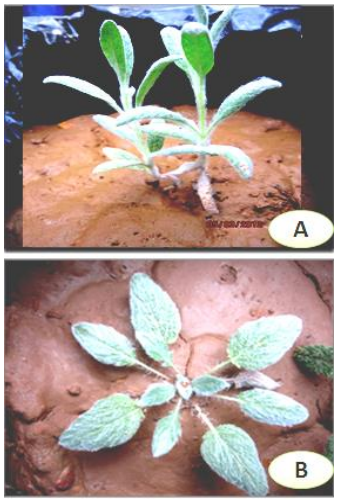


**Fig 2.** Twelve-week old seedlings with four nodes and eight opposite leaves. *S .mirzayanii* (A) *S. macrosiphon* (B).

logy, percentages that are mentioned above, the amino acid sequences of PAL have the highest similarity to other *Salvia* species. On the other hand, the moderate similarity between PAL from *S. mirzayanii* and *S. macrosiphon* with the monocotyledonous species such as *P. virgatum*, *O. sativa*, and *Z. mays* revealed that PAL sequences had been moderately conserved through the evolution. To investigate the evolutionary relationships among the isolated *PAL* genes and *PAL* from other plant species, the phylogenetic tree based on nucleotide sequences of the *PAL* was constructed (Fig. 4B). It was obvious that the phylogenetic tree was grouped in a family-specific manner. For example, three members of Poaceae *PAL* from *Saccharum officinarum*, *O. sativa*, and *P. virgatum* had a closer relationship than others. In addition, it was noticeable that *S. mirzayanii* and *S. macrosiphon* were clustered with two other *Salvia* species, *S. hypoleuca* and *S. virgata*. According to the traditional taxonomy and based on stamens characters (Jamzad, 2012), these species belong to the same group, and so the *PAL* of these plants cluster together, which means that they shared higher similarity than other plants at the molecular levels. Finally, based on the structural similarity and sequence characteristics including amino acid homologies and conserved motifs, it has been proposed that the genes encoding *Salvia PAL* and *PAL* from other plants diverged from a common ancestral gene.

***PAL expression, enzyme activity and total phenolic content under salinity stress***

Since the various classes of compounds such as phenolics, which are produced by the activity of the phenylpropanoids pathway, are important in many different biological situations including stresses, the regulation of phenylpropanoids metabolism at all cellular levels must be sensitive and specific. PAL, as a bridge between primary metabolism and natural product biosynthesis, is a potential site for phenylpropanoid pathway regulation (Bate et al., 1994). Therefore, the investigation on the characteristics as well as gene expression patterns of this crucial enzyme in the production of phenylpropanoid compounds in order to have a better understanding of defense mechanisms towards various stresses in medicinal plants appears significantly useful. In the present study, besides the partial isolation of coding sequences of *PAL1* in two species of *Salvia*, the effects of salinity stress on expression profiles of *PAL1*, enzyme activity and total phenolic content were investigated. The RT-PCR was used to determine *PAL1* gene expression during salinity stress. As shown in Fig. 5A, *PAL1* transcripts accumulation strongly increased in both species and reached the highest level 48 h after 100 mM NaCl treatment. These transcript accumulations were shown to be 18 times and 12 times higher than control plants in *S. mirzayanii* and *S. macrosiphon* respectively. Although the increase in *PAL1* transcript of *S. mirzayanii* was initiated strongly compared with *S. macrosiphon*, it must be noted that these transcript accumulations approximately reached the constant level between 24 h and 48 h in *S. mirzayanii* (Fig. 5A). PAL enzyme activity and total phenolic content were determined in the leaves of plants at 5 time points after initiation of salinity treatment similar to the selected time points for *PAL* expression investigations. Results showed that PAL activity was increased by 2.12 and 1.5 times 48 h after NaCl treatments in *S. mirzayanii* and *S. macrosiphon* respectively as compared to control plants (Fig. 5B). As shown in Fig. 5, the increase in PAL activity has been associated with the increase in total phenolic compound accumulations in these two *Salvia* species. These increases were shown to be 43% and 35% at 48 h after salinity treatment in *S. mirzayanii* and *S. macrosiphon* respectively as compared with the control plants. These results are similar to those observed in leaves of *Saccharum officinarum* (Hashemitabar et al., 2014), *Vitis vinifera* (Mohammadkhani et al., 2013), and *Hibiscus cannabinus* (Jeong et al., 2012) under salinity stress. Previous results have shown that within three hours of the initiation of an incompatible interaction between plants and stresses, the increased activity of the phenylpropanoids pathway is often observed. Enzymes activities are induced and in fact the genes encoding those enzymes are often transcriptionally activated, resulting in the formation of more enzymes and thus of newly synthesized phenolic compounds to relieve the stresses effects (Molitor, 1995). Since PAL acts as the gateway from primary to phenylpropanoids secondary metabolism, these findings confirm that PAL is a marker of stress conditions in plant species (Pinto et al., 2003) and it can be a good candidate for the technology of genetic engineering of crops towards salt tolerance. In the present study, three responses of *Salvia* species including *PAL* mRNA levels, PAL activity and total phenolic content were not completely coordinated under salinity stress (Fig. 5A and B; Fig. 6). For example, the *PAL1* transcripts accumulated 48 h after NaCl treatment were shown to be 10 times higher as compared with the control plants. However, there was a 3 fold increase in PAL activities and phenolic content accumulation in two *Salvia* species as compared to control plants. Although the regulatory mechanisms by which PAL is differentially controlled at transcriptional or functional levels remains unknown, it has been found that various biochemical and molecular effectors change the levels of enzyme transcription or activity (Chen et al., 2005). One of the major reasons, which might describe the difference between the *PAL* mRNA level and enzyme activity in some tissue could be the regulation of enzyme activity by allosteric regulators, which altered enzyme activity in spite of stable gene expression. It has been shown that trans-cinamic acid, as a product of PAL, lowers or inhibits the PAL activity at high concentration (Chen et al., 2005). Trans-cinamic acid is much more removed by the downstream pathways to synthesize other metabolites in the growing tissues in this development stage such as leaves and its lower concentration of trans-cinamic acid in the leaves and sheaths might cause lower PAL enzyme activity in these tissues without any changes in the quantity of its transcripts (Chen et al., 2005).

***Prediction of several hormones and environment related cis-elements on Salvia PAL promoter sequences***

Promoter analysis of the critical genes will help in the elucidation of the molecular mechanisms involved in the plants stress responses (Moghadam et al., 2012, 2013). The structures of the *PAL* promoters have been studied in several species and in certain cases; functions have been assigned to the identified sequence motifs (Molitor, 1995). In the present study, the pairwise comparison test between *PAL* promoters of *M. guttatus*, *Z. mays* and *A. thaliana*, detected common and different *cis*-acting elements within promoters of these plants. As shown in Table 3, there is the highest significant similarity in the number of occurrences of *cis*-acting elements between *M. guttatus* vs *A. thaliana*, *A. thaliana vs Z. mays* and *M. guttatus vs Z. mays* respectively. Therefore, these results showed that several hormones and environment-related *cis*-elements which were common between *M. guttatus* (a likelihood organism with *Salvia* species) and *A. thaliana* and were significantly over-represented in their *PAL* promoters could be predicted in *Salvia PAL* promoter sequ-

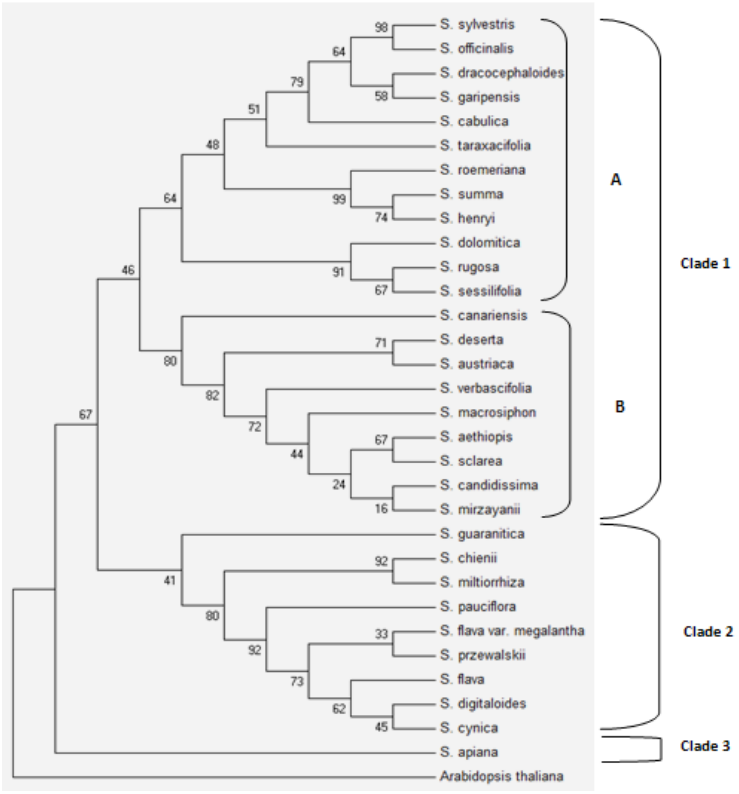


**Fig 3.** Maximum parsimony tree based on *18S rRNA* sequences. Number next to the branches is bootstrap values from Neighbor-joining (NJ) analysis; different clades are given on the right. Accession numbers: *Salvia x sylvestris* (KC473278.1); *Salvia officinalis* (DQ667225.1); *Salvia dracocephaloides* (DQ667265.1); *Salvia garipensis* (DQ667281.1); *Salvia cabulica* (DQ667287.1); *Salvia taraxacifolia* (DQ667209.1); *Salvia roemeriana* (DQ667211.1); *Salvia summa* (DQ667217.1); *Salvia henryi* (DQ667216.1); *Salvia dolomitica* (DQ667322.1); *Salvia rugosa* (DQ667290.1); *Salvia sessilifolia* (DQ667282.1); *Salvia canariensis* (DQ667256.1); *Salvia deserta* (DQ132865.1); *Salvia austriaca* (DQ667323.1); *Salvia verbascifolia* (DQ667264.1); *Salvia macrosiphon* (KM360154); *Salvia aethiopis* (DQ667272.1); *Salvia sclarea* (DQ667222.1); *Salvia candidissima* (DQ667261.1); *Salvia mirzayanii* (KM360153); *Salvia guaranitica* (KC473237.1); *Salvia chienii* (DQ132868.1); *Salvia miltiorrhiza* (EF373610.1); *Salvia pauciflora* (KC473253.1); *Salvia flava var. megalantha* (KC473236.1 ); *Salvia przewalskii* (KJ397260.1); *Salvia flava* (EF373624.1); *Salvia digitaloides* (KJ397259.1); *Salvia cynica* (EF373640.1); *Salvia apiana* (DQ667214.1); *Arabidopsis thaliana* (X16077.1).

ences (Table 2 and 3). Among these elements, it was found that the ABRE elements were involved in the responses to abscisic acid (ABA); TCA-elements in salicylic (SA) acid responses; the TC-rich repeat bases in defense and stress responses; ARE in anaerobic induction; HSE in heat stress responses; GA-motife in light responses; CGTCA and TGACG motifs in the MeJA-responses and WUN-motif in wound responses. In agreement with these findings, many researchers have shown the existence of TC-rich repeats (Zhao et al., 2012; Jiang et al., 2013), CAAT box, G box, CGTCA motif, TCA-element (Jiang et al., 2013) in *PAL* promoter coincide with stress hormones involved in environmental stress responses. Jiang et al. (2013) demonstrated that the presence of above *cis*-acting elements in *LrPAL2* caused a drastic response to MeJA and UV, moderate response to gibberellin (GA) and SA, and a slight increased response under wounding stress in *Lycoris radiate*. The highest transcript level of PAL ortholog was observed at an early time point (1 or 6 h) after treatments with wound (1 h), $H_2O_2$ (6 h) and SA (6 h), while the highest transcript level was detected at the late time point (24 h) after treatments with NaCl, cold and ABA (Jeong et al., 2012). These findings confirmed the impact of stress hormones such as ABA, SA, and JA on *PAL cis*-acting elements resulting in the increase in *PAL* transcript and activity. Previous studies have also shown that stress responses of plants are often mediated by phytohormones such as ABA, SA, GA, ethylene (ET) and jasmonic acid (JA) (Zhu et al., 2013; Moghadam et al., 2013). It is believed that crosstalk between stress adaptation/response and hormone regulation pathways exists, although the hormones may participate in stress reaction independently, synergistically or antagonistically (Zhu et al., 2013; Moghadam et al., 2013). Finally, the presence of these regulatory elements suggests that the promoter region of *Salvia PAL* might respond to several types of stresses, especially abiotic stresses, via a hormonal complex mechanism and might demonstrate its vital role in abiotic and biotic tolerance, especially in the early hours after stresses (Moghadam et al., 2012, 2013).

## Materials and Methods

### *Plant materials*

Seeds of *S. mirzayanii* and *S. macrosiphon* were provided by Research Center for Agriculture and Natural Resources, Shiraz, Iran. The seed collection sites of these two species are shown in the schematic map (Fig. 1). To break the seeds dormancy, they were soaked in distilled water for 10 min and then placed on water-soaked sterile filter papers and kept in refrigerator (4˚C) for 7 days. The germinated seeds were transplanted into plastic pots filled with prewashed sands and

Figure 4A

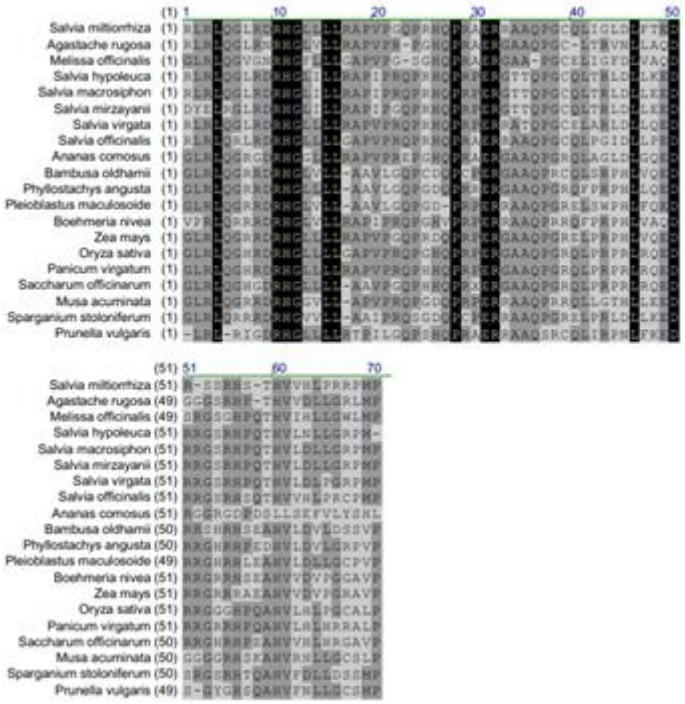


Figure 4B

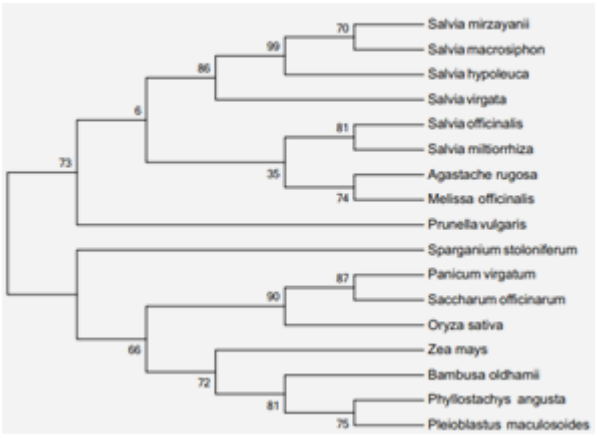


**Fig 4.** Amino acid sequence alignment (A) and phylogenetic analysis (B) of *Salvia* PAL orthologues. Sequence alignment and amino acids conservation profile for PALs orthologues were generated by ClustalW and vector NTI suit 10 software. The *PAL1* genes sequences showed highly conservative nucleotides, suggesting a key role for this protein in various plants. Constructions of the phylogenetic tree based on nucleotide sequence for *PAL* genes were carried out by MEGA4 software. Accession numbers for A and B: *Salvia miltiorrhiza* (DQ408636.1); *Agastache rugosa* (AF326116.1|AF326116); *Melissa officinalis* (FN665700.1); *Salvia hypoleuca* (KC182571.1); *Salvia virgata* (KC182572.1); *Salvia officinalis* (KC182570.1); *S. mirzayanii* (KP336898); *S. macrosiphon* (KP336899); *Ananas comosus* (AY098511.1); *Bambusa oldhamii* (HM009319.1); *Phyllostachys edulis* (HQ831347.1); *Pleioblastus maculosoide* (FJ596642.1); *Boehmeria nivea* (JX681144.1); *Zea mays* (NM_001153961.1); *Oryza sativa* (AY224546.1); *Panicum virgatum* (JX845715.1); *Saccharum officinarum* (EF189195.1); *Musa acuminata* (KF582546.1); *Sparganium stoloniferum* (KF633470.1); *Prunella vulgaris* (KJ010815.1).

humus (1:2) and kept in the greenhouse with the mean day/night temperature and relative humidity of 29±4°C, 38±5% and 17±2°C, 50±5% respectively and photoperiod of 16 h light/8 h dark.

***Stress treatment***

Twelve-week old seedlings with four nodes and eight opposite leaves were subjected to salinity stress (Fig. 2). Based on previous work on the effect of salinity stress on *S. mirzayanii*, an appropriate NaCl concentration was selected for salinity treatments (Valifard et al., 2014). The seedlings were irrigated with a solution of 100 mM NaCl. For this purposes, 17.53 g NaCl was added to 3 kg soil in each pot, except to control. In order to prevent osmotic shock, salt solutions were added gradually at two stages. In each stage, 8.8 g NaCl was dissolved in 310 ml (the amount of water held in pots at field capacity) distilled water, and was added

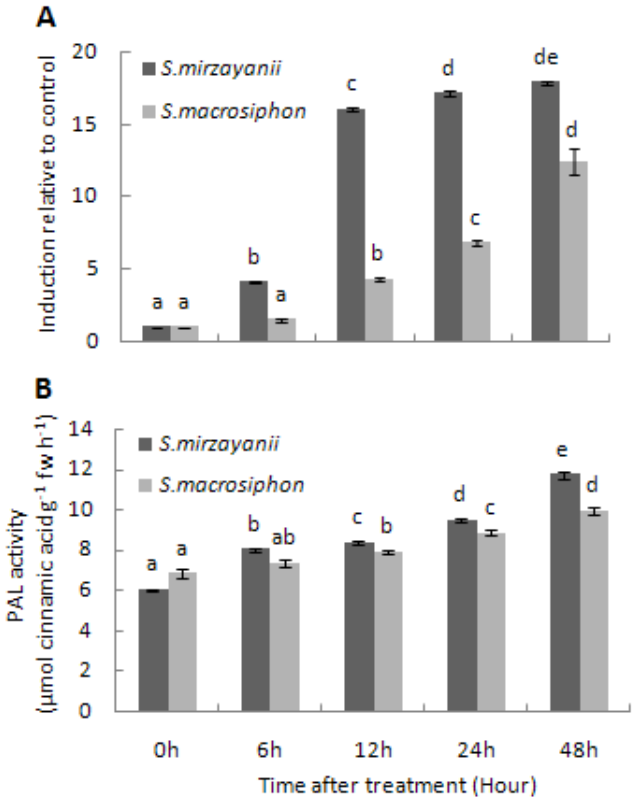


**Fig 5.** Real-time RT-PCR analysis of *PAL1* expression in leaves of *S. mirzayanii* (black columns) and *S. macrosiphon* (gray columns) treated with 100 mM NaCl salinity. The *Salvia 18S RNA* gene was used as an internal control. The control sample was normalized to 1. Data are the means±standard deviation (SD) from three independent experiments (**A**). PAL activity in leaves of *S. mirzayanii* (black columns) and *S. macrosiphon* (gray columns) after 100 mM NaCl treatment. Data are the means±SD from three independent experiments (**B**). In (**A**) and (**B**) statistical significances according to one way-ANOVA, Duncan test (P≤0.05) were performed.

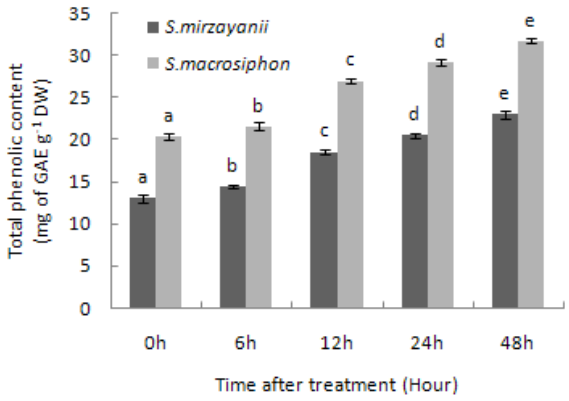


**Fig 6.** Total phenolic content in leaves of *S. mirzayanii* (black columns) and *S. macrosiphon* (gray columns) treated with 100 mM NaCl salinity. Data are the means±SD from three independent experiments. Statistical significances according to one way-ANOVA, Duncan test (P≤0.05) were performed.

to the pots. Control plants were irrigated with 310 ml distilled water at each stage. At the end of salt treatments, total soils electrical conductivities including control, were determined by EC meter (*Metrohm 344, Switzerland*). The values were as follows: 0.40 dS $m^{-1}$ for control soil and 9.1 dS $m^{-1}$ for salt treated soils. Leaves tissues were harvested at 0, 6, 12, 24 and 48 h after the end of treatments. Harvested leaves were frozen immediately in liquid nitrogen and stored in a -80˚C freezer until used. For each time point, three independent replicates of seedlings samples were collected. Plants irrigated with distilled water were also sampled at each time point as the respective control.

***Determination of phenylalanine ammonia-lyase activity***

For enzyme extraction, 2 g frozen fresh leaves sample was homogenized with 2.5 ml solution containing 100 mmol $L^{-1}$ $K_3PO_4$ buffer, pH 7.0, 2 mmol $L^{-1}$ of EDTA, 1% (m/v) PVP, and 1 mmol $L^{-1}$ phenyl- methylsulfonyl fluoride (PMSF). The homogenates were centrifuged at 12,000 g for 15 min at 4°C and the supernatant fractions were used for enzyme analysis. PAL activity was determined according to method described by Zheng et al. (2005). The reaction mixture contained 100 mmol $L^{-1}$ Tris-HCl buffer, pH 8.5, 1 mmol $L^{-1}$ 2-

mercaptoethanol, 15 mmol $L^{-1}$ L-phenylalanine, and 100 µl enzyme extract. The reaction mixture was incubated at 30°C for 15 min, and was terminated by the addition 6 mol $L^{-1}$ HCl and then its absorbance was measured at 290 nm by spectrophotometer (*Shimadzu UV-Vis spectrophotometer,* Japan). The amount of trans-cinnamic acid formed was evaluated by comparison with a standard curve and expressed as µmol cinnamic acid $h^{-1}g^{-1}$ dry weight (DW).

***Total phenolics determination***

Leaf extracts were prepared by stirring 1 g of frozen leaves tissues with 10 ml pure methanol. The extracts were then kept for 24 h at 4˚C, filtered through a Whatman no. 4 filter paper and stored at 4˚C until analysis (Ksouri et al., 2007). Total phenolic content was determined by Folin–Ciocalteu method (Singleton and Rossi, 1965). Two hundred microliters of 2-fold diluted (methanol) samples of the extract were added to 1.5 ml of 1:10 diluted Folin–Ciocalteu reagent in the test tubes. The mixture was shaken and allowed to stand for 6 min and then 1.5 µl of 7% $Na_2CO_3$ solution was added. After incubation for 90 min at 23˚C, the solutions absorbance was determined at 760 nm by spectrophotometer (*Shimadzu UV-Vis spectrophotometer,* Japan). All samples were prepared in triplicate. A mixture of 7% $Na_2CO_3$ solution, 1:10 diluted Folin–Ciocalteu reagent and the extracting solution instead of plant extract were used as blank. Solutions of gallic acid (0–500 µg $mL^{-1}$) was used to prepare the standard curves ($R^2$ = 0.997). Results were expressed as milligram gallic acid equivalent (mg GAE)/g leaf dry weight.

***Isolation of partial sequences of PAL1 and 18S rRNA***

Genomic DNA was extracted from frozen leaves by DENAzist Genomic DNA isolation kit-1 (*DENAzist*, Mashhad, Iran) according to manufacturer's instructions. The available coding sequences of *18S rRNA* and *PAL1* in GenBank for *Salvia* species were used to design the primers for isolation using Vector NTI Suite 10. Table 1 shows the sequences of primers used in this study synthesized by BIONEER, Seoul, South Korea. Amplicons of *18S rRNA* (720 bp) for isolation and *PAL1* (320 bp), and *18S rRNA* (110 bp) for RT-PCR were obtained by performing PCR on genomic DNA using 10 pmol of gene-specific primers. In the present study, the *18s rRNA* genes (720 bp) were used to investigate the phylogenetic status of *S. mirzayanii* and *S. macrosiphon* among other *Salvia* species published in the GenBank database. Also, the *18S rRNA* genes (110 bp) were used as reference genes to normalize the cDNA concentration among all samples for RT-PCR. Annealing temperature for *PAL1* and *18S rRNA* was 65 and 53˚C respectively. Isolated fragments of *PAL1* and *18S rRNA* were sequenced using M13 forward/reverse primes with ABI 3730× 1 DNA Sequencer of Macrogen Inc (Korea). All sequences obtained in this study were submitted to the NCBI database.

***Total RNA extraction, cDNA synthesis and quantitative real-time PCR***

Total RNA from frozen leaves tissues (100 mg) for each plant sample was extracted using DENAzist column RNA isolation kit-1 (*DENAzist, Mashhad, Iran*). The concentration and integrity of the total RNA were verified with nanodrop set (*Thermo Fisher Scientific, USA*) and agarose gel electrophoresis. Total RNA were treated with 1 µL DNase I (*RNase-free DNase I, Fermentas*) to remove residual genomic DNA according to the manufacturer's instructions. First-strand cDNA was synthesized from 1µg of purified RNA using a first-strand cDNA synthesis kit (Fermentas) based on the manufacturer's instructions. *18S rRNA* gene was used as a housekeeping gene and reference to normalize the cDNA concentration among all samples. Quantitative real-time PCR was performed by the Real-Time PCR System model Bioer-Analytic Jena 48. Real time PCR reactions were performed in a final volume of 20 µL containing 5 µL diluted cDNA, 0.5 µL of each gene specific primer, 10 µL SYBR Premix Ex Tag II (Tli RNaseH Plus) (TaKaRa, Dalian, China), and 4 µl DNase free water. Reactions with DNase free water (5 µL) instead of diluted cDNA were performed in parallel as negative controls. Each sample was run in duplicate with the following settings: 2 min pre-denaturation at 95ºC, followed by 40 cycles at 95ºC for 10 sec, annealing for 15 sec and 72ºC 15 sec. In order to verify the specificity of the amplified product, melting curve was analyzed at the end of the reaction. Threshold values were calculated by the internal software of system according to the method of $2^{-\Delta\Delta Ct}$.

***PAL promoter analysis***

Promoter analysis was performed to identify the environment related *cis*-elements, which have a critical role in the *PAL* expression under abiotic stress. To achieve this, three different plants, *Arabidopsis thaliana* as a dicotyledonous model organism, *Zea mays* as a monocotyledonous plant and *Mimulus guttatus* (Lamiales) as a likelihood organism with *Salvia* species were selected. The promoter region of *PAL* in these plants was compared against the genomic data of *S. mirzayanii* and *S. macrosiphon* by using the phytozome database (http://www.phytozome.net/). After identifying the genes on the chromosome using the BLAST-N algorithm, the region 2000 bp upstream of the transcriptional start point (ATG) of *M. guttatus, Z. mays* and *A. thaliana PAL* was considered as the promoter. Then, the upstream region of the *PAL* encoding a genomic fragment was analyzed using the PLANTCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/) database. For this purpose, upstream region sequences of *PAL* were applied to predict their key *cis*-acting elements and their precise location. The PLANTCARE promoter database focuses on *cis*-regulatory elements rather than on core promoter structure, aiming to reveal the regulatory mechanism that underlies the expression profiles (Lescot et al., 2002). While there are several studies indicating that the number of occurrences of *cis*-acting elements has a significant effect on the expression pattern of the target gene, the lack of powerful and reliable statistical method to compare *cis*-acting elements occurrences among promoters is a major drawback in this area of computational biology. Therefore, in the present study a pairwise statistical method was used for comparative promoter analysis. Comparative promoter analysis is a promising strategy for the detection of common and different *cis*-acting elements within promoters of the genes with similar or different expression profiles (Shamloo-Dashtpagerdi et al., 2015).

***Molecular evolution analyses***

Sequences obtained were analyzed using online bioinformatics tools from NCBI. The software Vector NTI Advance 10 was used for sequence multi-alignment. The isolated *PAL1* and *18S rRNA* and other known *PAL* and *18S rRNA* sequences retrieved from GenBank database were aligned with Clustral W. Subsequently, a phylogenetic tree

was constructed using neighbour-joining (NJ) method with MEGA 4 software (Tamura et al., 2007; Thompson et al., 1997). The reliability of the tree was confirmed by bootstrap analysis with 1,000 replicates (Felsenstein, 1985).

***Statistical analysis***

Three replications were used for each treatment and all data were expressed as means ± SE. The statistical analysis was performed using SPSS 20 software and the means were compared by using the one-way and multivariate analysis of variances (ANOVA) followed by Duncan's multiple range tests. The differences between individual means were deemed to be significant at $P \leq 0.05$. Excel 2013 program and Sigma plot 12 software were used to prepare the standard curves and graphs.

**Conclusion**

In order to meet the ever increasing demand of medicinal plants for traditional medicine as well as for the pharmaceutical industry, both traditional breeding and genetic engineering of medicinal plants could be utilized to improve stress tolerance with the goal of increasing productivity, antioxidant activity and valuable pharmaceutical properties. In the present study, *PAL1* from two *Salvia* species, which have many pharmaceutical properties, were isolated and characterized and their expression profiles, enzyme activities and total phenolic content under salinity stress were analyzed. Our results showed that there are significant positive correlations between gene expression induction, enzyme activities, and total phenolic content in *Salvia* species. Several hormone and environment-related *cis*-elements including ABRE elements, TCA-elements, TC-rich repeat, ARE, HSE, GA-motife, CGTCA and TGACG motifs, WUN-motif could be predicted on *Salvia PAL* promoter which respond to stress hormones and might show its vital role in the biotic and abiotic tolerance as an early-stress responsive gene. These results should be taken into consideration in the economic cultivation, breeding and genetic engineering of these valuable medicinal plants.

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