Differential inductions of phenylalanine ammonia-lyase and chalcone synthase during wounding, salicylic acid treatment, and salinity stress in safflower, *Carthamus tinctorius*

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**Synopsis**

Safflower (*Carthamus tinctorius* L.) serves as a reference dicot for investigation of defence mechanisms in Asteraceae due to abundant secondary metabolites and high resistance/tolerance to environmental stresses. In plants, phenylpropanoid and flavonoid pathways are considered as two central defence signalling cascades in stress conditions. Here, we describe the isolation of two major genes in these pathways, *CtPAL* (phenylalanine ammonia-lyase) and *CtCHS* (chalcone synthase) in safflower along with monitoring their expression profiles in different stress circumstances. The aa (amino acid) sequence of isolated region of *CtPAL* possesses the maximum identity up to 96% to its orthologue in *Cynara scolymus*, while that of *CtCHS* retains the highest identity to its orthologue in *Callistephus chinensis* up to 96%. Experiments for gene expression profiling of *CtPAL* and *CtCHS* were performed after the treatment of seedlings with 0.1 and 1 mM SA (salicylic acid), wounding and salinity stress. The results of semi-quantitative RT–PCR revealed that both *CtPAL* and *CtCHS* genes are further responsive to higher concentration of SA with dissimilar patterns. Regarding wounding stress, *CtPAL* gets slightly induced upon injury at 3 hat (hours after treatment), whereas *CtCHS* gets greatly induced at 3 hat and levels off gradually afterward. Upon salinity stress, *CtPAL* displays a similar expression pattern by getting slightly induced at 3 hat, but *CtCHS* exhibits a biphasic expression profile with two prominent peaks at 3 and 24 hat. These results substantiate the involvement of phenylpropanoid and particularly flavonoid pathways in safflower during wounding and especially salinity stress.

**Key words:** defence response, safflower, salicylic acid (SA), salinity, semi-quantitative RT–PCR, wounding

**INTRODUCTION**

As ground-anchored sessile organisms, plants have evolved diverse adaptive and defence mechanisms in order to survive in threatening environmental conditions. Growth-limiting factors including drought, salinity, cold, UV rays as well as pathogenic micro-organisms, e.g. fungi, bacteria, viruses, etc. can jeopardize the plant life if not negated by plant protective responses. In breeding programmes, identification of protecting factors in plants against challenging factors is a prerequisite. In this context, keeping our efforts in identification and characterization of

**Abbreviations:** 4CL, 4-Coumarate:CoA ligase; aa, amino acid; C4H, cinnamate 4-hydroxylase; CHS, chalcone synthase; hat, hours after treatment; PAL, phenylalanine ammonia-lyase; SA, salicylic acid.

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involved genes in plant responses to biotic and abiotic stresses [1–4], we report in this study the isolation as well as functional characterization of two genes in phenylpropanoid and flavonoid pathways, i.e. PAL (phenylalanine ammonia-lyase) and CHS (chalcone synthase) in safflower (Carthamus tinctorius) during salinity stress, wounding and SA (salicylic acid) treatment as an inducer of acquired resistance and PR genes expression [5].

We have been recently working on safflower [4] given that this industrial medicinal oil-seed plant has a rich germplasm collection in Iran and shows high levels of tolerance/resistance to environmental stresses. Safflower is a long-day, herbaceous, annual, self-compatible member of Asteraceae family and Carthamus genus. Having a well-developed root system, safflower is an ideal plant in arid and semi-arid climates [6,7]. Iran is one of the richest countries regarding safflower germplasms including domestic and wild species [8]. A variety of abiotic/biotic stresses challenges the safflower namely high-temperature, high relative humidity, long rainfalls, drought, cold and salinity as well as many fungal and a few bacterial and viral pathogens [9]. However, owing to high tolerance/resistance of safflower to environmental stresses, this plant might be considered as a reference plant for studying the defence mechanisms. Plant responses to environmental stimuli are governed by a complicated multi-player crosstalk among different defence pathways. In higher plants, phenylpropanoid biosynthetic pathway produces the important metabolites, e.g. flavonoids, isoflavonoids, lignin, anthocyanin, phytoalexins, antimicrobial furanocoumarins, hydroxyl cinnamate esters and phenolic esters, which are all critical players in development, structural protection, defence responses to microbial attacks and tolerance to abiotic stimuli [10,11]. As phenylpropanoid pathway is a gateway for production of many secondary metabolites [12,13], the investigation of characteristics as well as expression patterns of involved genes in production of these metabolites, e.g. PAL and CHS, for a better understanding of defence mechanisms towards various stresses appears significantly useful. PAL is the initial enzyme in phenylpropanoid pathway and the key participant in the lignification process [12], which converts the phenylalanine to trans-cinnamic acid via non-oxidative removal of ammonia as depicted in Figure 1. PAL is a critical enzyme for plant responses to environmental stresses as if its de novo synthesis is activated following pathogen attack, wounding, UV irradiation, as well as iron and phosphate depletion [19]. It is, also, responsive to phytohormones ethylene, jasmonic acid, SA and methyl jasmonate [20–24]. CHS is another important enzyme in phenylpropanoid cascade and the key enzyme in flavonoid biosynthesis (Figure 1). Flavonoids are the major groups of plant secondary metabolites with essential roles in physiological processes. Flavonoids have not only been considered for their significance in plants nutritional value [25], but are also important in terms of plant protection against UV rays, pathogen attacks and herbivores [26–29].

Since only one gene, i.e. C4H (cinnamate 4-hydroxylase), of phenylpropanoid pathway in safflower has been isolated and characterized so far [4], in this study the coding sequences of safflower PAL (CtPAL) and CHS (CtCHS), which are typically encoded by small multi-gene families, have been partially isolated and their expression profiles during SA treatment, wounding and salinity stress were monitored in order to further dissect the high levels
of resistance/tolerance of safflower to different environmental stresses.

**MATERIALS AND METHODS**

**Plant material and growth condition**

Seeds of safflower var. 22-191 (kindly provided by Dr Maham-madinejad, Department of Agronomy, Shahid Bahonar University of Kerman, Iran) were sterilized with 70% (v/v) ethanol and sodium hypochlorite [5% (w/v) active chlorine] for 2 and 15 min, respectively. Having vernalized at 4 °C for 2 h, seeds were sown on water-soaked sterile filter papers. The germinated seeds were transplanted into 15-cm-diameter pots filled with prewashed sand and kept in the greenhouse at 26 ± 2 °C and photoperiod of 16 h with every other 2 days irrigation regime. Fertilization by Hoag-1213ity of Kerman, Iran) were sterilized with 70% (v/v) ethanol and

**Isolation of partial sequences of CtPAL and CtCHS genes**

Isolation of genomic DNA from leaves was carried out after Saghai-Marof et al. [30]. The available coding sequences of PAL orthologues in members of Asteraceae family, i.e. Helianthus annuus, Rudbeckia hirta, Cynara scolymus and Gynara bicolor, were used to design the isolating primer pair for CtPAL. Likewise, the coding sequences of CHS genes in R. hirta, Lactuca sativa, G. bicolor and Sillybum marianum were considered to design the isolating primers for CtCHS. Table 1 shows the sequences of primers used in this study, which were synthesized by Eurofin MWG Operon (Germany). Amplicons of CtPAL (872 bp) and CtCHS (559 bp) were obtained by performing PCR on genomic DNA using 1 pmol of gene-specific primer pairs. Temperatures of annealing for CtPAL and CtCHS were 51 and 56 °C, respectively.

**Cloning of CtPAL and CtCHS amplicons into sequencing vector**

To clone the amplicons of CtPAL and CtCHS into pTZ57R/T vector, InsTAclone™ PCR Cloning Kit (Thermo SCIENTIFIC, # K1213) and competent cells of Escherichia coli strain JM107 were recruited. In brief, based upon blue/white screening, recombinant colonies were selected for DNA extraction by GF-1 Plasmid DNA Extraction Kit (Vivantis). Sequences of isolated region of CtPAL and CtCHS genes were obtained using M13 universal primers (Faza Pajooh Biotech). Sequences were certified by means of Chromas Lite 2.01 (Technelysium) after clipping the vector sequence.

**Conserved domains, homology and phylogenetic analyses**

Bioinformatics analysis of CtPAL and CtCHS aa (amino acid) sequences were performed in conserved domain platform [31,32] at http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml. The aa sequences of CtPAL and CtCHS were analysed for homology using ClustalW [33]. Constructions of phylogenetic tree based on nucleotide sequence for CtPAL and CtCHS genes were carried out using Phylogeny.fr program [34–36]. Briefly, sequences were aligned with the highest accuracy by MUSCLE [37]. Phylogenetic trees were constructed based upon the maximum likelihood approach executed in PhyML 3.0 software [38,39]. Graphical demonstration of trees was completed by TreeDyn [40].

**Gene expression analyses**

Wounding, salinity and SA treatments were all performed on 14-day-old seedlings. For wounding, leaves were comparably equally pressed with sterile blunt-nosed thumb forceps. For salinity, seedlings were drenched with 150 mM sodium chloride solution. For SA treatment, two experimental groups of 0.1 and 1 mM SA were considered. SA solutions were applied on leaves using sprayer. Following each treatment, samplings were done in a time course, i.e. 0, 3, 6, 12, 24 and 48 hat (hours after treatment). Taken into account the potential diurnal rhythm in the gene expression patterns, all treatments were started at 8 am.

**RNA extraction and cDNA synthesis**

RNAs were extracted by means of RNX™ Plus Kit (Cinnagen) from the treated seedlings according to manufacturer’s instructions. Next to DNasel treatment of RNA samples, 1 μg of RNAs, using RevertAid First Strand cDNA Synthesis Kit (Thermo SCIENTIFIC, # K1691), was reverse transcribed to corresponding
Sequence alignment and aa conservation profile for PAL orthologues were generated by ClustalW. Constructions of phylogenetic tree based on nucleotide sequence for PAL gene was carried out by Phylogeny.fr program. In brief, sequences were aligned with the highest accuracy by MUSCLE. Phylogenetic tree was constructed based upon the maximum likelihood approach executed in PhyML 3.0 software. Graphical demonstration of tree was completed by TreeDyn. Accession numbers for (A): Carthamus tinctorius (AFK25796); Cynara cardunculus (CAL91171); Lactuca sativa (AAL55242); Chrysanthemum boreale (AGU91428); Rudbeckia hirta (ABN79671); Gynura bicolor (BAJ17655); Helianthus annuus (CAAT3065); Ageratina adenophora (ACT53399); Platycodon grandiflorus (AEM63670); Lonicera japonica (AGE10589); Angelica gigas (AEAL72280).

Figure 2  Amino acid sequence alignment (A) and phylogenetic analysis (B) of CtPAL orthologues

Sequence alignment and aa conservation profile for PAL orthologues were generated by ClustalW. Constructions of phylogenetic tree based on nucleotide sequence for PAL gene was carried out by Phylogeny.fr program. In brief, sequences were aligned with the highest accuracy by MUSCLE. Phylogenetic tree was constructed based upon the maximum likelihood approach executed in PhyML 3.0 software. Graphical demonstration of tree was completed by TreeDyn. Accession numbers for (A): Carthamus tinctorius (AFK25796); Cynara cardunculus (CAL91171); Lactuca sativa (AAL55242); Chrysanthemum boreale (AGU91428); Rudbeckia hirta (ABN79671); Gynura bicolor (BAJ17655); Helianthus annuus (CAAT3065); Ageratina adenophora (ACT53399); Platycodon grandiflorus (AEM63670); Lonicera japonica (AGE10589); Angelica gigas (AEAL72280).
cDNAs, which were later used as templates for semi-quantitative RT–PCR.

**Semi-quantitative RT–PCR**

To normalize the cDNA amounts of different time points in each treatment, we considered the PCR product intensity of 18S rRNA as the house-keeping gene. The primer pairs for CtPAL and CtCHS are given in Table 1. The PCR thermal profile was: 98 °C (5 min) followed by 35 cycles of 98 °C (10 s), 52 °C (15 s) and 72 °C (1 min), and a final extension time at 72 °C for 10 min. An independent experiment was carried out to verify the linear amplification in such setting. The interpretation was based on the intensity of PCR products, corresponding to gene transcription levels.

**RESULTS AND DISCUSSION**

In this study, besides the partial isolation of coding sequences of PAL (CtPAL) and CHS (CtCHS) in safflower, the consequences of salinity stress, wounding, as well as SA treatment, as an stimulus of plant defence against pathogen attacks, on expression profiles of these genes were investigated. Very little information, at the molecular level, is available in safflower, thereby keeping our work on safflower [4], we focused, in this study, on CtPAL and CtCHS genes, two critical genes in phenylpropanoid and flavonoid pathways (Figure 1). These pathways have been proved to be highly critical in plant protective reactions during biotic and abiotic stresses [2].

**Conserved domains, homology and phylogenetic analyses of CtPAL**

According to the results of conserved domain analysis, the isolated region of safflower PAL, CtPAL, contains the conserved domain of Lyase class I_like superfamily (cl00013) accommodating HAL (histidine ammonia-lyase) and PAL. PAL–HAL conserved domain (cd00332) is present in plants, fungi, several bacteria and animals [41]. Phenylalanine and HALs, which are active as homotetramers [42], catalyse the beta-elimination of ammonia from respective phenylalanine and histidine [43]. Like other homotetrameric enzymes in this family, safflower PAL possesses four active sites, as detected in conserved domain platform. PAL, present in plants and fungi, catalyses the conversion of L-phenylalanine to E-cinnamic acid. The aa sequence of the isolated CtPAL fragment comprising 291 aa (GenBank: AFK25796) was used as an initial query to search, using the protein–protein BLAST tool, against the non-redundant protein sequences. As a result, the isolated region of CtPAL shows the maximum identity up to 96 % to PAL of C. scolymus, followed by lettuce PAL (L. sativa) up to 95 %, G. bicolor and R. hirta up to 94 % and sunflower PAL (H. annuus) and Ageratina adenophora up to 92 %, which are all in Asteraceae family as shown in Figure 2(A). The inferred evolutionary history of PAL nucleotide sequences from several plant species and the corresponding phylogenetic tree bring to light a rather conserved PAL orthologues in Asterids with low genetic distance (0.2) as depicted in Figure 2(B). The coding sequence of CtPAL was deposited in GenBank under the accession number JN998699.

**Conserved domains, homology and phylogenetic analyses of CtCHS**

Bioinformatics analysis of CtCHS aa sequence in conserved domain platform confirmed that the isolated region of CtCHS possesses the CHS_like (cd00831) conserved domain [44] including chalcone and stilbene synthases (Figure 3A). As well, a malonyl-CoA binding site delivering the substrate to the active site cysteine is detected in safflower CHS. In fact, the members of condensing enzymes superfamily (cl09938), which are capable of catalysing a claisen-like condensation reaction, are engaged in metabolism of fatty acids and biosynthesis of natural products polyketides [47,48] suggesting a similar activity for safflower CHS. From the homology point of view, CtCHS (GenBank: AFI57883) retains considerable identities to its orthologues in C. chinensis (96 %), L. sativa, S. marianum, G. bicolor, R. hirta (95 %), Dahlia pinnata (94 %), Chrysanthemum nankinense (93 %) and A. adenophora (92 %). The aa sequences of CHS orthologues in Asteraceae show, as well, a very considerable conservation (Figure 3B), which is rooted from the rather conserved nucleotide sequences of CHS orthologues in this family, forming a distinct branch in corresponding phylogenetic tree (Figure 3B). Likewise, CHS looks highly conserved in members of Brassicaceae family (Rorippa islandica, Cardamine maritime, Barbarea vulgaris, Arabis setosifolia, Brassica oleracea) making a separate branch (Figure 3B). Musa acuminate, Hypericum hookerianum and Zingiber officinale were as well summoned together in a discrete branch to disclose a more conservation in CHS gene in monocots (Figure 3B). CtCHS partial coding sequence was deposited in GenBank with accession number JQ425086.
Figure 3  Amino acid sequence alignment (A) and phylogenetic analysis (B) of CbCHS orthologues

Sequence alignment and aa conservation profile for CHS orthologues were generated by ClustalW. Constructions of phylogenetic tree based on nucleotide sequence for CHS gene was carried out by Phylogeny.fr program. Briefly, sequences were aligned with the highest accuracy by MUSCLE. Phylogenetic tree was constructed based upon the maximum likelihood approach executed in PhyML 3.0 software. Graphical demonstration of tree was completed by TreeDyn. Accession numbers for (A): Carthamus tinctorius (AFI57883); Pericallis cruenta (ACF75870); Silybum marianum (AFK65634); Lactuca sativa (BAJ10380); Gynura bicolor (BAJ17656); Rudbeckia hirta (ABN79673); Dahlia pinnata (BAK08888); Ageratina adenophora (ACQ84148); Vitis vinifera (BAA31259); Litchi chinensis (ADB44077); Lilium speciosum (BAE79201);
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Effects of SA treatment on CtPAL and CtCHS gene expression profiles

Following treatment of safflower with 0.1 mM SA, as a stimulus of plant responses to pathogen attacks [5], CtPAL transcription levels in different time points were monitored. Accordingly, a slight induction of CtPAL gene was observed at 3-6 hat and levelled off thereafter (Figure 4A). On the other hand, for CtCHS gene only at 3 hat, a noticeable induction was observed (Figure 4A). In contrast, treatment of safflower plants with 1 mM SA had a dramatic influence on both genes expression. Indeed, 1 mM SA treatment led to a biphasic induction pattern of CtPAL gene in 3-6 as well as 24 hat, out of which the latter was much stronger, followed by a fast decline in expression (Figure 4B). A slight increase in CtCHS expression was also observed at 24 hat.

We treated the safflower plants with two different concentrations of SA, 0.1 and 1 mM, in order to investigate SA-dependency of CtPAL and CtCHS expressions. Seeing that one of the metabolic pathways for SA biosynthesis is succored by PAL activity, the latter stronger induction in PAL transcription might be related to induction of CtPAL by exogenous SA treatment. Generally, plants respond to environmental stresses, e.g. wounding, pathogen attacks, etc. in three main phases [49,50], i.e. (i) development of a physical barrier in the immediate vicinity of wounding or penetrating micro-organism, (ii) activation of defence genes, transiently, neighbouring the stressed site, and (iii) comparatively late systemic activation of defence genes in a rather long-lasting way, of which the first two are almost concomitant. In other word, biphasic induction of gene activation proposes that those distinct phases might be triggered by distinctive signalling events; a quick initial induction in response to immediate imposed stress and the slow subsequent response to a generated stress signal [49]. This general pattern is also observed in this study for CtPAL and CtCHS in response to 1 mM SA (Figure 4B). A comparable expression pattern for both PAL and CHS in alfalfa cell suspension culture treated with yeast elicitor was also observed as such the CHS expression maximized at 3 hat and continued with half strength till 24 hat, whereas expression of PAL was transient [51]. Similarly, 1 mM SA treatment caused a biphasic induction of C4H in safflower [4] supporting that expressions of PAL and C4H are coordinated in safflower in response to environmental stresses. As observed in the present study, higher concentration of SA has a more drastic effect on responsiveness of CtPAL and CtCHS than lower concentration. This observation substantiates the crucial role of SA in triggering the phenylpropanoid pathway, which per se leads to activation of flavonoid biosynthetic pathway, denoted in induction of respective CtPAL and CtCHS. In fact, elevation of SA level triggers the SAR (systemic acquired resistance), which immunizes the plants towards upcoming pathogen attacks [52].

Effects of wounding stress on CtPAL and CtCHS gene expression profiles

As phenylpropanoid pathway takes clear task in plant responses to wounding [4,52,53], to characterize the engagement of PAL and CHS in safflower response to wounding, their expression patterns were checked in a 48-h time-frame after leaf injury. Consequently, a slight induction of CtPAL was observed at 3 hat, which lasted in a half strength level till 24 h (Figure 5A).
nalling molecule in plant immunity by induction of (Figure 5A) to boost up (i) the biosynthesis of SA as a crucial sig-n...ning occurs, becomes activated soon after injury that the phenylpropanoid pathway in safflower, through which...of secondary metabolites, which contribute to cell wall fortifica-tion (i) is the biosynthesis of SA as a crucial sig-nalling molecule in plant immunity by induction of CIPAL (this...man (Iran) to M.R. M.R. and A.V. acknowledge the Ministry for

Effects of salinity stress on CtPAL and CtCHS gene expression profiles
To our knowledge, there is minute information available, at mo-lecular level, on involvement of phenylpropanoid pathway in plant responses to salinity. We recently reported the engagement of C4H gene in safflower response to salinity stress [4]. To more scrutinize the key players of safflower in this pathway, we monitored the expression profiles of CIPAL and CtCHS genes in saline condition. Consequently, CIPAL expression got slightly induced at 3 h and decreased later (Figure 5B). This pattern has also been observed by Gao et al. [56] in cotyledon, hypocotyls, and rootlets of Jatropha curcas after treatment with 150 mM sodium chloride; however, the highest induction of PAL was detected in roots. Higher induction of PAL may be a defensive reaction to cellular damages due to high salinity level [56]. In inbred lines stressed with salinity, PAL gene expression elevated transi-ently, similar to the antioxidant genes expression patterns in these plants, suggesting a comparable role for PAL in decreasing the oxidative stress imposed by salinity [57]. For CtCHS, a biphasic strong induction pattern at 3 and 24 h after salinity stress was observed (Figure 5B). As discussed earlier, this biphasic pattern in CtCHS expression might reflect the safflower responses to (i) the immediate salinity and (ii) the later produced stress signal, suggesting that CtCHS takes a considerable task in safflower response to salinity. This probably indicates the real involvement of flavonoid defence pathway in salinity stress condition. We could not find any report on involvement of CHS in plant responses to salinity stress; however, this prominent biphasic induction of CtCHS gene expression clearly substantiates a distinctive role for this gene in safflower tolerance to salinity. This hints at the suit-ability of CtCHS gene for recruitment in breeding programmes headed for salinity tolerance in other plants. Overall, in this study, we provide molecular evidence for the involvement of CIPAL and CtCHS genes in safflower responses to abiotic stresses. In particular, CtCHS might be considered as a promising candidate for improvement of salinity tolerance in plant breeding programmes.

AUTHOR CONTRIBUTION
Mohammad Rahnamaeian designed the experiments. Sara Dehghan, Mahnaz Sadeghi, and Anne Poppel performed the experiments; Sara Dehghan and Mohammad Rahnamaeian analysed the data; Mohammad Rahnamaeian, Andreas Vilcinskas, Rainer Fischer, Reinhard Lakes-Harlan and Hamid Reza Kayoussi contributed reagents/materials/analysis tools. Mohammad Rahnamaeian and Sara Dehghan wrote the manuscript.

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