RESEARCH PAPER

Plant Signaling & Behavior 8, e27335; November; © 2013 Landes Bioscience

Isolation and characterization of isochorismate synthase and cinnamate 4-hydroxylase during salinity stress, wounding, and salicylic acid treatment in *Carthamus tinctorius*

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Keywords: Cinnamate 4-hydroxylase, Isochorismate synthase, Safflower, Salicylic acid, Salinity, Wounding

Salicylic acid (SA) is a prominent signaling molecule during biotic and abiotic stresses in plants biosynthesized via cinnamate and isochorismate pathways. Cinnamate 4-hydroxylase (C4H) and isochorismate synthase (ICS) are the main enzymes in phenylpropanoid and isochorismate pathways, respectively. To investigate the actual roles of these genes in resistance mechanism to environmental stresses, here, the coding sequences of these enzymes in safflower (*Carthamus tinctorius*), as an oilseed industrial medicinal plant, were partially isolated and their expression profiles during salinity stress, wounding, and salicylic acid treatment were monitored. As a result, safflower ICS (*Ct*ICS) and C4H (*Ct*C4H) were induced in early time points after wounding (3–6 h). Upon salinity stress, *Ct*ICS and *Ct*C4H were highly expressed for the periods of 6–24 h and 3–6 h after treatment, respectively. It seems evident that ICS expression level is SA concentration dependent as if safflower treatment with 1 mM SA could induce ICS much stronger than that with 0.1 mM, while C4H is less likely to be so. Based on phylogenetic analysis, safflower ICS has maximum similarity to its ortholog in *Vitis vinifera* up to 69%, while C4H shows the highest similarity to its ortholog in *Echinacea angustifolia* up to 96%. Overall, the isolated genes of *Ct*ICS and *Ct*C4H in safflower could be considered in plant breeding programs for salinity tolerance as well as for pathogen resistance.

Introduction

Safflower (*Carthamus tinctorius*) belonging to Asteraceae is mainly cultivated as an oilseed plant; however, its petals and kernels are rich in natural antioxidants.[1] It is evident that safflower extracts of leaves, roots, kernels, and especially petals are able to scavenge the reactive oxygen species (ROS) and contain the active compounds such as carthamin and polyphenols.[2] According to FAO statistics in 2012, the world production of safflower in 2010 reached to more that 6×10^5 tons out of about 8×10^5 hectares.

Plant immune responses are modulated by a fine-tuned crosstalk among the major signaling molecules, i.e., salicylic acid (SA), jasmonic acid (JA), and ethylene (Et).[3] Shikimate pathway, also present in bacteria and fungi, is one of the most crucial pathways for primary and secondary metabolite biosynthesis in plants.[4] This is a part of the biosynthetic pathway of volatile organic compounds and is recognized as the biosynthetic pathway of aromatic compounds.[5] This metabolic pathway converts the carbohydrates, sequentially, to produce the ultimate product, chorismate. Chorismate per se is the last common precursor for phenylalanine, tyrosine, and tryptophan.[4] Aromatic amino acids, consumed during protein synthesis, are precursors for a vast range of secondary metabolites pivotal for plant growth, development, and defense responses.[6,7]

Besides being involved in plant growth and development, photosynthesis, transpiration, etc., salicylic acid is a key signaling molecule in response to environmental stresses. SA biosynthesis in plants occurs via two pathways, i.e., cinnamate (phenylpropanoid) and isochorismate. The cinnamate pathway can be further split into the paths involving benzoic acid[8] and *o*-coumarate as precursors (**Fig. 1**). Genomic studies in *Arabidopsis* have revealed that the major portion of SA synthesis befalls via isochorismate pathway.[6] However, plant species capable of SA biosynthesis via isochorismate pathway are relatively minor compared with the

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Submitted: 10/17/2013; Revised: 11/25/2013; Accepted: 11/25/2013
Citation: Sadeghi M, Dehghan S, Fischer R, Wenzel U, Vilcinskas A, Kavousi HR, Rahnamaeian M. Isolation and characterization of isochorismate synthase and cinnamate 4-hydroxylase during salinity stress, wounding, and salicylic acid treatment in Carthamus tinctorius. Plant Signaling & Behavior 2013; 8:e27335; PMID: 24309561; http://dx.doi.org/10.4161/psb.27335

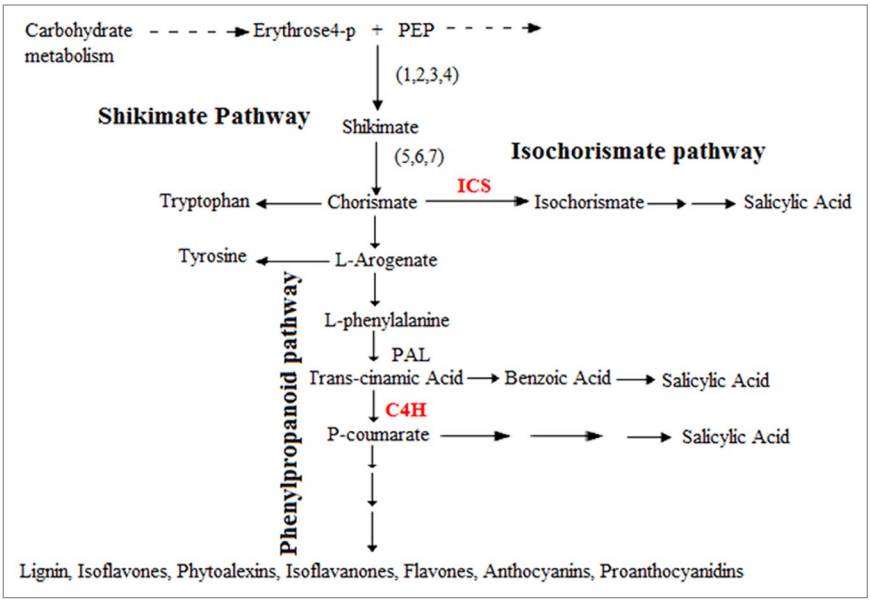

Figure 1. Salicylic acid biosynthesis pathways. Isochorismate synthase (ICS) in isochorismate pathway. The 1–7 in shikimate pathway refers to 7 steps to produce the final product chorismate. Isochorismate synthase (ICS) and cinnamate 4-hydroxylase (C4H) enzymes in respective isochorismate and phenylpropanoid pathways are shown in red. The scheme was adapted after refs 61–64.

species sharing the cinnamate pathway. Isochorismate synthase (ICS) catalyzes the conversion of isochorismate to finally yield SA.[7] Phenylpropanoid pathway is, as well, one of the main plant pathways for secondary metabolites biosynthesis.[6] It is estimated that 20 percent of carbon fixation by photosynthesis is directly contributing to this pathway.[9] The products of this pathway include different classes of flavonoids, e.g., chalcones, flavonones, flavones, flavonoles, anthocyanins, and isoflavonones (**Fig. 1**). Flavonoids are generally biosynthesized in many plants and take diverse tasks as flower pigments, UV protectants, and antimicrobials.[6,9,10] Phenolic compounds are synthesized in the cells in unstressed condition; however a/biotic stresses alter their synthesis rates. In reality, alterations in activities of biosynthesizing or degrading enzymes of these products affect their quantities in the cell.[11] Activity of phenylpropanoid pathway is exceptionally crucial for plant protection against stress damages.[12] Cinnamate 4-hydroxylase (C4H) is one of the leading enzymes in phenylpropanoid pathway, which catalyzes the second step of the pathway in converting the *trans*-cinnamate to coumarate. C4H is the member of P450 protein family and catalyzes the monooxygenation of vast variety of substrates.[13] The flavonoid pathway is a part of phenylpropanoid pathway, synthesizing also other secondary metabolites like alkaloids, lignin, suberin, and monolignols. It is evident that flavonoids act as antimicrobials and antioxidants during microbial challenges in some plants.[14,15]

Mechanical wounding in plants might be resulted from many environmental stress sources, e.g., wind, sand, rainfall, herbivores, etc. As a rule, plants react to wounding through induction of diverse genes given that an open wound is well prone to microbial infections. Therefore, local expression of defense genes in wound periphery prevents the entry of assaulting microorganisms.[16] Wound signaling in plants is a complex process including many regulatory molecules and other inducible defense responses.[17] Among the molecular components of wound signal perception and transduction, jasmonate and MAP kinases are of high significance. Plants activate the phenylpropanoid metabolism as a critical defense response against wounding and pathogenic attacks. As a matter of fact, augmentation of carbon flow via this pathway leads to the accumulation of defense compounds with structural and/or antimicrobial properties.[18,19]

Abiotic stresses are within the main causes of yield loss, worldwide, and salinity, in particular, is becoming a global agricultural crisis.[20] Salinity imposes a double-loss on agriculture by limiting both the yield and the arable lands, thereby necessitating a deep understanding of plant responses to this abiotic stress.[21] Tolerance to salinity is a complicated trait and is governed by several quantitative trait loci (QTLs). These QTLs are involved in many pathways, concurrently, including salinity signal perception and transduction culminating in the overall response of plant.[20] High soil salinity results in ion toxicity as well as hyperosmosis, which by itself leads to ROS production and, ultimately, programmed cell death.[22] Different abiotic stresses pathways share common components in their signaling cascades with a sophisticated interplay[23] for maintenance of the homeostasis and lowering the fitness cost.[24] In *Arabidopsis*, for instance, salinity and drought stress signaling cascades involve the phenylpropanoid pathway.[25] The cinnamate-benzoate path is more common than cinnamate-coumarate one for SA biosynthesis in stress condition in different plant species, e.g., water deficit as well as UV irradiation in barley,[26] ozone in tobacco,[27] thermal stimuli in pea,[28] and salt stress in rice.[29] In this study, regardless of possible involvement of cinnamate-benzoate pathway, we report the molecular cloning and functional analyses of 2 important genes, isochorismate synthase and cinnamate 4-hydroxylase, in safflower, involved in responses to stresses, i.e., salinity stress, wounding, as well as salicylic acid treatment.

Results and Discussion

Isolation of *Ct*ICS from safflower

Several ICS cDNAs have been isolated from prokaryotes and eukaryotes. Isochorismate synthase (EC 5.4.4.2) belonging to the family of isomerases is known to catalyze the conversion of chorismate to isochorismate. It is implicated that ICSs mediate production of salicylate from chorismate,[30] synthesis of phenolate siderophore amonabactin,[31] biosynthesis of menaquinone,[32] etc. in prokaryotes. In plants, very few peer-reviewed reports on ICS isolation and characterization are available. In *Arabidopsis*, it is demonstrated that ICS is involved in the synthesis of salicylic acid

required for both local and systemic acquired resistance.[33] As well, it is involved in phylloquinone (vitamin K1) synthesis.[34] To look for ICS gene in *C. tinctorius* as an alternative source for salicylic acid biosynthesis, the available ICS coding sequences in GenBank were considered to design isolating primers (**Table 1**). The validated partial sequence of *Ct*ICS was deposited in GenBank under accession number of JQ772500. The inferred amino acid sequence of *Ct*ICS (Accession: AFI57884) was blasted against the non-redundant protein sequences database, revealing that ICS in safflower (Asteraceae) shows considerable identities to orthologs in *Vitis vinifera* (Vitaceae), *Ricinus communis* (Euphorbiaceae), *Populus tremuloides* (Salicaceae), *Solanum lycopersicum* (Solanaceae), *Glycine max* (Fabaceae), *Capsicum annuum* (Solanaceae), *Medicago truncatula* (Fabaceae), and *Arabidopsis thaliana* (Brassicaceae) as shown in **Figure 2A**. The maximum identity, however, of *Ct*ICS is to chloroplastic isochorismate synthase 2 (Accession: XP_002267681) of grape (*Vitis vinifera*) up to 69%. Phylogenetic analysis of deduced amino acid sequence of *Ct*ICS in parallel with its eukaryotic and prokaryotic orthologs demonstrated that ICS gene is rather conserved in prokaryotes and eukaryotes (**Fig. 2B**). However, two main clusters are constructed each devoted to either plants or bacteria. As briefly mentioned earlier, isochorismate synthase orthologs are extensively characterized in bacteria, believed to be involved in siderophore and salicylic acid biosyntheses. As well, isochorismate synthases in plants are involved in salicylic acid biosynthesis, required for both local and systemic acquired resistance, while SA synthesized through phenylpropanoid pathway seems to potentiate the plant cell death.[33] As expected, the plant ICSs cluster together aside from bacterial ICSs indicating that although being rather conserved in terms of involving in SA biosynthesis in bacteria

Table 1. Sequences of primes used in this study. Primers for each gene were designed according to the most conserved domains of orthologs in close species. The cDNA sequences of orthologs in GenBank were used for primer design

Gene	Primer	Sequence (5` – 3`)	Amplicon size
Cinnamate 4-hydroxylase	*Ct*-C4H-fwd	ATGGGGCAGC GCAACCT	343
	Ct-C4H-rev	ACATGTTGTT GTACATCATC AGCTG	
Isochorismate synthase	*Ct*-ICS-fwd	CTCAGGTTGA GTTTGATGAG CT	419
	Ct-ICS-rev	CTCTGGAGTG TTTCCAATGA A	
Isochorismate synthase (internal)	*Ct*-ICS2-fwd	ATGGACATTT GGGAAAGCAC	195
	Ct-ICS2-rev	TTGGTAAGGG GTGAGTCGTC	
18S rRNA	18S-fwd	ACTCACCTCA AGACT	205
	18S-rev	CTTTGGCACA TCC	

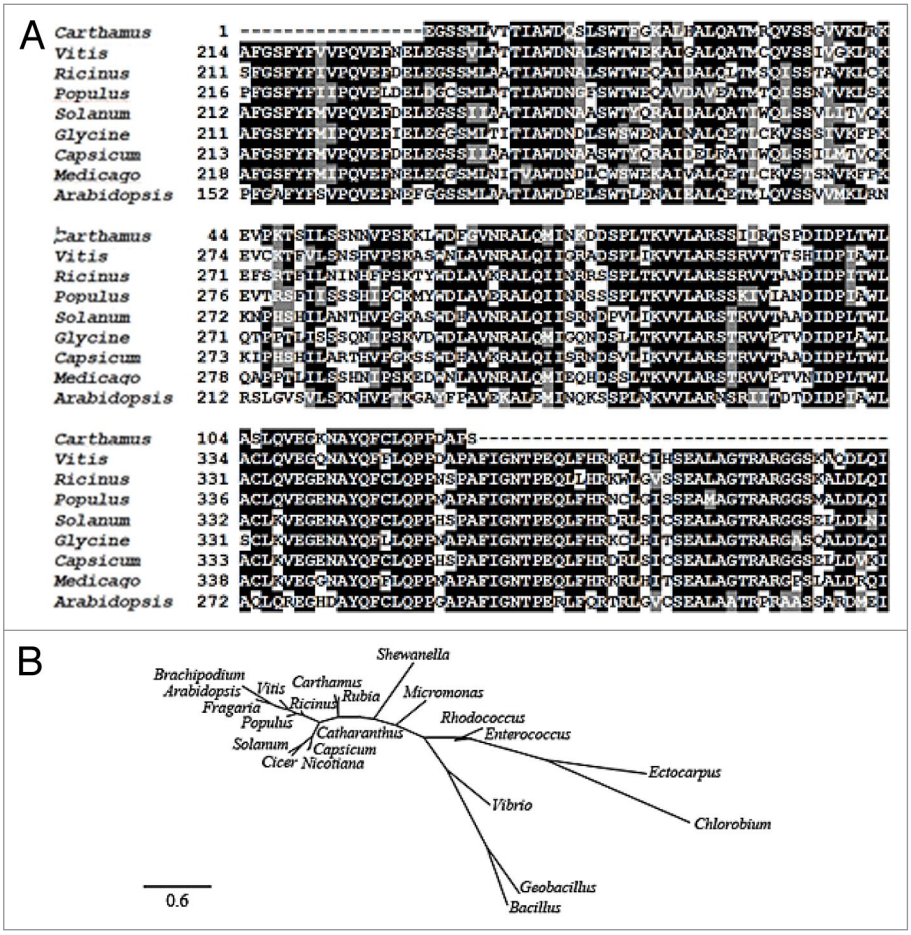

Figure 2. Amino acid sequence alignment (**A**) and phylogenetic analysis (**B**) of *Ct*ICS orthologs. The known ICS sequences fall into 2 main clusters each devoted to either plants or bacteria. The evolutionary history was inferred using the Neighbor-Joining method. The evolutionary distances were computed using the Poisson correction method and are in the units of the number of amino acid substitutions per site. All positions containing gaps and missing data were eliminated from the data set. Phylogenetic tree was constructed at Phylogeny.fr. Accession numbers of sequences are: *Carthamus tinctorius* (AFI57884), *Ricinus communis* (XP_002511526), *Vitis vinifera* (XP_002267681), *Populus tremuloides* (ACX46384), *Fragaria vesca* subsp. *vesca* (XP_004301164), *Solanum lycopersicum* (NP_001234794), *Glycine max* (XP_003522193), *Capsicum annuum* (AAW66457), *Arabidopsis thaliana* (AAC97926), *Cicer arietinum* (XP_004514127), *Nicotiana tabacum* (AAW67000), *Catharanthus roseus* (Q9ZPC0), *Brachypodium distachyon* (XP_003578021), *Rubia cordifolia* (ABK79678), *Rhodococcus rhodnii* (WP_010837681), *Ectocarpus siliculosus* (CBN77562), *Bacillus cereus* (WP_001003908), *Micromonas pusilla* (XP_003064233), *Chlorobium limicola* (YP_001944055), *Geobacillus thermoglucosidasius* (YP_004586325), *Enterococcus gallinarum* (WP_003127529), *Shewanella frigidimarina* (WP_011639322), and *Vibrio anguillarum* (NP_943589). The scale bar indicates 0.6 substitutions per site.

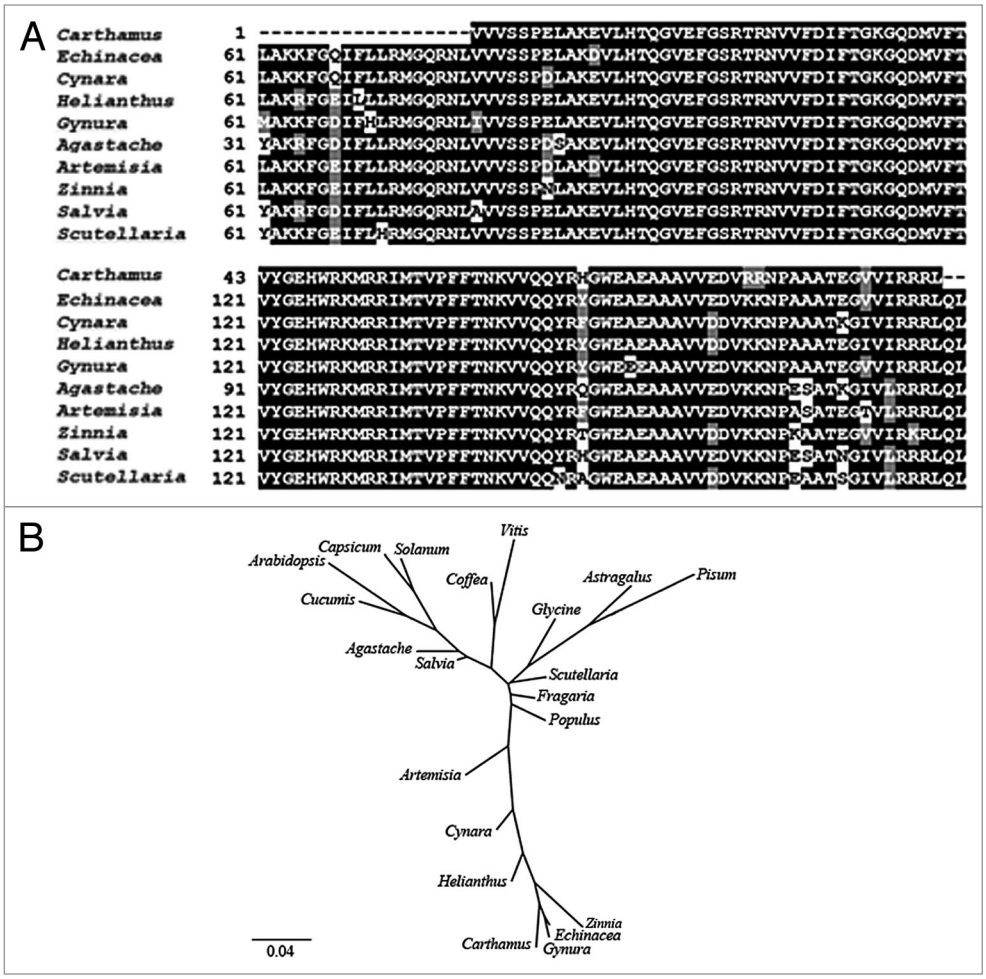

Figure 3. Amino acid sequence alignment (**A**) and phylogenetic analysis (**B**) of *Ct*C4H with other orthologs in GenBank. The evolutionary history was inferred using the Neighbor-Joining method. Evolutionary distances were computed using the Poisson correction method and are in the units of the number of amino acid substitutions per site. All positions containing gaps and missing data were eliminated from the data set (Complete deletion option). Phylogenetic analyses were conducted at Phylogeny.fr. The sequences for C4H genes: *Carthamus tinctorius* (AFK25795), *Cynara cardunculus* var *scolymus* (CAM84301), *Echinacea angustifolia* (ACF74449), *Helianthus tuberosus* (CAA78982), *Gynura bicolor* (BAJ17666), *Agastache foeniculum* (BAJ22964), *Coffea arabica* (CAJ41419), *Populus kitakamiensis* (BAA11578), *Artemisia annua* (ADO16181), *Zinnia violacea* (AAB42024), *Salvia miltiorrhiza* (ABC75596), *Solanum lycopersicum* (XP_004242478), *Glycine max* (ACR44227), *Fragaria vesca* subsp. *vesca* (XP_004294725), *Scutellaria baicalensis* (ADN32769), *Astragalus chrysochlorus* (ACY06865), *Vitis vinifera* (CAN77208), *Capsicum annuum* (ACF19421), *Cucumis sativus* (CAK95273), *Pisum sativum* (AAG09205), and *Arabidopsis thaliana* (CAP08837). The scale bar indicates 0.04 substitutions per site.

and plants, plant ICSs are separate from their bacterial orthologs from the evolutionary point of view.

Isolation of safflower C4H (*Ct*C4H)

Using conserved sequences of C4Hs, *Ct*C4H partial sequence (343 bp) was cloned from safflower. The validated sequence of *Ct*C4H was deposited in GenBank (Accession: JN998608). The inferred amino acid sequence of *Ct*C4H (Accession: AFK25795) was blasted against non-redundant protein sequences database, revealing that the *Ct*C4H shows the highest identity to its ortholog in *Echinacea angustifolia* up to 96% (**Fig. 3A**). The phylogenetic analyses based on amino acid sequences of C4H orthologs in different plant species disclosed that C4H gene is much conserved in Asterales, i.e., *Carthamus tinctorius*, *Cynara cardunculus*, *Echinacea angustifolia*, *Gynura bicolor*, *Zinnia violacea*, *Helianthus tuberosus*, and *Artemisia annua* constructing a separate cluster in the phylogenetic tree (**Fig. 3B**). However, as depicted in **Figure 3B**, cinnamate hydroxylases genes are considerably conserved in plants. The multi-enzymatic pathway of phenylpropanoid is in charge of biosynthesis of many secondary metabolites essential for many developmental processes in plants including disease resistance,[3] and cinnamate 4-hydroxylase is the second enzyme in this pathway catalyzing the hydroxylation of *trans*-cinnamic acid to *p*-coumaric acid. In plant physiology, cinnamate hydroxylases control carbon flux to: 1) pigments influential on pollination and UV protection, 2) numerous phytoalexins synthesized by plants when challenged by pathogens, and 3) lignins,[35] presenting it as one of the most critical enzymes in plants growth and development.

***Ct*ICS and *Ct*C4H responses to wounding**

Induction of *Ct*C4H in response to wounding stress takes place at 3 h after treatment (hat) and levels off thereafter, while *Ct*ICS highly induces a bit later at 6 hat with a decrease afterward (**Fig. 4A**). In *Arabidopsis*, C4H expression is clearly higher in roots than in leaves.[36] Several lines of evidence substantiate the participation of phenylpropanoid pathway in wound signaling.[18,37-39] Wounding affects the phenylpropanoid pathway by inducing PAL[40,41] and C4H.[42] PAL as the just upstream gene of C4H in this cascade is proved to be upregulated by wounding via different mediatory signals including, but not limited to, ethylene, methyl jasmonate, and abscisic acid.[38] According to Mizutani et al. (1997),[36] a C4H promoter region of 907 bp contains all of the 3 *cis*-acting elements (boxes P, A, and L) conserved among the PAL and 4CL genes, which per se substantiates that C4H expression is coordinated in *Arabidopsis* with PAL1 and 4CL in response to light and wounding. In this context, while wounding induces the PAL promoter, which is the starting gene in phenylpropanoid pathway and results in PAL mRNA accumulation and elevated expression, inhibitors of PAL transcription can reduce the responses to wounding.[38] It is documented in lettuce that the PAL activity level

correlates with the wound severity and contributes to wounding signal transduction and accumulation of phenolic compounds involved in secondary processes like tissue browning.[38] As well, in parsley PAL and 4CL expression levels are locally boosted in response to wounding. Likewise, in tobacco plants transgenic for parsley 4CL promoter fragment, wounding results in the reporter gene induction.[18] PAL transcript accumulation pattern in lettuce in response to wounding looks like a slight induction during the first 6 h with a climax at 12 h and leveling off at 24 h accompanied by ethylene production. Methyl jasmonate is one of the wound signal transducers, which increases the PAL mRNA transcript in cell culture. In addition, ABA can enhance the PAL activity after wounding in potato cell culture during suberization of cell wall.[38] Mechanical wounding in leaf periphery of pine leads to abundant expression of phenylpropanoid and flavonoid genes. In this study, accordingly, *Ct*C4H, induces significantly at 3 h after wounding and levels off later in a steady-state manner (**Fig. 4A**). Batard et al. (1997)[43] reported that in Jerusalem Artichoke tubers wounding caused a biphasic induction of C4H at 10 and 20 h after wounding followed by steady-state content of C4H messenger decreasing until 50 h and continuing rather constantly for the next 2 days. C4H protein and enzymatic activity clearly showed the comparable pattern of transcript induction; however the peaks were shifted 10–20 h later than that observed for mRNA. Likewise, activity was to some extent delayed compared with protein accumulation. Comparably, in tea, the expression levels of C4H and PAL in response to wounding was induced at 12 h after treatment up to 45% relatively to 0 h and decreased thereafter.[44] Here, the highest induction of *Ct*ICS, in contrast, is observed at 6 h after wounding (**Fig. 4A**). Flavonoid pathway produces, via phenylpropanoid pathway, certain secondary metabolites, which contribute to cell wall fortification as a defense response. According to present study, phenylpropanoid pathway in safflower is activated rapidly after wounding followed by activation, with delay, of isochorismate pathway, showing that both pathways get activated soon after wounding stress to eventually contribute to SA biosynthesis, which is generally approved as a fundamental signal transducer in plant defense.[3] On the other hand, phenylpropanoid pathway contributes to production of a broad range of secondary metabolites crucial for plant protection against environmental threats. After 24 h, the gene transcriptions of both *Ct*C4H and *Ct*ICS decrease (**Fig. 4A**) to prevent the unnecessary loss of resources after activation of downstream pathways, which could as such be the consequence of a negative feedback from downstream signals.

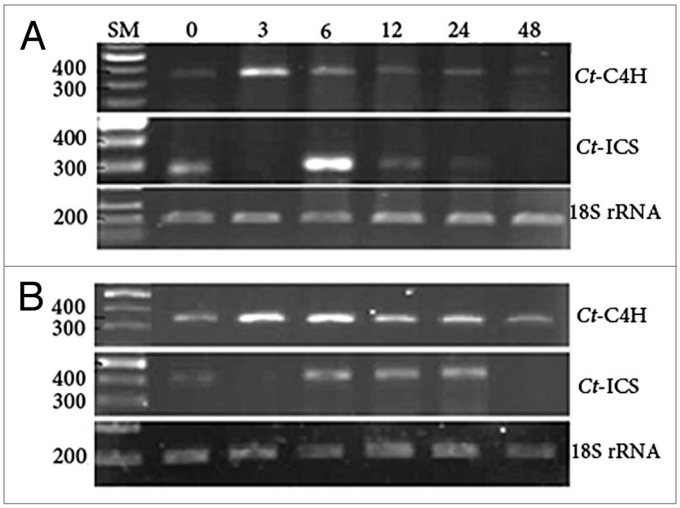

Figure 4. Expression profiles of *Ct*C4H and *Ct*ICS genes after wounding (**A**) and during salinity stress (**B**). Samplings were performed at 0, 3, 6, 12, 24, and 48 h after treatment. The RNA was extracted from all treated plants and after DNaseI treatment, total RNA was reverse transcribed to cDNA. The cDNA amounts were first normalized by 18S rRNA PCR product intensity. For *Ct*ICS, the internal primers based on isolated sequence were designed and used for RT-PCR. Intensity of PCR products at different time points refers to as temporal expression level of the genes. 18S rRNA transcription levels serve as internal control.

Expression of *Ct*ICS and *Ct*C4H in response to salinity stress

During salinity stress, *Ct*C4H induces at 3 h after treatment (hat) and slowly settles down after 12 h. *Ct*ICS, however, is induced by salinity stress from 6 to 24 hat and calms down thereafter (**Fig. 4B**). It is proven that the salinity stress causes the induction of aromatic amino acids biosynthesis. Concurrent induction of both genes implicates that the lignin biosynthesis should also be boosted up by salinity stress, which by itself contributes to cell wall fortification.[45] Among aromatic amino acids, tryptophan is the activator of positive feedback for phenylalanine (forerunner of phenylpropanoid pathway) and tyrosine biosyntheses. It is documented that expression of tryptophan biosynthesizing enzymes is induced in response to biotic as well as abiotic stresses including salinity.[45] Since shikimate pathway triggers the tryptophan biosynthesis in plants and chorismate is the last common precursor of tryptophan, phenylalanine and tyrosine, when challenging with salinity, the higher activation of shikimate pathway and consequent higher chorismate production can lead to a higher activity of chorismate-driven pathways, e.g., phenylpropanoid and isochorismate. It has been recorded that phenylpropanoid pathway gets induced in salt-sensitive genotypes of rice, IR29, in response to salinity stress. On the other hand, *Arabidopsis* mutant *uvs66*, deficient in one of the genes in phenylpropanoid pathway and highly sensitive to UV, is highly sensitive to salinity.[46] Association of phenylpropanoid pathway with salinity, ozone, and UV stresses concerns the oxidative stress.[46] It is acknowledged that the long-term salt accumulation in plants leads to diminishing photosynthetic activity, increasing oxidative stress, and finally imposing an anaerobic condition as well as metabolic damages.[45] This pathway retains a dual function in: 1) secondary metabolites production, e.g., lignin for cell wall fortification, and 2) scavenging the oxidative stress factors in consequence of salinity stress. Moreover, this pathway contributes to SA production. In wheat, the salinity-derived growth impairments could be evaded by exogenous application of SA. It results in augmented activation of antioxidant enzymes catalase, peroxidase, glutathione reductase, ascorbate peroxidase, and superoxide dismutase.[47] Lee et al. (2010)[48] also reported that although SA is not essential for *Arabidopsis* seed germination under normal growth condition, it does play a promotional role in seed germination under high salinity circumstances

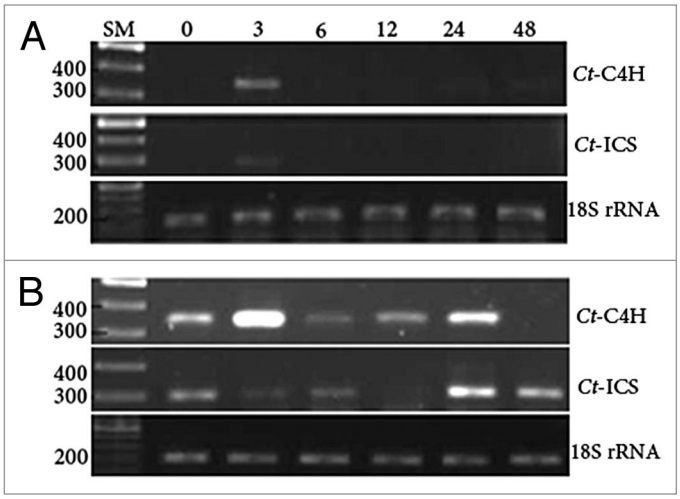

Figure 5. Expression profiles of *Ct*C4H and *Ct*ICS genes after SA treatment of 0.1 mM (**A**) and 1 mM (**B**). Samplings were performed at 0, 3, 6, 12, 24, and 48 h after treatment. Total RNA was prepared from all plants followed by DNaseI treatment. Then, RNA was reverse transcribed to cDNA. For *Ct*ICS, the internal primers were used for RT-PCR. Intensity of PCR products at different time points refers to as temporal expression level of the genes. 18S rRNA transcription levels serve as internal control.

by decreasing the oxidative damage. Hence, induction of the corresponding components in those pathways namely C4H and ICS during salinity stress looks inevitable. Accordingly, results of this study revealed that in salinity-stressed plants, *Ct*C4H gene is induced early after stress in 3–6 h and settles down subsequently (**Fig. 4B**). Salinity, on the other hand, induces the *Ct*ICS fairly later at 6–24 hat (**Fig. 4B**). Thus, after salinity stress, the phenylpropanoid pathway is quickly activated followed by activation of isochorismate pathway; altogether giving rise to SA and other metabolites biosyntheses and culminating in the overall protection of plant.

Responses of *Ct*C4H and *Ct*ICS to SA treatment

In contrast to *Ct*C4H, induction of *Ct*ICS gene upon SA treatment seems to be concentration-dependent. In 0.1 mM SA condition, *Ct*C4H is rather highly induced in a biphasic manner at 3 hat and to some extent at 24 and 48 hat (**Fig. 5A**), which gets more intensified upon treatment with 1 mM SA (**Fig. 5B**). In contrast, the induction patterns for *Ct*ICS are different upon treatment with different SA concentrations. Treatment of plants with 1 mM SA results in a comparatively stronger induction of *Ct*ICS, even at 0 h, however in the following time points until 18 hours after treatment (hat) a decline in gene expression is observed. Again, at 24 h, an intense induction is visible, which repeats at 48 hat. This pattern is not evident when 0.1 mM SA is applied on plants. In plants treated with 1 mM SA, *Ct*C4H gene was more transcribed at 3 hat followed by a drop at 6 hat, a second phase of high transcription from 12 to 24 hat, and the final drop at 48 hat (**Fig. 5B**). In return, the *Ct*ICS gene with a very slow ramp during the first 12 h gets induced at 24–48 hat (**Fig. 5B**), indicating that in safflower SA biosynthesis is controlled probably by phenylpropanoid rather isochorismate pathway. In *Nicotiana benthamiana*, Catinot et al. (2008)[49] showed that ICS is, like PAL, crucial for SA biosynthesis as if ICS-silenced plants do not accumulate SA following UV exposure or pathogenic attack. Interestingly, the lower concentration of SA (0.1 mM) induces both genes weakly at only 3 hat (**Fig. 5A**), suggesting the SA responsiveness of both genes. In real life, pathogenic attacks elicit the de novo SA biosynthesis, which per se triggers the immune responses, particularly expression of pathogenesis-related (PR) genes.[50] Accordingly, exogenous SA can amply induce the *Ct*C4H and *Ct*ICS genes in safflower leading to higher supply of pivotal precursors for defense compounds, i.e., propanoids, flavonoids, and lignins. Salicylic acid has a crucial role in defense responses against pathogens and abiotic stimuli as such higher SA content can induce more strongly and much durably the defense arsenals. Evidently, *Arabidopsis* ICS1 expression is influenced by a sort of positive feedback from SA, which is for itself the ultimate product of ICS1 gene.[51] It is as well documented that transgenic *Brassica rapa* plants expressing bacterial isochorismate synthase accumulate SA significantly higher than the corresponding control plants.[52] In contrast, plants deficient in responsiveness to SA are super susceptible to environmental stresses. For instance, *Arabidopsis* SA mutant, i.e., *eds1*, *eds2*, *eds5*, *pad4*, and *sid2* are highly susceptible to fungal and bacterial infections.[53] Mutant plants of *sid2* line are impaired in SA synthesis due to a mutation in the gene of isochorismate synthase isozyme, ICS1; a crucial factor in induction of SAR in *Arabidopsis*. It has been recently shown that *Arabidopsis* ICS1 is required for SA biosynthesis.[51] Moreover, ICS1 gene expression analysis using transgenic plants expressing ICS1 promoter:reporter gene constructs reveled that SA positively regulates ICS1, however ICS2 is unaffected by SA. Auto-regulation of *Arabidopsis* ICS1 by SA is inferred by sustained elevated level of ICS1 during 6–48 h after SA treatment. Interestingly, supplying exogenous SA to *ics1*-mutant plants does not induce the downstream defense genes to the same extent as seen in wild type counterparts.[51] In this study, treatment of safflower plants with SA elevated the ICS gene expression rather soon and maintained it high for approximately 48 h. This is confirmed when higher concentration of SA is applied (**Fig. 5B**). In another study,[54] ICS-silenced tomato plants were recruited to investigate the role of SA in delaying nonhost hypersensitive response (HR) cell death. As a result, it was shown that likely due to SA pathway suppression in ICS-RNAi plants, a late (48 h) and mild HR was observed in response to infiltration of *Pseudomonas syringae* pv *tabaci* alone or *P. syringae* pv *tabaci*+phytotoxin coronatine.[54] SA concentration-dependency is evident not only in defense responses but also in other developmental phenomena. SA treatment of seeds before germination improves the germination rate, seedling growth, and the net assimilation, while high SA concentration has inhibitory effects.[55] Priming of rice seeds with low SA concentration before planting decreases the ROS production level rooted in cadmium. As well, it increases the content of different antioxidant enzymes namely catalase, guaiacol peroxidase, glutathione reductase, and superoxide dismutase resulting in a protection against oxidative damages. Additionally, SA induces the H_2O_2 accumulation in tissues infected with microbial pathogens leading to onset of hypersensitive response. Furthermore, SA affects the lipid peroxidation, which per se plays a role in defense response and

systemic acquired resistance (SAR) in plants during pathogenic attacks.[55] SAR immunizes the plants to a wide range of pathogens, even less similar to the primary infecting pathogen. As a matter of fact, SA is a significant signal for HR and SAR responses.[24] However, this might not be excluded that differences in sensitivities of *Ct*C4H and *Ct*ICS to SA might be attributed to their differences in affinity to SA, given that generally high affinity mechanism is likely inhibited at relatively higher concentrations of signaling molecules.

To conclude, we determined in safflower the partial coding sequences of 2 important genes in phenylpropanoid and isochorismate pathways, i.e., cinnamate 4-hydroxylase and isochorismate synthase, which are very critical in plant resistance and adaptation to biotic and abiotic stress circumstances. Both genes get highly induced after wounding, salinity stress, and salicylic acid treatment to contribute to safflower fitness. The findings of this study regarding the molecular cloning and functional characterization of these genes may be applicable in breeding programs for salinity tolerance as well as for pathogen resistance in safflower and other plants.

Materials and Methods

Plant materials and growth condition

Seeds of safflower var. 22–191 (kindly provided by Dr Mahammadinejad, Shahid Bahonar University of Kerman) were sterilized with 70% ethanol and sodium hypochlorite (5% chlorine) for respective 2 and 15 min. Prior to germination, seed were vernalized for 2 h and then seeded in petri dishes on sterile, water-soaked filter papers. The germinated seeds were transplanted into pots filled with sand and grown in glasshouse at 26 ± 2 °C and photoperiod of 16 h with irrigation regime of every 3 days. The plants were fertilized by Hoagland solution once a week.

Isolation of partial sequences of *Ct*ICS and *Ct*C4H genes

Genomic DNA isolation was performed using leaf samples according to Saghai-Maroof et al. (1984).[56] The isolating primers were designed using the highly conserved domains, in taxonomically close species, of coding sequences of C4H (*Cynara scolymus*, *Zinnia violacea*, and *Gynura bicolor*) and ICS (*Solanum lycopersicum*, *Capsicum annuum*, *Ricinus communis*, *Catharanthus roseus*, and *Rubia cordifolia*). The primers were synthesized by Eurofin MWG Operon (Germany) with the given sequences (**Table 1**). Amplification of *Ct*ICS and *Ct*C4H partial coding sequences was performed using genomic DNA. Polymerase chain reactions (PCR) were performed in final volume of 25 µL including 10 pmol of specific primers for each gene. The annealing temperature for *Ct*ICS and *Ct*C4H were 50 and 54 °C, respectively.

Cloning of *Ct*ICS and *Ct*C4H amplicons for sequencing

The InsTAclone™ PCR Cloning Kit (Thermo SCIENTIFIC, # K1213) was used to clone the PCR products of *Ct*ICS and *Ct*C4H into sequencing plasmid according to manufacturer instruction. The *E. coli* strains for cloning of *Ct*ICS and *Ct*C4H were HD5α and JM107, respectively. Briefly, bacterial strains were transformed with pTZ57R/T cloning vector harboring the isolated fragments. Next, the recombinant colonies were picked based on blue/white screening and plasmid DNA was extracted using GF-1 Plasmid DNA Extraction Kit (Vivantis). Sequencing of isolated coding region of genes was performed by Faza Pajooh Biotech (Tehran, Iran) using M13 universal primers. The sequencing results were checked using Chromas Lite 2.01 (Technelysium) after clipping the flanking sequences of cloning vector.

Homology and phylogenetic analyses

Homology analyses of amino acid sequences of isolated genes were performed using ClustalW.[57] The evolutionary history was inferred using the Neighbor-Joining method.[58] The evolutionary distances were computed using the Poisson correction method.[59] All positions containing gaps and missing data were eliminated from the data set (Complete deletion option). Phylogenetic analyses were conducted at Phylogeny.fr.[60]

Gene expression experiments

Mechanical wounding of leaves was done on 14-d-old seedlings. The wounding was performed by sterile blunt-nosed thumb forceps punched rather equally onto the leaves. Salinity stress was applied, as well, on 14-d-old seedlings. The plants were irrigated with 150 mM NaCl solution. Similar seedlings were used for salicylic acid treatments in 2 groups of 0.1 mM, and 1 mM SA solutions. The solutions were sprayed onto the leaves. Samplings for all 3 assays were performed at 0, 3, 6, 12, 24, and 48 hat. All experiments were initiated at 8 AM to consider the possible diurnal rhythm in the genes expression profiling.

RNA extraction and cDNA synthesis

Total RNA was extracted using RNX™ Plus Kit (Cinnagen) from all treated plants following the manufacturer instruction. After DNaseI treatment of all RNA samples, 1 µg of total RNA was reverse transcribed to cDNA by RevertAid First Strand cDNA Synthesis Kit (Thermo SCIENTIFIC, # K1691).

Semi-quantitative RT-PCR

The cDNA amounts were first normalized by 18S rRNA PCR product intensity. For *Ct*ICS, the internal primers based on isolated sequence were designed and used for RT-PCR. PCR thermal profile was as follows: 98 °C (5 min) followed by 35 cycles of 98 °C (20 s.), 52 °C (20 s.), and 72 °C (1 min), and finally 10 min incubation at 72 °C for final extension. A separate experiment was performed to confirm the linear amplification in the condition used. More and less intensity of PCR products at different time points were judged for respective more and less transcription level of each gene.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

The authors thank the anonymous reviewers for their valuable comments on the manuscript. Rahnamaeian M acknowledges the research grant from Shahid Bahonar University of Kerman (Iran). Rahnamaeian M and Vilcinskas A acknowledge the Ministry for Science and Art of the State of Hesse (Germany) for funding both the LOEWE research focus "Insect Biotechnology" and the Fraunhofer project group "Bioresources."

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