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Identification of hydrogen peroxide responsive ESTs involved in phenylethanoid glycoside biosynthesis in *Cistanche salsa* cell culture

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Abstract

Hydrogen peroxide is an effective abiotic elicitor that can induce secondary metabolite biosynthesis in plants. We show that in cell suspension culture of a salt-tolerant medicinal plant *Cistanche salsa*, the production of bioactive components phenylethanoid glycosides (PeGs) was increased after an H_2O_2 treatment. To identify genes related to PeGs biosynthesis affected by H_2O_2, we constructed a suppression subtractive hybridization library of H_2O_2 responsive genes using a *C. salsa* cell line and identified 105 expressed sequence tags (ESTs) and 85 genes. EST library functional annotation and gene ontology analyses showed genes related to various stress responses, biosynthesis of secondary metabolites, and transcriptional regulation. Among them we identified two genes related to the PeGs biosynthesis pathway (4-coumarate coenzyme A ligase and cinnamate 4-hydroxylase), and two WRKY type transcription factors. The expressions of selected genes after the H_2O_2 treatment were analyzed by RT-qPCR. An early increased transcription of PeG biosynthesis pathway genes after the treatment revealed that H_2O_2 induced PeGs biosynthesis *via* up-regulation of its key genes.

Additional key words: cinnamate-4-hydroxylase, 4-coumarate coenzyme A ligase, suppression subtractive hybridization.

Introduction

Cistanche salsa is a perennial plant parasite on the root of *Haloxylos ammodendrun*, and it grows in the desert area of western China. For centuries, *Cistanche* species has been used as traditional herbal medicine (Wang *et al*. 2012). Bioactive components are phenylethanoid glycosides (PeGs) which have a remarkable effect on reactive oxygen species (ROS) clearance, radiation protection, immunity enhancement, and anti-aging (Nan *et al*. 2013). PeGs biosynthesis in *Cistanche* is initiated from phenylalanine (Fig. 1 Suppl.) and synthesized caffeic acid is further glycosylated to form various PeGs (Wang *et al*. 2012). Three main PeGs, echinacoside, acteoside, and 2'-acetylacteoside, are considered to be the most active components in *Cistanche* (Kobayashi *et al*. 1984). In order to enhance their content in *Cistanche* cell culture, various elicitors have been tested including chitosan, putrescine, Ag^+, and osmotic stress (Ouyang *et al*. 2005a,b, Cheng *et al*. 2005, 2006, Liu *et al*. 2007, Liu and Cheng 2008). However, genes involved in PeGs biosynthesis in *Cistanche*, mainly phenylalanine metabolism pathway genes, have been rarely identified. Only a gene encoding phenylalanine ammonia-lyase (PAL) was recently identified in *Cistanche deserticola* (Hu *et al*. 2011). Other genes encoding, for example, cinnamate-4-hydrosylase (C4H), which could catalyze conversion of cinnamate to cumaric acid, and 4-coumarate coenzyme A ligase (4CL), an essential gene for lignin and flavanoids biosyntheses, have not been found in *Cistanche*.

We used cell culture of *Cistanche salsa* to study PeGs biosynthesis and related genes expression. We also investigated the effect of an effective elicitor H_2O_2 on PeGs biosynthesis. Furthermore, to find early responsive genes involving in PeGs biosynthesis, we carried out suppression subtractive hybridization (SSH) to construct a cDNA library of H_2O_2-responsive genes.

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Abbreviations: C4H - cinnamate-4-hydroxylase; 4CL - 4-coumarate coenzyme A ligase; ESTs - expressed sequance tags; GA - gibberellin; GO - gene oncology; KEGG - Kyoto Encyclopedia of Genes and Genomes; PAL - phenylalanine ammonia lyase; PeGs - phenylethanoid glycosides; ROS - reactive oxygen species; RT-qPCR - real time quantitative polymerase chain reaction; SSH - suppression subtractive hybridization.

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Materials and methods

The CS2001 cell line was established from petals of *Cistanche salsa* (C.A. Mey.) Beck collected at Neimenggu province and subcultured. The culture method was similar to a protocol published previously (Liu *et al*. 2007). Generally, a solid Murashige and Skoog (1962; MS) medium was used for subculture and a liquid medium for PeGs biosynthesis (it was the modified MS medium supplemented with 30 g dm^{-3} glucose and 2.0 mg dm^{-3} indole-3-acetic acid). For H_2O_2 treatment, 5.0 ± 0.2 g of cells in a fast growth period, usually 20 to 30 d after they were subcultured in the solid medium, were inoculated into a 50 cm^3 liquid medium in a 250 cm^3 Erlenmeyer flask and cultured in the dark with or without different H_2O_2 concentrations. The PeGs content after 10 d induction by H_2O_2 was analyzed according to a method published previously (Liu *et al.* 2007) based on reverse phase high performance liquid chromatography with methanol gradient elution.

The cells 2 h after the H_2O_2 treatment were collected from the medium by filter paper using a Büchner funnel, and then they were frozen in liquid nitrogen and ground to powder. Every 2 g of cell powder was lysed using the mixture of 1 cm^3 of 2-mercaptoethanol, 1 cm^3 of 200 g dm^{-3} sarcosyl, and 8 cm^3 of a GTC buffer (5 M guanidinium isothiocyanate, 62.5 mM Tris-HCl, pH 8.0, 6.25 mM EDTA, pH 8.0, 5 mM thiourea, and 10 mM dithiotreitol). The cells were then incubated on ice for 15 min, then 1/3 volume of 8.5 M CH_3COOK, pH 6.0, was added, and the cells were incubated on ice for 15 min and then centrifuged at 15 000 *g* for 15 min. The supernatant was added to an equal volume of isopropanol to precipitate total RNA. The total RNA was further purified by an RNeasy mini kit (*Qiagen*, Hilden, USA) according to the manufacturer's instructions. Messenger RNA was isolated from total RNA using an *Oligotex* mRNA midi kit (*Qiagen*).

An SSH library was generated from control and 30 % H_2O_2-elicited cell samples 2 h after the elicitation using a PCR-select cDNA subtraction kit (*Clontech*, Mountain View, USA). For library construction, the products were purified separately using a gel purification kit (*Qiagen*) and cloned into pGEM vector using *TA* cloning kit (*Tiangen*, Beijing, China).

Plasmids from independent clones were isolated and sequenced using T7 primer. All expressed sequence tag (EST) sequences from the library (with the vector sequences removed) were used for contig assembly with CAP3 (http://doua.prabi.fr/software/cap3). The ESTs were grouped into contigs and singletons; they were used for homology search using the *BLASTx* program at *NCBI* (http://blast.ncbi. nlm.nih.gov). Sequences were then annotated, analyzed, and classified according to gene ontology (GO) terms using *Blast2GO* (http://www. blast2go.com).

Real time quantitative polymerase chain reaction (RT-qPCR) was performed according to Bustin *et al.* (2009). The cDNA samples were synthesized using a *PrimeScript* RT reagent kit (*Takara*, dalian, China) from 0.5 μg of total RNA. RT-qPCR was carried out using *SYBR Premix Ex Taq II* and *ROX plus* kits (*Takara*) on an *ABI 7300* real-time PCR system *(Applied Biosystems,* Foster City, USA) with three technical replicates. Primers were designed using the online *Primer3* software according to the instruction supplied by *Takara*; primers used in this study are listed in Table 1 Suppl. The gene expressions were normalized to 18S RNA, the expression of which was stable in all the samples.

The results were analyzed using *R version 3.1.1*, the significance of differences was verified by the Student's *t*-test.

Results

Hydrogen peroxide induced the biosynthesis of all three main PeGs in *C. salsa* suspension cells (Table 1) but with no obvious effect on cell growth (Fig. S2). The content of echinacoside increased already after the 10 μg dm^{-3} H_2O_2 treatment ($P < 0.05$), a higher increase was recorded after the 20 μg dm^{-3} H_2O_2 treatment ($P < 0.01$), and the highest increase was after the 40, 80, and 160 μg dm^{-3} H_2O_2 treatments. Similar results are shown for acteoside, but its content peaked after the 80 μg dm^{-3} H_2O_2 treatment. The biosynthesis of 2'-acetylacteoside was less sensitive to H_2O_2 treatment; a significant increase of its content ($P < 0.05$) was at H_2O_2 concentrations over 40 μg dm^{-3}. After the 80 μg dm^{-3} H_2O_2 treatment, the amount of all the three main PeGs significantly increased ($P < 0.01$) over the control. Therefore, this concentration was used in further experiments.

To generate a library enriched for early responsive genes that are up-regulated by H_2O_2, we used cDNA from *C. salsa* suspension cells treated with 80 μg dm^{-3} H_2O_2 for 2 h. Single-pass sequencing 129 recombinant clones in the SSH library yielded 105 clean ESTs after vector sequence exclusion. These clear ESTs were used for assembly by CAP3, and 18 contigs and 67 singletons were generated (Table 2). Thus, 85 genes were isolated in this library. The mean length of the clean 105 ESTs was 527 bp, which is adequate for a sequence similarity-based functional classification.

All 85 genes obtained in the library were retrieved by *BLASTx* against the *GenBank* non-redundant protein database for sequence similarity (E-value ≤ 0.001).

Table 1. Effect of H_2O_2 on content of PeGs in cell culture of *Cistanche salsa*. Means ± SD of at least five samples within three biological independent repetitions; * - $P < 0.05$, ** - $P < 0.01$.

H_2O_2 [μg dm^{-3}]	Echinacoside [mg dm^{-3}]	Acteoside [mg dm^{-3}]	2'-Acetylacteoside [mg dm^{-3}]	Total PeGs [mg dm^{-3}]
0	480.1±13.7	380.4±15.9	17.6±3.5	878.1±13.6
10	520.6± 9.5*	407.8±15.2	19.8±1.6	948.2±23.9*
20	549.0±12.2**	459.6±16.3**	20.3±1.4	1028.9± 7.2**
40	589.3±18.5**	498.4±10.5**	25.6±0.8*	1113.3±19.2**
80	553.5±12.0**	510.3±12.8**	30.3±2.0**	1094.1±23.2**
160	567.4±12.3**	480.3±19.6**	30.6±0.7**	1078.3±23.6**

Table 2. An overview of ESTs in the CS2001 SSH library after H_2O_2 treatment.

Features	H_2O_2 up-regulated
Number of sequenced clones	129
Number of clean ESTs	105
Average ESTs length [bp]	527
Number of contigs	18
Number of singletons	67
Number of unigenes	85

Twenty-one genes showed no hits in the database, they might be specific to *C. salsa*. The results with *BLAST*hits were imported to the *Blast2GO* software for further *GO* and *KEGG* pathway enrichment analyses. Of the 64 genes with *BLAST* hits, 50 were annotated according to the description of homologous genes from other species. The annotated genes were named according to the description of *BLAST* hits. The GO analysis (Fig. 3 Suppl.) shows that among these 50 genes, there were 14 genes involved in responses to stimuli, and over 30 genes related to metabolic processes (Tables 1 and 2). Among these genes, two PeG biosynthesis pathway gene homologues were isolated as expected: 4-coumarate coenzyme A ligase (named *Cs4CL1*: Contig03) and cinnamate 4-hydroxylase (named *CsC4H1*: Contig14). Further, RT-qPCR was performed to confirm induced expressions of genes from the library after the H_2O_2 treatment.

To confirm that essential genes identified from the SSH library were responsive to the H_2O_2 treatment, the expressions of two PeGs biosynthesis pathway related genes (*Cs4CL1*, *CsC4H1*), two ROS induced genes (*CsSOD1*: Contig 16, *CsHSP1*: CISTH099), and one WRKY family transcription factor like gene (*CsWRKY1*: Contig 07) were measured by RT-qPCR after the treatment with H_2O_2 (Fig. 1). *CsC4H1*, *Cs4CL1*, *CsSOD1*, and *CsWRKY1* expressions were up-regulated more than 15-fold, 3-fold, 45-fold, and 4-fold, respectively 2 h after the H_2O_2 treatment. Expression of *CsHSP1* was altered only 1.8 fold after the H_2O_2 treatment.

The time-course of the expressions of *CsWRKY1*, *CsC4H1*, and *Cs4CL1* after the H_2O_2 treatment was further analyzed by RT-qPCR (Fig. 2). The *CsC4H1* and *Cs4CL1* expressions were highly increased (more than 80-fold and 50-fold, respectively) after the 16-h treatment and then down-regulated after 32 h. The expression of CsWRKY1 was up-regulated more than 20-fold at 4 h and then decreased from 8 h afterwards (Fig. 2).

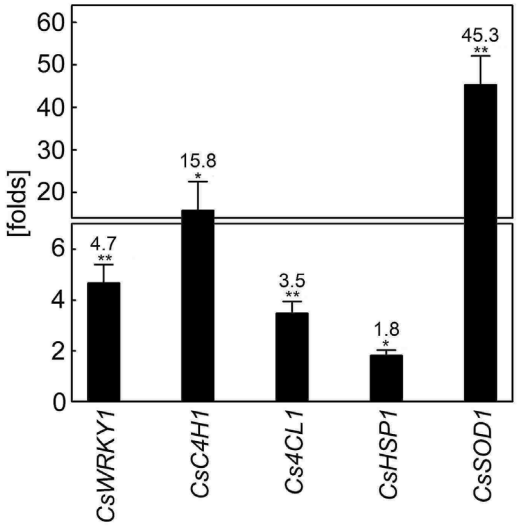

Fig. 1. Relative gene expression determined by RT-qPCR after H_2O_2 treatment. *CsWRKY1* - WRKY transcription factor (Contig07), *CsC4H1* - cinnamate 4-hydroxylase (Contig09), *Cs4CL1* - 4-coumarate: ligase (Contig03), *CsHSP1* - heat shock protein 70 kDa (Contig 01), and *CsSOD1* - superoxide dismutase (Contig16). Cells were treated with 80 μg dm^{-3} H_2O_2 in a liquid medium for 2 h and expressions were compared with a control treated with water. Means ± SD of three biological repetitions; * - $P < 0.05$, ** - $P < 0.01$.

Discussion

Hydrogen peroxide is a well-known plant elicitor that shows various effects on plants. Like ROS, it can induce the ROS scavenging system composed of superoxide dismutase, catalase, peroxidases, *etc*., and it serves as the downstream signal of salt, osmotic, and dehydration stresses (Wi *et al*. 2006, Kar 2011, Furlan *et al*. 2013, Sathiyaraj *et al*. 2014). It was suggested that H_2O_2 generation in plants is accompanied with induced

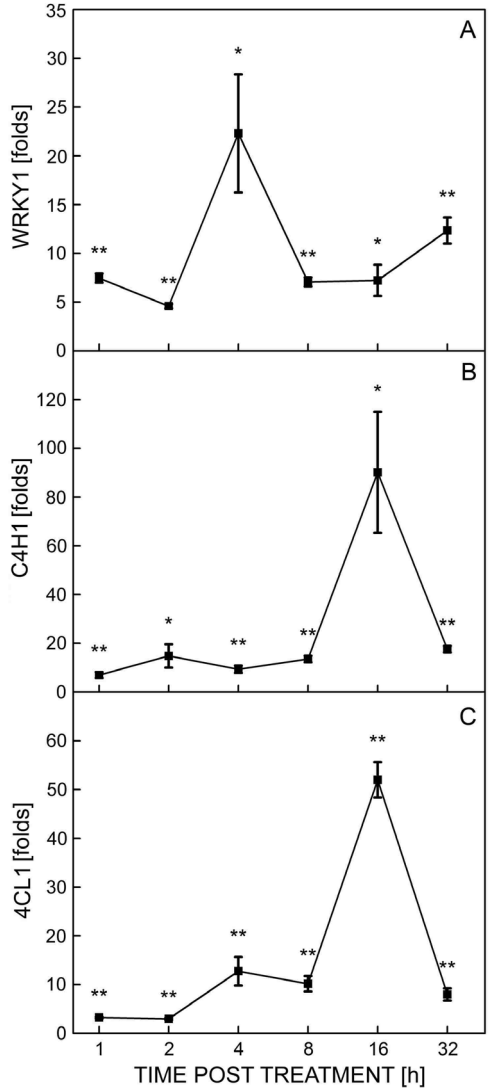

Fig. 2. The relative expression of *CsWRKY1* (*A*), *CsC4H1* (*B*), and *Cs4CL1* (*C*) at different times after treatment with 80 μg dm^{-3} H_2O_2. The expressions were normalized to a control; (treated with water). Means ± SD of three biological repetitions; * - $P < 0.05$, ** - $P < 0.01$.

PAL pathway gene expression (Salcedo-Morales *et al*. 2014), which is important for PeGs biosynthesis. Given that naturally *C. salsa* grows in saline and alkaline soils and contains a high amount of PeGs, H_2O_2 might induce secondary-metabolite biosynthesis in the *C. salsa* cell culture. As expected, our results reveal that PeGs biosynthesis in the *C. salsa* cell culture was induced after the H_2O_2 treatment (Table 1). The content of three main PeGs increased after the H_2O_2 treatment indicating its pivotal role in PeGs biosynthesis. Genes enriched in the SSH library further suggested its effect on PeGs biosynthesis induction in *C. salsa.*

Many known H_2O_2-responsive genes, such as ascorbate peroxidase (*CISTH069*, Karyotou and Donaldson 2005), h-quinone oxidoreductase (*CISTH125*, Bello *et al*. 2001), *CsSOD1*, *CsHSP1* (Baruah *et al*. 2014), and disease resistance response genes (Contig10, Nanda *et al*. 2010), were enriched in the SSH library (Table 3, Table 2 Suppl.). Some of these typical ROS responsive genes with an induced expression after the H_2O_2 treatment were further confirmed by RT-qPCR (Figs. 1 and 2) indicating that a qualified SSH library was constructed here. Two PeG biosynthesis pathway genes, *CsC4H1* and *Cs4CL1*, were also found in this library and these two gene expressions were induced by the H_2O_2 treatment in a time dependent manner (Fig. 2). It suggests that the H_2O_2 treatment induced PeGs biosynthesis at the genetic level *via* the regulation of PAL pathway genes. However, *PAL* and other downstream PeGs biosynthesis genes (Wang *et al*. 2012) were not identified in the SSH library; it might be because of the library have not been sequenced sufficiently. A relation between *CsC4H1* and *Cs4CL1* induced expressions and an increased PeGs biosynthesis after the H_2O_2 treatment should be clarified by a further analysis.

In addition to the PAL pathway genes induced by the H_2O_2 treatment, two WRKY family transcription factors (*CsWRKY1*, *CsWRKY2*) were found in the SSH library (Table 3 and Table 2 Suppl.), and a *CsWRKY1* induced expression was confirmed by RT-qPCR. Its expression was responsive to the H_2O_2 treatment earlier than the expression of two PAL pathway genes, *Cs4CL1* and *CsC4H1* (Fig. 2). From previous studies, co-expression of some WRKY transcription factors and PAL pathway genes has been observed in rice (Gupta *et al*. 2012). Furthermore, PAL gene expression is up-regulated in an *OsWRKY03* over-expression mutant (Liu *et al*. 2005). The above mentioned two WRKY family transcription factors might be also involved in PeGs biosynthesis in *C. salsa*. Interestingly, dehydration related ESTs (CISTH067, CISTH064) were found in the SSH library, which may suggest that the H_2O_2 treatment was related to dehydration responsive genes expression in *C. salsa* as observed in other plants (Schmidt *et al*. 2013, Zhou *et al*. 2013). One gibberellin (GA) signalling related gene, *gibberellin receptor gid1b-like* (*CISTH092*), was identified in the SSH library implying the connection of GA signalling and H_2O_2 treatment in *C. salsa,* which was also reported in barley (Bahin *et al*. 2011). However,

Table 3. Annotations of contigs isolated from the CS2001 SSH library.

Seq. name	EST accession No.	Matched gene acc. No.	Seq. description	Min. E-value
Contig03	JZ712396	ACL31667	4-coumarate: ligase	9.61E-180
Contig12	JZ712416	NP_001275197	eukaryotic translation initiation factor 2	2.48E-151
Contig05	JZ712400	ACN94266	somatic embryogenesis receptor kinase	9.75E-122
Contig08	JZ712406	EPS69014	pleiotropic drug resistance protein 1-like	6.39E-109
Contig10	JZ712411	XP_002297997	disease resistance response protein 206	2.13E-100
Contig16	JZ712424	AEO27875	superoxide dismutase	7.14E-94
Contig04	JZ712398	XP_004299750	mitochondrial alternative oxidase 2a	1.96E-88
Contig09	JZ712408	XP_003519372	class I heat shock protein	1.42E-72
Contig18	JZ712427	XP_004289713	proline iminopeptidase-like	7.59E-70
Contig14	JZ712420	AGW27201	cinnamate-4-hydroxylase	9.71E-64
Contig01	JZ712392	CAD12247	heat shock protein 70 kDa	5.17E-63
Contig07	JZ712404	AFL91071	wrky 7 transcription partial	3.89E-61
Contig02	JZ712394	NP_001275197	eukaryotic initiation factor 4a	7.48E-60
Contig06	JZ712402	XP_002520560	rho gdp-dissociation inhibitor 1-like	6.93E-46
Contig11	JZ712413	XP_004515867	ubiquitin-associated domain-containing	4.16E-34
Contig13	JZ712418	CAO99123	zinc transporter of isoform 1	7.55E-34
Contig15	JZ712422	-	-	-
Contig17	JZ712428	-	-	-

more studies are needed to confirm whether these genes are related to PeGs biosynthesis. Other than genes mentioned above, the SSH library constructed here also presents genes with or without *BLAST* hits. It improves our understanding of H_2O_2 effect on *C. salsa* and firstly supplies the information of its genes involved in PeGs biosynthesis.

In conclusion, we assessed the effect of H_2O_2 on PeGs induction in the *C. salsa* cell culture. The SSH library was constructed after the H_2O_2 treatment, and the expression of selected genes was confirmed by RT-qPCR. Two PeG biosynthesis pathway-related genes and other H_2O_2-responsive genes were identified. These findings will extend our understanding of H_2O_2-induced PeGs biosynthesis in *C. salsa* to the molecular level, providing the information for gene manipulation of *C. salsa* cell cultures to improve PeGs production.

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