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Analysis of elicitor inducible cytochrome P450 induction in *Astragalus chrysochlorus* cells

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Abstract

In this study, elicitor-inducible cytochrome P450 biosynthesis in *Astragalus chrysochlorus* was investigated for further analysis on phenylpropanoid metabolism. In order to analyse cytochrome P450s under yeast extract elicited conditions, we used non-radioactive P450 targeted differential display method. The P450 targeted differential display of mRNA technique was performed with upstream primers based on the conserved heme-binding region [PFG] of P450s, as a result 56 clearly differential bands were revealed; 37 of the bands were correctly analysed, and one of the PCR products was contained the P450 fingerprint. This sequence has been confirmed to be up-regulated and subsequently cloned and sequenced. Homology analysis of the 400 bp long sequence revealed that 81 % similarity with cinnamate 4-hydroxylase in the manner of amino acid. Quantitative real-time-PCR analysis showed that putative C4H gene was up-regulated 13,24-fold by 6h yeast extract treatment unlike untreated control. 1338 bp long cDNA fragment (Accession no: GQ844863) of *A. chrysochlorus C4H* (AcC4H) has been obtained by PCR with degenerate primers. Bioinformatics analyses revealed that putative AcC4H (1338 bp) was highly similar (95 %) to trans-cinnamate 4-monooxygenase (EC 1.14.13.11). As a result, we have isolated a putative *C4H* fragment from *A. chrysochlorus* suspension cells under yeast extract elicited conditions. This knowledge will use for obtaining whole *C4H* sequence, and to manupulate phenylpropanoid metabolic pathway of this medicinal plant.

Key words: Cell suspension; Cinnamate 4-hydroxylase (C4H); Non-radioactive differential display; Phenylpropanoid pathway; Yeast extract.

Introduction

Plant phenylpropanoids confer various physiological functions in accordance with environmental perturbations, therefore most of secondary metabolites are synthesized through phenylpropanoid pathway. The core reactions of phenylpropanoid metabolism involve three enzymes, phenylalanine ammonia-lyase (PAL; EC 4.3.1.24), cinnamate 4-hydroxylase (C4H; EC 1.14.13.11), and 4-coumarate coenzyme A ligase (4CL; EC 6.2.1.12) (Fig.1) (Hahlbrock and Scheel 1989). C4H constitutes the CYP73 family of the large group of cytochrome P450 monooxygenases (Teutsch et al., 1993). Plant cytochrome P450 monooxygenases (P450s) are membrane-bound heme proteins, and they are member of super family catalyzing a variety of reactions in plant secondary metabolism. The first P450 enzyme in the phenylpropanoid pathway is cinnamate 4-hydroxylase (C4H), which catalyzes the hydroxylation of cinnamic acid to 4-coumaric acid in the general phenylpropanoid pathway (Werck-Reichhart 1995; Batard et al., 1997). There is considerable interest in the regulation of phenylpropanoid biosynthesis: both as a model for understanding flux control in such a complex biosynthetic pathway and also for the identification of targets for biotechnological manipulation of phenolic products accumulation. Thus, the best studied example of plant P450s is cinnamate 4-hydroxylase (C4H).

In order to understand the cell regulation networks, one has to identify and characterize differentially expressed genes. Standard methods currently used to isolate differentially expressed genes include substractive hybridization, differential hybridization, and single-cell polymerase chain reaction (PCR). These methods are rather time consuming (Yamazaki and Saito 2002). Repeated retrieval of abundant cDNAs can become a substantial handicap when screening for different members of a gene family that is as large as the P450 super family (Schuler 1996). The increasing number of plant P450 sequences in the databases allowed the recognition of conserved sequence elements that can be used to deduce polymerase chain reaction (PCR) primers. Therefore, PCR-based screening techniques have become the method of choice (Schuler 1996; Bolwell et al., 1994). The differential display (DD) PCR was first reported by Liang and Pardee (Liang and Parde 1992). This method can be used to amplify low abundance transcripts by PCR. This technique possesses the following advantages over other similar techniques: it is based on a simple and established method; it does not require sequence information, more than two samples can be compared simultaneously, and only a small amount of starting material is needed. In the last 15 years, DD has been used to isolate genes from plants which are involved in physiological events, signal transduction, stress response and secondary metabolism (Yamazaki and Saito 2002; Hu et al., 2004; Gao et al., 2008). So, it seems to be an appropriate method for the investigation of gene expression on the plants which has not got completely sequenced genome such as *A. chrysochlorus*. Some secondary products

are induced by addition of elicitors or signal compounds in cell suspension cultures (Schopfer and Ebel 1998; Latunde-Dada et al., 2001; Ghorpade et al., 2011). In soybean, production of pterocarpans such as glyceollin is inducible by pathogen infection or elicitor treatment. Also, cinnamate 4-hydroxylase, dihydroxypterocarpan 6-hydroxylase and another novel flavonoid 6-hydroxylase were isolated from yeast extract treated cells by using DD technique (Schopfer and Ebel 1998; Latunde-Dada et al., 2001). In these studies, yeast extract was used to stimulate the gene expression of the phenylpropanoid pathway. *Astragalus* is one of the most widely used traditional Chinese medicine. It possesses many biological activities including hepatoprotective, antioxidative, diuretic, antihypoxic, and immunostimulant functions, and has been used for general debility, chronic illness, and spleen deficiency by Chinese doctors for centuries. It has also been proven to be effective for clinical treatment of nephritis, cardiovascular diseases, hypertension, diabetes, and cancers (Miller 1998; Sinclair 1998). The known beneficial components of *Radix astragali* (*Astragalus membranaceus*) are mainly flavonoid, triterpene saponins, and polysaccharides, of which phenylpropanoid pathway metabolite flavonoids are associated with its antioxidant and cytotoxic activities (Ma et al., 2002; Xiao et al., 2004). A great deal of research on *Astragalus* has been carried out on its active components and clinical performance in the worldwide; however, little is known about genetics or molecular biology of this plant species. *A. chrysochlorus* Boiss. and Kotschy (2n = 16) is one of the endemic species in Turkey, growing in 32-36° meridian of northern Anatolia (Aytac 1997). In previous studies, antioxidant, cytotoxic, phagocytic effects and Selenium accumulation capacity of *A. chrysochlorus* was determined (Hasancebi 2003; Karagoz et al., 2007; Ari et al., 2010). To increase the bioactive phenolic metabolites of *A. chrysochlorus,* investigation of the main enzymes of biosynthetic pathway must be the first step. Therefore, in this study we focused on one of the main enzymes of the phenylpropanoid pathway. We cloned a cDNA fragment of putative C4H which was obtained by using mRNA differential display, and was upregulated in yeast extract treated *A. chrysochlorus* suspension cells.

Results

Differentially expression of the genes

By using DD technique, 56 clearly differential bands were revealed but 37 of the bands were correctly analysed, and one of the PCR products (400 bp long) (Fig. 2) has contained the P450 fingerprint. A comparison of the deduced amino acid sequence with the known P450 sequences revealed that the isolated cDNA belonged to already known plant cytochrome P450 family. Homology analysis revealed that this 400 bp long sequence was 81% similar with cinnamate 4-hydroxylase. The P450 similar fragment showed positive signal in dot blot (Fig. 3). After that it was cloned into the pCR®2.1-TOPO® vector and sequenced.

Isolation and analysis of cDNA fragment encoding cinnamate 4-hydroxylase

A pair of degenerate primers based on other known C4Hs was designed and an 1338 bp long fragment was amplified by revers transcription-PCR from *A. chrysochlorus* (Accession number: GQ844863). The tblastx results demonstrated that the deduced amino acid sequence of the 1338 bp long fragment shares the highest identity with complete coding

Table 1. P450-specific upstream PFG primers (Schopfer and Ebel 1998).

Primer name	Primer sequence (5′-3′)
PFG1	C G C C A T T T G G
PFG2	C G C C A T T C G G
PFG3	C G C C C T T T G G
PFG4	C G C C C T T C G G
PFG5	C G C C G T T T G G
PFG6	C G C C G T T C G G
PFG7	C G C C T T T T G G
PFG8	C G C C T T T C G G

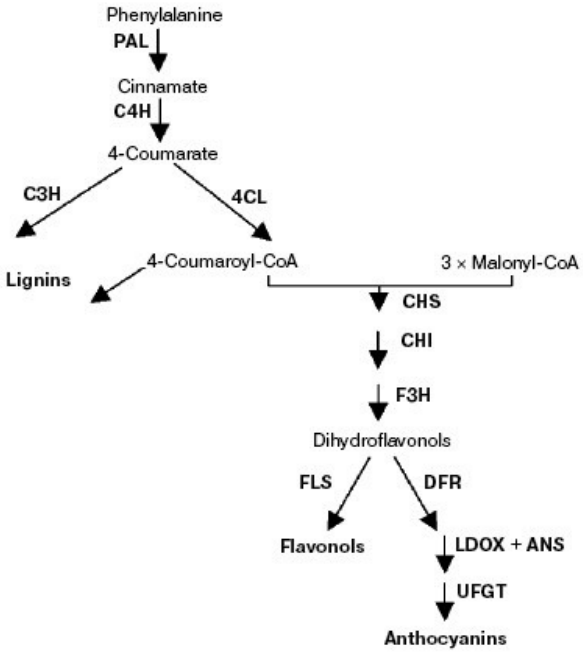

Fig 1. General phenylpropanoid pathway. PAL phenylalanine ammonia-lyase, C4H cinnamate 4-hydroxylase, C3H *p*-coumarate 3-hydroxylase, 4CL 4-coumaroyl CoA ligase, CHS chalcone synthase, CHI chalcone isomerase, F3H flavanone-3-hydroxylase, DFR dihydroflavonol 4-reductase, FLS flavonol synthase, LDOX leucoanthocyanidin dioxygenase, ANS anthocyanidin synthase, UFGT UDP glucose-flavonoid 3-O-glucosyl transferase (revised from Singh et al., 2009).

sequence of C4H from *Glycyrrhiza echinata* (up to 95%). Also deduced amino acids sequence of this PCR product showed similarities with 92% *Pisum sativum* (U29243), 90 % *Nicotiana tabacum* (DQ350353) and 86% *Arabidopsis thaliana* (U37235) complete coding sequences (Fig. 4). After that we named this sequence as AcC4H, and it was deposited in the GenBank database (Accession no. GQ844863) (Fig. 4). Deduced amino acid analyses showed that AcC4H is highly similar [385/444 amino acid (86%)] to trans-cinnamate 4-monooxygenase (EC 1.14.13.11). Besides, it contains featured sequences found in plant P450 proteins and conserved residues found in C4H proteins. SOPMA analysis indicated that the deduced putative AcC4H contained 207 α-helix, 26 β-turns, 64 extended strands, and 163 random coils in its secondary structure. Geno3D analysis also indicated

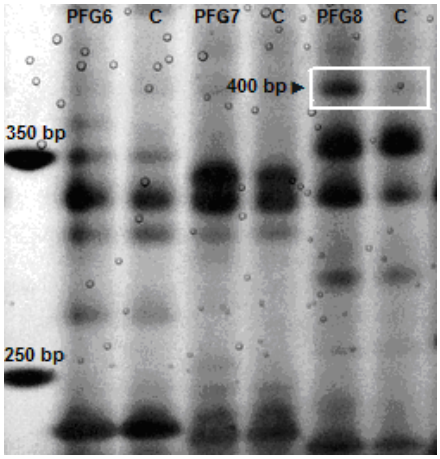

Fig 2. Representative band patterns on differential display (DD) analysis showing differentially regulated PCR fragments. Total RNA was extracted from *A.chrysochlorus* cell suspension cultures incubated with the yeast extract for 6 h or water (as control) and subjected to DD analysis. A non-denaturing 6 % polyacrylamide gel post-stained with silver staining. Primer combinations used were as: PFG6-8 and T11VC; C: Control (Table 1). Arrow in 400 bp indicates the signal demonstrating altered expression of putative AcC4H. DNA size standards are shown in bp on the left.

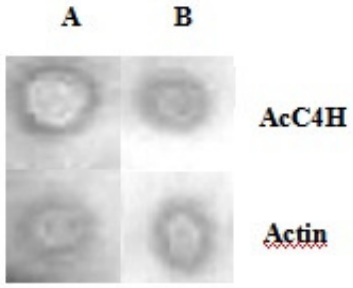

Fig 3. Conformation of the differentially expressed cDNA fragments by dot blot analysis. (A) RNA dot blot of 6h yeast extract untreated control cells, (B) RNA dot blot of 6h yeast extract treated cells. *Actin* gene is included as a housekeeping gene control.

that AcC4H belongs to cytochrome P450 monooxygenase family.

Elicitor-induced expression of AcC4H

Quantitative RT-PCR analysis was used to study expression patterns of putative C4H gene in *A. chrysochlorus* under yeast extract stress. Elicitor treatment caused 13,24-fold higher accumulation of the AcC4H transcript in cell cultures (Fig. 5). This result was concurring with those obtained by differential display and dot blot.

Discussion

In this work, we have isolated a gene fragment encoding C4H from 6 hour yeast extract treated *A. chrysochlorus* suspension cells by using mRNA differential display. We used [PFG] motif specific primer as described in Schopfer and Ebel (1998) to obtain predominantly P450 specific cDNAs. [PFG] motif is located 300-400 nucleotides upstream of the polyadenylation site. Using of this specific primer allow rapid confirmation of the P450 fingerprint. Different biotic and abiotic stressors may cause the up regulation of P450 genes around 6-12 h incubation time (Suziki et al., 1996; Yamada et al., 2000). Thus, the corresponding transcript level was expected to be elevated at about 6 h post-elicitation. Between the biotic elicitors, yeast extract plays an essential role in the elicitation procedure, causing either immediate or circuitous activation of the genes involved in secondary metabolism. For example, using P450 targeted DD technique yeast extract treated cells resulted in induction of glyceollin through up-regulation of phenylpropanoid pathway enzymes such as C4H (Schopfer and Ebel 1998; Latunde –Dada et al., 2001). In Taxus, the cDNA encoding a cytochrome P450 enzyme, taxane 10 *b*-hydroxylase, involved in taxol biosynthesis was isolated using DD to examine cell cultures (Jennewein et al., 2001). C4H plays a key role in both lignin biosynthesis and plant defense through flavonoid biosynthesis (Liu et al., 2009). cDNA encoding C4H have now been cloned for the first time from *A. chrysochlorus*. The transcripts of putative AcC4H in *A. chrysochlorus* was induced in response to yeast extract as an elicitor treatment.

The expression of putative AcC4H was examined after 6 h following treatment with 10 g/L concentration of yeast extract. The expression of AcC4H gene was obviously increased after treatment with yeast extract, with the expression 13,24-fold changes being observed in the 6h yeast extract treated cells. So far, only *Astragalus membranaceus* var. Mongholicus (DQ371297) and *Astragalus mongholicus* (HQ339960) C4H gene sequences were isolated. The tblastx result demonstrated that the deduced amino acid sequence of putative AcC4H shares 96% identity with C4H from *A. membranaceus* and *Astragalus mongholicus*. According to the bioinformatic analysis, putative AcC4H contains featured sequences found in plant P450 proteins and conserved residues found in C4H proteins. Similarity in secondary structure with functional P450s, as indicated by Geno3D, revealed putative AcC4H could be functional and catalyses the required reactions. Developmentally regulated C4H expression in plants is correlated with lignification and other sites of active phenylpropanoid metabolism. Such results are in agreement with reports exhibiting a positive relationship between the accumulation of the flavonoids and in the activity of respective enzymes and genes (Singh et al., 2009). In conclusion, the sequence and expression analyse of putative AcC4H presented in this study establish the foundation for elucidating the phenylpropanoid biosynthesis pathway in *A. chrysochlorus* in more detail. Further researches such as the investigation of the function of the putative AcC4H in an appropriate host and after that overexpression of this gene could lead to not only a better understanding of plant metabolism but also potential applications to increase production of bioactive compounds.

Materials and methods

Plant material and elicitor treatment

A. chrysochlorus primary cell suspension cultures were propagated in the dark as described by Cakir and Ari (2009). For induction experiments, 5 ml culture (with 1 ml packet

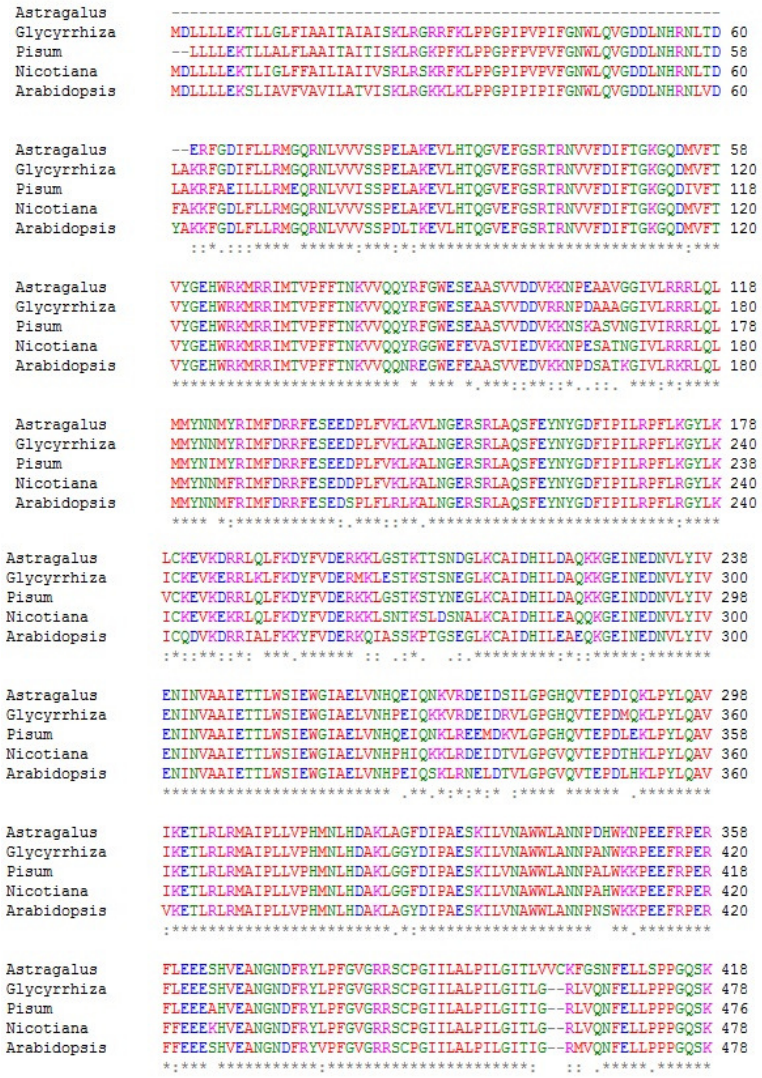

Fig 4. Sequence alignment of the deduced amino acids of AcC4H (Accession no. GQ844863) with those of releated proteins. Conserved residues are shaded in the same colour. "*" means that the residues in that column are identical in all sequences in the alignment. ":" means that conserved substitutions have been observed. "." means that semi-conserved substitutions are observed. *Astragalus*, *A. chrysochlorus* C4H (GQ844863); Glycyrrhiza, *Glycyrrhiza echinata* C4H (D87520); Pisum, *Pisum sativum* C4H (U29243); Nicotiana, *Nicotiana tabacum* C4H (DQ350353).

cell volume) from the primary cell suspension culture was subcultured in 25 ml fresh MS medium (Murashige and Skoog 1962) consisted of 3% sucrose and 1 mg/L 2,4-Dichlorophenoxyacetic acid (2,4-D) in 100 ml Erlenmeyer flask. Elicitor yeast extract was prepared by ethanol precipitation as described by Chen and Chen (2000). 10 g/L concentration of yeast extract was added on the 13th day (first day of log phase) of cell culture according to Cakir and Ari (2009). The same amount of water was added to the control cultures. Cultures were treated with yeast extract for 6 hours.

Non-radioactive Differential Display PCR

Transcriptional changes were investigated using modified differential display technique (Schopfer and Ebel 1998) which was originally developed by Liang and Pardee 1992. Total RNA was isolated from yeast extract treated and untreated cell suspension cultures and treated with DNaz I (Promega, Z 3100). Complementary DNA was synthesized from total RNA by using reverse transcriptase Superscript II (Invitrogen, 11917-010). As described by Schopfer and Ebel (1998) P450-specific upstream primers (Table 1) instead of arbitrary primers were used in combination with three anchored oligo(dT) primers (T11VA, T11VC, T11VG; V indicates a mixture of A, C and G). PCR reaction contained 1x reaction buffer [10 mM Tris-HCl (pH 8.8), 50 mM KCl, 0.1 %Triton X-100], 0.2 mM dNTPmix, 2 mM $MgCl_2$, 0.5 µM PFG primer, 2.5 µM oligo (dT) primer, 3 % DMSO, 2 µl cDNA, 1 U Taq DNA polymerase and add MQ water was added up to 40 µl. The PCR cycling parameters were set as follows: 94 ºC for 30 s, 40 ºC for 2 min, 72 ºC for 30 s, 40 cycles; 72 ºC for 5 min, 1 cycle. PCR products were separated on 6 % non-denaturing polyacrylamide gels. After the gel was pre-run for 30 min, electrophoresis was performed in 1XTBE buffer at 160 V for 18 h. cDNA bands were visualized by silver staining as described by Tullo and

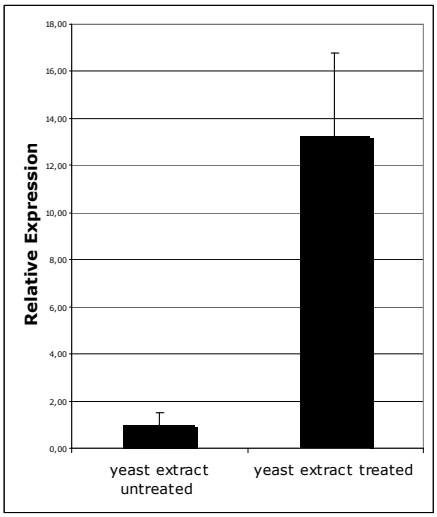

Fig 5. Quantitative RT-PCR result of putative AcC4H. Effect of 6 hour yeast extract treatment on the expression of putative AcC4H gene. The average fold changes in the expression levels was calculated relative to *actin*. Statistical significance was evaluated by Student's *t*-test ($P < 0.05$).

Sbisa (2002). Bands of interest were excised from the polyacrylamide gels and DNAs were recovered as described by Abbaszadegan and his colleagues (2004). Eluted DNA (5 µl) was re-amplified directly under identical conditions as in the initial PCR (except for the number of cycles, 35 instead of 40) with the corresponding anchored oligo(dT) and P450-specific upstream primers. PCR products were checked on 2% agarose gel and purified by gel extraction method using High Pure PCR Product Purification Kit (Roche, 11732668001).

Dot Blot and Cloning

Dot blot hybridization was performed using the DNA Labeling and Detection (DIG) Kit according to the manufacturer's instructions (Roche Diagnostics, Germany, 11175041910). A DIG-11-dUTP labeled DNA fragment carrying the *actin* was used as inner control to normalize labeling reaction. Differentially expressed putative P450 was used for probe synthesis. The RNA samples (20 µg) were blotted on a nylon membrane. After air drying, the membrane was baked at 80°C for 15 min. The probe labeling, hybridization and immunological detection were performed according to the instruction manual of DIG DNA labeling and detection kit. The cDNA fragments of interest were cloned into pCR®2.1-TOPO® vectors according to the manufacturer's protocol (Invitrogen, K4560-01).

Searching the full length of the AcC4H by using comparative approach

The comparative approach works with the alignment of cDNA sequences from phylogenetically related species and outputs a list of possibly degenerate primer pairs. Using this approach, primer pairs could be designed for PCR amplification of cDNAs of species which have not got any available sequence information. Such PCR-based strategies for the identification of unknown gene sequences require the design of primers from multiple sequence alignments, focusing on conserved and variable regions (Fredslund et al., 2005). According to this knowledge, we made multiple alignment between legume C4Hs (*Glycine max, Glycyrrhiza echinata, Pisum sativum, Vigna radiata*). By using that data and the sequence data coming from differential display, we designed a C4H specific primer pair (AcC4HP1: 5'-AACTGGCT(C/G)CA(A/G)GTCGGCGA(C/T)GA-3' and AcC4HP2: 5'-AGGAGGAGACAAAAGCTCGA-3'). This primer pair makes it possible to obtain 1338 bp length cDNA of AcC4H. The PCR cycling parameters were set as follows: 94 ºC for 5 min, 1 cycle; 94 ºC for 30 s, 53 ºC for 30 s, 72 ºC for 1 min, 30 cycles; and 72 ºC for 10 min, 1 cycle. The band of proper size was purified from 1.5 % agarose gel, and cloned into the pCR®2.1-TOPO® vector and sequenced.

Quantitative expression analysis by Real-Time PCR (qRT-PCR)

For qRT-PCR analyses, first-strand cDNA was used as template. RT-PCR was performed with the DyNAmo™ HS SYBR® Green qPCR Kit (Finnzymes) following the manufacturer's instructions: Reaction mixtures consisting of 12,5 µl SYBR Green Master, 0.2 µM of each of forward and reverse primers, 5 µl cDNA were brought to 25 µl with ultra pure nuclease-free water. The PCR conditions were set as follows: 50°C for 2 min (UNG incubation), 95°C for 15 min (pre-incubation), followed by 40 cycles of 94°C for 10 sec, 60°C for 30 sec, and 72°C for 30 sec. The final step included gradual temperature increase from 55°C to 95°C at the rate of 1°C/10 seconds to enable melting curve data collection. A non-template control was run and serial dilutions (1, 1:10 and 1:100) of the reference (*actin*) and the target genes were included with every assay. Amplification specificity of each reaction was verified by melting curve analysis. Expression levels were normalized against the reference gene, *actin*. Relative gene expression was determined according to the method of Pfaffl (2001). Triplicate qRT-PCR experiments were performed for each sample.

Bioinformatics analysis

Sequencing reactions were performed commercially by Iontek A.S. /Turkey, on ABI PRISM 310 Genetic Analyzer using DYEnamic ET Terminator Cycle Sequencing Kit (Amersham). The nucleotide sequences were subjected to BLASTX and BLASTN searches at the NCBI database. Sequence alignments with E value less than 10^{-4} were considered significant. Secondary structure of deduced protein sequence was predicted by SOPMA (http://npsa-pbil.ibcp.fr). Secondary structures similarity was predicted by Geno3D (http://pbil.ibcp.fr).

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