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Isolation and Characterization of CPRgene Promoter from *Artemisia annua* by Thermal Asymmetric Interlaced Polymerase Chain Reaction (TAIL-PCR)

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ABSTRACT

Malaria, an infectious diseases, couses in almost 1 million deaths each year over the world. Artemisinin, a sesquiterpene lactone endoperoxide, is one of the most effective antimalarial drugs purified from *Artemisia annua* L. in China, 1970s. As the low content of this compound in plant many of studies have been focused on using elicitors affecting gene expression involved in artemisinin synthesis pathway. The main step to enhance artemisinin content in plant by using elicitors is characterization of key genes promoter. Cytochrome p450 Reductase (CPR) is one of the key enzymes that plays an important role in artemisinin synthesis pathway. Promoter sequence of key genes involving in artemisinin biosynthesis pathway included ADS, CYP71AV1 and DBR2 was isolated except CPRgene. We used standard Thermal Asymmetric Interlaced Polymerase Chain Reaction (TAIL-PCR) with some modification in thermal cycle numbers to isolate the unknown 5' flanking region of the CPRgene from *Artemisia annua*. Subsequent bioinformatics analysis to characterize functional cis-acting elements inside the promoter was performed. The 5' flanking sequence of CPR was cloned in pGEM-T Easy vector and sequenced. Subsequent sequence analysis for characterize functional motifs using bioinformatics software indicated a group of putative cis-acting elements such as TATA box, CAAT box, G box, W box and etc., inside the CPR promoter. This sequence was submitted in GenBank databases under the accession number KC243135. Present study demonstrated that characterization of cis-acting response elements can facilitate using elicitors to enhance artemisinin production in plant.

Key words: *Artemisia annua*, artemisinin, TAIL-PCR, CPR

INTRODUCTION

Artemisia annua, a plant of composite family, is one of the most important plants traditionally used in China to treat fevers and as a source of essential oils for therapeutic purposes (Ferreira and Janick, 2009). Medical importance of *Artemisia annua* is mostly because of the well-known secondary metabolite, artemisinin (Teoh *et al*., 2006; Duke *et al*., 1987). In addition, *A. annua* leaves and subcuticular space of the glands have been reported to be a good source of essential oils include artemisinin and its derivates (Zheng and Wang, 2001; Van Nieuwerburgh *et al*., 2006). Artemisinin, known Qinghaosu in China, is an important natural sesquiterpene lactone with antimalarial effect (Klayman, 1985) against susceptible and multi-drug resistant *Plasmodium* spp.

(White, 2008). Current researchs also indicates that artemisinin drugs have effect against cancer (Nakase *et al*., 2008; Singh and Lai, 2004; Noori *et al*., 2013), Trypanosoma (Mishina *et al*., 2007), Leishmania (Sen *et al*., 2007), Schistosomiatic (Borrmann *et al*., 2001), Bacteria (Goswami *et al*., 2012) and some viruses like Hepatitis B (Romero *et al*., 2005) and HIV (Jung and Schinazi, 1994). The structure of artemisinin was determined by X-ray analysis (Misra *et al*., 1993). Artemisinin's structure suggests that antimalaria activities of this compound is because of an endoperoxide bridge (Sarina *et al*., 2013). The Endoperoxide Bridge inartemisinin is cleaved by free iron, leading to the generation of an unstable radical of the drug and in next step causes selective alkylation of malarial proteins, leading to death of the parasite (Robert and Meunier, 1998). In contrast to traditional antimalarial drugs, artemisinin acts quickly and causes minimal adverse side effects (Meshnick, 2002; Yamachika *et al*., 2004). As a response to the widespread antimalariadrug resistance to chloroquine, WHO recommends combination therapies, especially those containing an artemisinin derivatives (Artemisinin-based Combination Therapy, ACT) (WHO, 2008). The combination of artemisinin with other antimalarial drugs are widely used in the world. This eventuates one of the most significant public health developments in malaria control (Zurovac *et al*., 2007). In order to enhance artemisinin content in the plant, there have been many efforts to increase the production of artemisinin. Such methods of increasing artemisinin content, e.g., *A. annua* organ culture and suspension cultures (Ali *et al*., 2012; Baldi and Dixit, 2008), stimulating hormone medium (Weathers *et al*., 2005) and metabolic engineering have been performed (Zhang *et al*., 2009).

In recent years, many researchers have focused their efforts on investigating the use of elicitors to enhance artemisinin biosynthesis and the genes expression coding for the key enzymes involving in the artemisinin biosynthesis (Yin *et al*., 2012; Putalun *et al*., 2007; Zheng *et al*., 2008). To increase the yield of artemisinin by elicitors, it is necessary to study the promoter cis-acting response elements of key genes contributing in artemisinin biosynthesis pathway. Characterization of these sequences lead to an understanding about functional motifs affecting gene expression in response to specific elicitors. The Artemisinin biosynthesis pathway is well established (Towler and Weathers, 2007) and CPRgene is one of the most important genes in artemisinin biosynthesis pathway (Zeng *et al*., 2008). Therefore identification of CPR promoter cis-acting elements is a useful step toward determining elicitors that play a key role in the signal transduction pathway leading to increase gene expression in artemisinin biosynthesis pathway.

The purpose of this study is to characterize cis-acting response elements of CPRgene promoter from *Artemisia annua* and subsequent analyzing with bioinformatics software.

MATERIALS AND METHODS

Plants material and extraction of total DNA: Seeds of *A. annua* were collected from Biological Resource Center of Iran (Accession No. P1000060) and the plants were grown in the greenhouse. Total genomic DNA was isolated from young fresh leaves of greenhouse-grown plants by common CTAB buffer and purified with phenol-chlorophorm-isoamylalchol described by Kump and Javornik in 1996 and directly used in PCR-mediated DNA amplifications.

TAIL-PCR: Thermal Asymmetric Interlaced Polymerase Chain Reaction (TAIL-PCR) developed by Liu and Whittier (1995) uses reverse nestedthree gene-specific primers with shorter arbitrary degenerate primers as forward. We used six degenerate 10 mer primers instead of degenerate 16 mer short primers as an alternative procedure (Table 1). Reverse nested gene-specific

Table 1: Amplification primers used in TAIL-PCR reactions

Sequence of primer (5'→3')	Primer name
TCGGCACAACAATCACCGGCGA	TAIL-1
ACAAGCACCACAACGCATCCGA	TAIL-2
ACGATTCTCCATAAACACGGCT	TAIL-3
GGTGCTCCGT	AD-1
CGATGAGCCC	AD-2
CTATCGCCGC	AD-3
CGCAGACCTC	AD-4
GTGTGCCCCA	AD-5
ACGGTG CCTG	AD-6

Table 2: Thermal condition for TAIL-PCR cycles

Reaction	Program No.	Cycle No.	Thermal parameters
Primary round PCR	1	1	95°C, 4 min
(AD/TAIL-1)	2	3	94°C, 1 min, 58°C, 1 min, 72°C, 2 min
	3	2	94°C, 29 sec, 30°C, 2 min, ramping to 72°C over 3 min, 72°C, 2.5 min
	4	15	94°C, 30 sec, 62°C, 1 min, 72°C, 2 min, 94°C, 10 sec, 62°C, 1 min, 72°C, 2 min, 94°C, 30 sec, 29°C, 1 min, 72°C, 2.5 min
	5	1	72°C, 5 min
Secondary round PCR (AD/TAIL-2)	6	12	94°C, 30 sec, 60°C, 1 min, 72°C, 2 min, 94°C, 30 sec, 60°C, 1 min, 72°C, 2 min, 94°C, 30 sec, 29°C, 1 min, 72°C, 2.5 min
	5	1	72°C, 5 min
Tertiary round PCR	7	20	94°C, 30 sec, 29°C, 1 min, 72°C, 2.5 min
(AD/TAIL-3)	5	1	72°C, 5 min

primers,TAIL-1:5'-TCGGCACAACAATCACCGGCGA-3', TAIL-2: 5'-ACAAGCACCA CAACGCATCCGA-3' and TAIL-3: 5'-ACGATTCTCCATAAACACGGCT-3' were designed according to the known 5' sequence of CPR cDNA of *A. annua*.

Three rounds of PCR were carried out on a Biorad Thermal Cycler using the product of the previous PCR as template for the next PCR reaction and performing a common arbitrary degenerate primerand other nested gene-specific primers respectively. The primary PCR was carried out in a 20 μL volume containing 100 ng of genomic DNA, 0.2 μM gene-specific primer (TAIL-1 primer), 2.0 μM 10 mer primer, 200 μM of each dNTP, 0.2 U high fidelity LA Taq DNA polymerase (TaKaRa CN: RR02AG) and 1×Taq polymerase buffer supplied with the enzyme. The secondary PCR was carried out with gene-specificTAIL-2 primer in combination with the same arbitrary degenerate primer as used in the primary PCR. The reaction solution for PCR was the same as for the primary PCR, except that 2 μL of a 1/20 dilution of the primary PCR product was used as template. For the tertiary PCR, the reaction solution for PCR was the same as for the primary PCR except that 1 μL of a 1/10 dilution of the secondary PCR product was used as template.

Standard TAIL-PCR (Liu and Whittier, 1995) with some modification (Table 2) amplifies region flanking known sequence through three amplification reactions using nested primers complementary to known CPR5' region sequence and Arbitrary Degenerateprimers (AD). Specific product yield of primary TAIL-PCR was not enough to be visible on the gel and the high specific product obtained at the secondary and tertiary reactions.

The products of the secondary and tertiary PCR were separated in adjacent lanes on 1% agarose gels and when it was found that their size difference corresponded to gene-specific primerpositions (39 bp), the target fragment was recovered and cloned into pGEM-T Easyvector (Promega Corporation, Madison, WI) and sequenced.

After sequencing fragment, promoter and putative transcription start site was predicted using Neural Network Promoter Predictions. (http://www.fruitfly.org/seq_tools/promoter.html).

Analysis for cic-acting response elements was done with PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html) (Lescot *et al*., 2002) and PLACE (http://www.dna.affrc.go.jp/PLACE/signalscan.html) (Higo *et al*., 1999) databases. The isolated sequence has been submitted in GenBank databases under the accession number KC243135.

RESULTS

Isolation of upstream DNA sequence of CPR: The 802 bp fragment isolated by Thermal Asymmetric Interlaced Polymerase Chain Reaction (TAIL-PCR) using three gene-specific primers as reverse and six arbitrary degenerate 10 mer primers as forward. Aligned DNA sequences shows that it was about 107 bp at 3' end of this fragment overlapped with the 110 bpat 5' end of CPR cDNA (data not shown). This confirmed that the upstream 5' region of CPRc DNA was isolated correctly.

Primary confirmation was obtained with size difference between secondary and tertiary TAIL-PCR products corresponded with gene-specific TAIL-2 and TAIL-3 primers distance at exon one of CPRc DNA sequence (Fig. 1). One of the tertiary TAIL-PCR reactions (TAIL3-AD1) as a sharp band, indicated by a white arrow, selected and after cloning in pGEMT-Easy vector sequenced.

Sequence bioinformatics analysis: Isolated sequence was analyzed with Neural Network Promoter Predictions and putative transcription start site, +1 position, predicted at 67 bp upstream to the initiation translation code. This fragment contained 695 bp upstream to the initiation translation ATG-codon, include da group of cis-acting response elements. Most important cis-acting elements predicated by PlantCARE and PLACE databases inside CPRgene promoter with their function are shown in Table 3. The position of indicated cis-acting elements in CPRgene promoter also shown in Fig. 2.

Table 3: Cis-acting elements inside CPRgene promoter

Cis-acting element	Function
TATA box	Core cis-acting element of eukaryotic promoters
CAAT box	Common cis-acting element in eukaryotic promoters
ABRE	Cis-acting element involved in the abscisic acid responsiveness
ARE	Cis-acting regulatory element essential for the anaerobic induction
G box	Cis-acting regulatory element involved in light responsiveness
I box	Part of a light responsive element
TCA element	Cis-acting element involved in salicylic acid responsiveness
TGA element	Auxin-responsive element
MBS	MYB Binding Site
CCAAT box	MYBHv1 binding site
GT1 motif	Light responsive element
GATA box	Light responsive element
LTRE	Low-temperature-responsive element
W box	Fungal elicitor responsive element

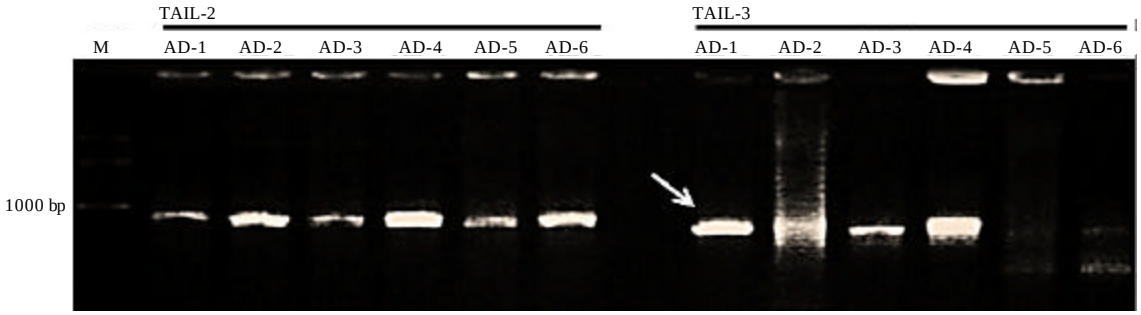

Fig. 1: 1.1% Agarose gel analysis of secondary and tertiary TAIL-PCR. Size difference between secondary and tertiary products was related with TAIL-2 and TAIL-3 primers position. The band indicated with white arrow was isolated from gel and sequenced, M: Size maker, AD: Arbitary digemerit primers

Fig. 2: The 5' flanking region of CPRgene with cis-acting elements position inside promoter, "+1" indicates the predicated transcription start site, 1: TATA box, 2: CAAT box, 3: W box, 4: G box, 5: I box, 6: Myb Binding Site (MBS), 7: ARE, 8: LTRE, 9: TGA elements and 10: ABRE

DISCUSSION

The TATA box motif with consensus TATAA sequence which is fundamental in eukaryotic promoters, located at -29 bp upstream transcription start sequence. This motif usually locates about -25 to -30 bp upstream of the transcription start site in eukaryotic promoters (Kiran *et al*., 2006) involving in the initiation process of transcription by RNA polymerase. There was six CAAT box at -50, -98, -159, -198, -313 and -481 positions. CAAT box is a common regulatory motif in eukaryotic promoters that is critical in regulation of transcription initiation (Song and Wang, 2009). One of the cis-acting regulatory founded by PlantCARE database, was G box. The G box (CACGTG) is

Table 4: Common motifs in ADS, CYP71AV1, DBR2 and CPRgenes

Cis-acting element	Function
TATA box	Core cis-acting element of eukaryotic promoters
CAAT box	Common cis-acting element in eukaryotic promoters
W box	Fungal elicitor responsive element
G box	Cis-acting regulatory element involved in light responsiveness
TGA element	Auxin-responsive element

a well-conserved motif found in plant genes promoter and contribute in light response gene expression (Menkens *et al*., 1995). This sequence can efficiently bound by a family of transcription factors known GBF (G box Binding Proteins) that have well-known bZIP domains (Donald and Cashmore, 1990). There was several other light responsive elements in CPR promoter such as I box, GT1 motif, GATA box and TCT motif that indicated light is one of the most important signal in CPR expression in artemisinin biosynthesis pathway. Wang *et al*. (2001) found that light spectrum would influence biomass and artemisinin content of transformed hairy roots of *Artemisia annua*. The 5' flanking region of CPR was also contained several functional W box motifs involved in fungal elicitors response gene regulation (Rushton *et al*., 1996). W boxe motifs have interaction with WRKY transcription factors and frequently cluster within eukaryotic short gene promoters (Fukuda and Shinshi, 1994). Stimulation of artemisinin production by fungal elicitors have been reported (Liu *et al*., 1999; Wang *et al*., 2001). It was found a TGA motif in CPR promoter by PlantCARE database that play an important role in response to auxin hormone (Liu *et al*., 1994). Our results also showed that MBS motif, bound by MYB domains family, was located at -373 upstream to transcription start site. MYB transcription factors family are related in the control of cell cycle in animals and higher eukaryotics (Lyon *et al*., 1994) and in plants they have a key role in response to viral infection (Yang and Klessig, 1996) and phytohormones e.g., Gibberellin and Abscisic acid (Gubler *et al*., 1995; Abe *et al*., 2003). The stimulating effect of these phytohormones in artemisinin production have been reported (Weathers *et al*., 2005).

The cis-acting elements of ADS (Kim *et al*., 2008), CYP71AV1 (Wang *et al*., 2011) and genes involving in artemisinin production, have been isolated and further characterization by bioinformatic analysis indicated that there are some funtional response elements in these genes promoter. Finally in this study we compared these genes cis-acting elements and CPR promoter together. In addition to TATA box and CAAT box, we found three other functional motifs which are common in these four genes (Table 4). This fact can help us to use elicitors affecting the four ADS, CYP71AV1, DBR2 and CPR key genes expression contributing in artemisinin biosynthesis pathway in *Artemisia annua*.

CONCLUSION

As the importance of cis-acting elements located inside promoter regions, it is necessary to find and characterize functional motifs responding special elicitors to regulate gene expression in eukaryotic organisms. *Artemisia annua* is a worthwhile plant for engineering artemisinin production. There are some information about regulatory regions of key genes involved in artemisinin biosynthesis in databases bank but not about CPRgene. In this study we isolated 695 bp upstream to initiation translation codon of CPRgene from *Artemisia annua* using modified TAIL-PCR procedure. Subsequent analysis with bioinformatic databases such as PlantCARE and PLACE indicates that there are several functional motifs involved in specific elicitors responding e.g., light, hormones, salicylic acid etc. It is a helpful step to understand which elicitors are affecting

gene expression enhancement in artemisinin biosynthesis pathway and we can use these elicitors to increase artemisinin production in *Artemisia annua* plant. The isolated sequence has been submitted in GenBank databases under the accession number KC243135.

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