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The presence of amorpha-4, 11-diene synthase, a key enzyme in artemisinin production in ten *Artemisia* species

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ABSTRACT

Background and the purpose of the study: Artemisinin is one of the most effective medicine against malaria, which is produced naturally by *Artemisia annua* in low yield. It is produced in a metabolic pathway, in which several genes and gene products are involved. One of the key genes in this pathway is *am1*, which encodes amorpha-4, 11-diene synthase (ADS), a key enzyme in artemisinin biosynthesis pathway. The aim of this study was to determine the presence of this gene in ten *Artemisia* species in order to increase the yield of production of Artemisinin.
Methods: The experiments were carried out using PCR. Specific primers were designed based on the published *am1* gene sequence obtained from *A. annua* (NCBI, accession number AF327527).
Results: The amplification of this gene by the specific primers was considered as a positive sign for the potentiality of artemisinin production. Since the entire *am1* gene was not amplified in any of the 10 species used, four parts of the gene, essential in ADS enzyme function, corresponding to a) pair site of Arg10-Pro12 in the first 100 amino acids, b) aspartate rich motif (DDXXD), c) active site final lid and d) active site including farnesyl diphosphate (FDP) ionization sites and catalytic site in the ADS enzyme, were investigated.
Major conclusion: The sequence corresponding to ADS active site was amplified only in *A. annua, A. aucher*i and *A. chamaemelifolia*. The negative results obtained with other species could be due to some sequence alteration, such as point mutations or INDELs. We propose *A. aucheri* and *A. chamaemelifolia* as two potential candidate species for further characterization, breeding and transferring *am1* gene for artemisinin overproduction.

Keywords: amorpha-4,11-diene synthase, *Artemisia* (sweet wormwood), artemisinin, Asteraceae, PCR.

INTRODUCTION

Human malaria is an important cause of mortality in tropical regions and have been reported to affect 300–500 million people worldwide annually. Infection with *Plasmodium falciparum* accounts for 200–250 million cases, of which around 2 million are fatal (1). *Artemisia annua* L. is the main source for Artemisinin extraction. At the present, the World Health Organization (WHO) has recognised this sesquiterpene 3-oxane and its derivates (arteether, artesunate, artemether) obtained from *A. annua* as one of the most effective medicines for curing malaria. Artemisinin is a novel drug in the treatment of malaria, especially in those areas where resistance to quinine derivatives is common (2, 3). Artemisinin is the last effective drug against deadly resistant strains of *P. falciparum* (4). The first step in artemisinin biosynthesis is the cyclisation of farnesyl diphosphate (FDP) by amorpha-4,11-diene synthase (ADS) (5). At the present time the extraction of artemisinin from *A. annua* plants seems to be the only source of this medicine (6). The chemical synthesis of artemisinin is not cost effective and the toxicity of by-products is also another problem (7, 8). Employment of cell and callus culture has not been promising, since a certain level of differentiation is required for artemisinin production (9). The yield of Artemisinin production by the plant *A. annua* is relatively low ranging from 0.01% to 0.5% on the basis of dry weight (6). An alternative for the production of artemisinin in higher amounts have been proposed as the selection and breeding of high artemisinin yielding cultivars (10). In this investigation presence of ADS encoding gene (*am1*) in ten different species of *Artemisia* growing in Iran was examined to discover potential candidate species for further characterization, and possibly *am1* gene transfer to increase artemisinin.

MATERIALS AND METHODS

Plant materials

The seeds of all *Artemisia* species except *A. draconculus,* were gifted by Dr. Razban (Research

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Table 1. *Artemisia* species used in this study.

Species	Collection place	Specimen* No.	Species	Collection place	Specimen No.
A. annua	Gorgan	1595	*A. fragrance*	Jolfa	19969
A. aucheri	Yazd	4500	*A. cina*	Karaj	923
A. siberi	Isfahan	10132	*A. austerica*	Sarab	2953
A. vulgaris	Urmiah	3709	*A. draconculus***	---------	----------
A. scoparia	Zanjan	1298	*A. chamaemelifolia*	Tabriz	15385

* The specimen numbers are available in the Research Institute of Forests and Rangelands of Iran.
** This species was purchased from local shops.

Table 2. Primers designed according to *am1* gene sequence.

Primer	Sequence 5' to 3'	Amplification region	Tm ºC
F1	CCTCCTTCAACCGTTACCCCG	Arg_{10}-Pro_{12} site	75
R1	GCGAGAAGGATACCAAGGCAG	Conserved sequence	73.2
F2	CTTCTCGCCAGTGGTAGGGTCA	Conserved sequence	75
R2	GAAGATACTCCCATCGACCCCT	Conserved sequence	73.2
F3	GCTAACGAACTTGCGAGGTAGA	FDP ionization site	71.3
R3	CGTTTCCTCCCTTCTTGTCTAG	Conserved sequence	71.3
F4	CGGACTTGGATCAGGGGTTTTC	Active site	73.2
R4	ATGGTTAGGAAGCACGTATCGG	Catalytic site	71.3
F5	GCTTAAAGGGAAACGGCAAC	Start point	68.9
R5	CATGATGTGTATAGCGTGC	Catalytic site	65.4

Institute for Forests and Rangelands; RIFR). The seeds had been collected during summer 2008, from different parts of Iran (Table 1). Seeds were sterilized in 5% (v/v) bleach for 10 min, followed by 3 times washings in double sterilised distilled water. For breaking seed dormancy, they were stored at 4°C for 72 hrs, then germinated in a 0.5 X MS medium (11), supplemented with 3% (w/v) sucrose of pH 5.9 and 0.8% (w/v) plant agar. After germination, seeds were subcultured in 1 X MS medium containing 1.5% (w/v) sucrose, 1.5% (w/v) glucose and incubated at 25°C ± 1 and 16/8 h light/dark photoperiod with 55.5 µmol m^{-2} s^{-1} illumination, according to the report of Weathers *et al*. (12).

DNA extraction

Genomic DNA extraction from fresh medicinal plants such as *Artemisia* is a difficult task due to the presence of high amounts of secondary metabolites. A method (13) was chosen and carried out with some modifications to reduce the secondary metabolites and polysaccharides contents.

About 0.1 g of the leaf tissue was grounded to a fine powder by liquid nitrogen in a pestle and mortar. The powder was transferred into a microtube containing 350 µl of the extraction buffer [2% (w/v) CTAB, 100 mM Tris-HCl, 20 mM EDTA, 1.4 M NaCl, 2% (w/v) PVP, 2% (v/v) 2-mercaptoethanol] and 350 µl 8 M lithium chloride, both pre-warmed at 65 °C and incubated for 45 min, with occasional inversions at 10 min intervals. An equal volume of chloroform: isoamyl alcohol 24 : 1 was added and tubes were shaken at 50 rpm for 20 min. Microtubes were centrifuged at 13000 rpm (Labnet, UK) for 5 min at room temperature and supernatant was transferred into a new tube. To each tube were added, 0.15 M sodium acetate (5 M) and 0.6 vol ice cold isopropanol and then inverted gently, untill DNA bundles appeared. Tubes were transferred to a -20 °C freezer and left for one hour and then centrifuged at 13000 rpm (Labnet, UK) for 10 min and supernatant was poured out. An aliquot of 300 µl of 70% ethanol (v/v) was added to the pellets and centrifuged at 13000 rpm (Labnet, UK) for 5 min. Supernatant was poured out, pellet were air dried and 10-50 µl sterilized double distilled water was added to each tube. DNA samples were stored at -70 °C for future uses.

Primers

Primers were designed purely based on the DNA sequences (NCBI accession number AF327527) on the *am1* gene (with the length of 4392 bp) corresponding to amino acid residues, important for ADS function (14). Also conserved sequences in mono and sesquiterpene synthases (3, 4), and sesquiterpene cyclases in dicotiledons (2) were considered in primer design (Table 2). Position of the primers on the *am1* gene is shown in figure 1.

Table 3. The summary of results obtained with different primer pairs on the *am1* gene.

	Artemisia species									
primer	*annua*	*austrica*	*aucheri*	*scoparia*	*chamaemelifolia*	*vulgaris*	*siberi*	*cina*	*fragrance*	*draconculus*
F1-R1	+	+	+	-	-	+	+	+	+	+
F3-R3	+	-	+	-	+	+	+	+	-	-
F3-R4	+	+	+	+	+	+	+	+	+	+
F3-R5	+	-	+	-	+	-	+	-	-	-
F4-R4	+	+	+	-	+	+	+	-	-	-
F4-R5	+	-	+	-	+	-	-	-	-	-

Following 4 parts were investigated in *am1* gene:
a) Pair site of Arg10-Pro12 in the first 100 amino acids using primers F1-R1. The expected amplification product was a 412 bp fragment.
b) Aspartate rich motif (DDXXD), using primers F2-R2. This motif is the binding site of substrate. Primers F2 and R2 lie on exons 2 and 3, respectively. The expected amplification fragment was 1870 bp long and includes the intronic part of the gene.
c) Active site, using primers F3-R3, F3-R4, F4-R4 and F3-R5. This site is formed by three amino acids; Asp 444, Tyr 520 and Asp 524 and includes FDP ionization and catalytic sites. Primers F3-R3, F3-R4 and F4-R4 amplify the FDP ionization site and primer F3-R5 amplify the catalytic site; the expected amplification bands are 1196 bp, 1750 bp, 997 bp and 2144 bp respectively.
d) Active site final lid, using F4 and R5 primers. This site is formed by three amino acids; Trp 271, Tyr 520 and Asp 524. Primer F4 sits around Trp 271 and primer R5 sits around Asp 524. The expected amplification band is 1544 bp long.

PCR conditions

PCR reactions were carried out in a total volume of 20 µl reaction mixture containing: 2 µl DNA polymerase buffer (Sib enzyme, Russia), 0.2 mM dNTPs (Sib enzyme, Russia), 20 pmol of each primer, 2 units of Taq DNA polymerase (Sib enzyme, Russia) and 50 ng of genomic DNA. Thermal cycler apparatus (Techne, Model TC512, UK) was programmed at 94°C for 4 min, 25 cycles of 94°C for 40 s, 56°C for 60 s, 72°C for 2 min and the final extension at 72°C for 8 min. Different annealing temperatures were used for each pair of primers according to Gene Runner software recommendations (Table 1). PCR products were run on a 1% (w/v) agarose gel along with a 1kb DNA size marker (Cinnagen), stained by ethidium bromide (0.5 µg/ml) and visualized in a gel documentation system (UVP, USA)

Digestion of PCR products

PCR products were digested by *Hin*dII and *Bgl*II enzymes. The cutting sites and the expected number of fragments were obtained using Gene Runner software. Reactions were carried out in a 20 µl final volume. Each reaction contained 1 µg DNA, 2 µl of buffer (10 X), 0.2 µg BSA, 1 unit of each of restriction enzymes (Sibenzyme, Russia) and distilled sterile water.

RESULTS

Amplification of entire *am1* gene in the 10 mentioned *Artemisia* species, was the main aim of this study. Nevertheless, despite applying different annealing temperatures, extension times and PCR components concentrations, this amplification did not happen.
In the amplification of aspartate rich motif (DDXXD), all species gave a 412 bp amplification band except *A. scoparia* and *A. draconculus* (Fig 2a). Aspartate rich motif is the binding site of substrate to ADS enzyme (14). The expected band (1870 bp) was not observed in any of the species. Several unspecific bands were amplified in *A. annua, A. austrica* and *A. chamaemelifolia* (data not shown).
The F3 primer position was designed around Arg 262 and R5 primer position around Tyr 520 and Asp 524 residues to find catalytic site. The expected amplification band was observed in *A. annua*, *A. aucheri*, *A. chamaemelifolia* and *A. siberi* (Fig. 2b). To investigate FDP ionization site, primers F3 and F4 were used. Primer F3 contains the Arg 262 position and Primer R4 corresponds to the positions of Arg 440 and Asp 444 on the ADS enzyme. R3 primer sits on a conserved sequence in terpene synthase genes (14). Primers F3-R4 amplified the 1750 bp fragment in *A. annua*, *A. siberi*, *A. austrica*, *A. vulgaris*, *A. scoparia*, *A. draconculus* and *A. cina* (Fig. 2c). F3-R3 primers could amplify the 1196 bp fragment in *A. annua*, *A. aucheri*, *A. chamaemelifolia, A. vulgaris, A. siberi and A. cina* (Fig. 2d). Primers F4-R4 amplified the 997 bp fragment in *A. annua*, *A. aucheri*, *A. chamaemelifolia*, *A. siberi*, *A. vulgaris* and *A. austrica* (Fig. 2e). By using primers F4 and R5, the expected 1524 bp fragment was observed only in *A. annua, A. aucheri* and *A. chamaemelifolia* (Fig. 1f). To ensure that the PCR fragments contained the correct sequences, the products of F3-R4 (FDP ionization site) and F3-R5 (catalytic

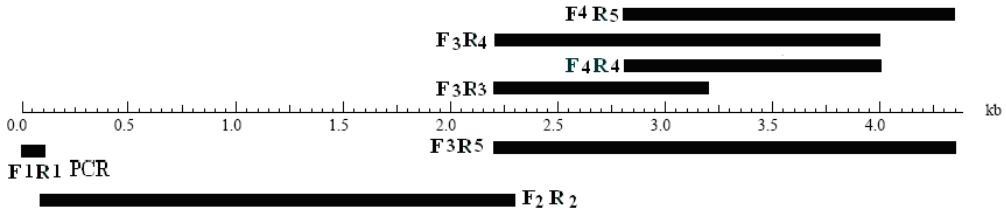

Figure 1. Primers positions in *am1* gene sequence and their expected products.

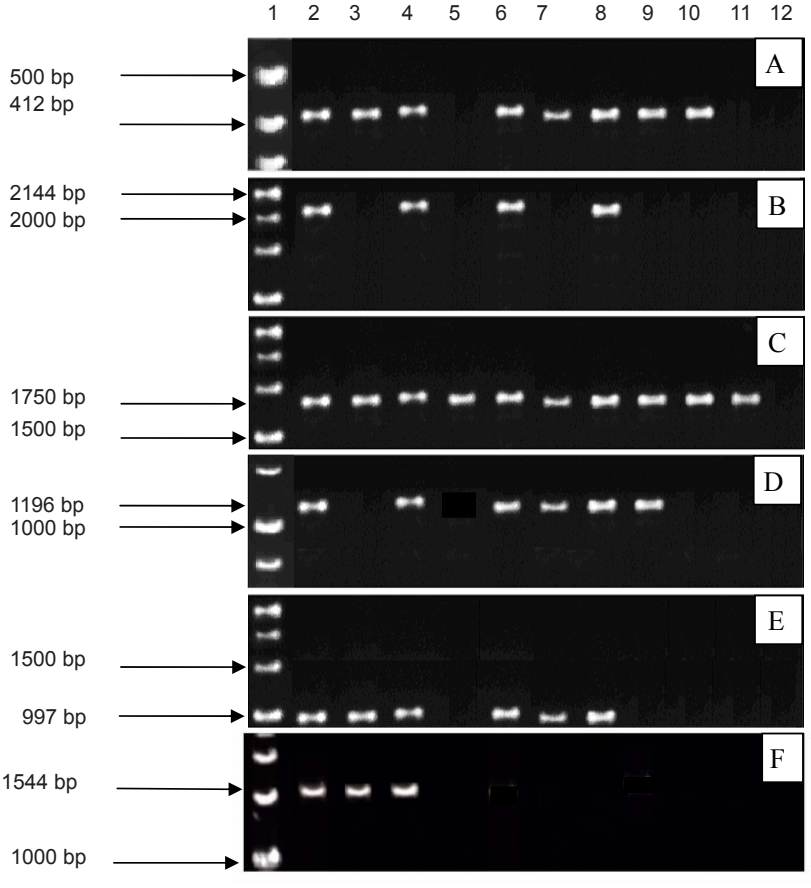

Figure 2. PCR products obtained from the designed primers based on the *am1* gene sequence with the following primer pairs: A) F1-R1 B) F3-R5 C) F3-R4 D) F3-R3 E) F4-R4 F) F4-R5 in 10 wild *artemisia* species. The lanes show in order (1) 1kb DNA size marker, (2) *A. annua*, (3) *A. austerica*, (4) *A. aucheri*, (5) *A. scoparia,* (6) *A. chamaemelifolia,* (7) *A. vulgaris*, (8) *A. siberi,* (9) A. *cina*, (10) A. *fragrance*, (11) *A. draconculus* and (12) negative control.

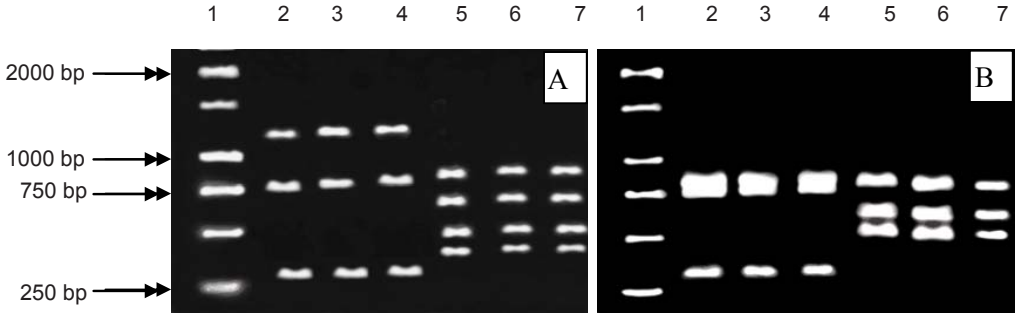

Figure 3. Restriction digestion of F3-R3 (A) and F3-R5 (B) amplification products. Lane (1), 1 kb DNA size marker, lanes 2-4, PCR products from *A. annua*, *A. aucheri* and *A. chamaemelifolia*, digested with *Bg*lII and lanes 5-7 PCR products from *A. annua*, *A. aucheri* and *A. chamaemelifolia* digested with *Hin*dII, respectively.

site) were amplified in *A. annua*, *A. chammelifoli* and *A. aucheri* were digested with *Bg*lII and *Hin*dII. The restriction fragments expected from F3-R4 product digestion with *Bgl*II were 802, 728 and 264 bp and the expected restriction fragments obtained from *Hin*dII digestion were 761, 575 and 458 bp long. The restriction fragments expected from F3-R5 digestion with *Bgl*II and *Hin*dII which also obtained were 1151, 728, 264 bp, and 575, 480, 327 bp long respectively. Indicating that correct sequences had been amplified (Figs. 3A, B). Results are summarized in (Table 3).

DISCUSSION

There are different medicines for treatment of malaria. A novel medicine on this respect is artemisinin, obtained from *A. annua*. This study was conducted to discover Artemisia species containing the *am1* gene, an important gene in artemisinin synthesis pathway. Since most of the studied species grow wildly in different areas, it was not possible to investigate artemisinin production in those species, especially at different growth stages. In addition, some of the species needed a stage of adaptation to the glasshouse or field conditions to grow. To solve each one of these problems, further studies were required. Therefore an initial study was conducted to investigate the presence of *am1* gene. PCR was employed and primers were designed to amplify the entire *am1* gene as well as 4 important sequences, essential for the correct ADS enzyme function. Positive results were obtained from several pairs of primers, amplifying 5', 3' and the middle part of the *am1* gene in some of the *Artemisia* species. Nevertheless, despite applying a wide range of PCR conditions, the whole gene (4392 bp) was not amplified. However, the primers designed to amplify both 5' and 3' ends of the *am1* gene, showed positive results. This means that forward and reverse primers were matching the corresponding sequences at the two ends of the gene, but probably changes in some sequences in the gene would not allow whole gene amplification. This may be as a result of INDELs somewhere in the *am1* gene sequence that prevented the whole gene amplification.

CONCLUSION

Result of this investigation showed that two *Artemisia* species contain the DNA sequences to encode the important parts of ADS enzyme for correct folding and function. In conclusion two *Artemisia* species, *A. aucheri* and *A. chamaemelifolia* are recommended for further investigation and possibly gene transfer with the aim of increasing artemisinin production.

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