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Original Article

Sequence characterized amplified region marker as a tool for selection of high-artemisinin containing species of *Artemisia*

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Abstract

Malaria is currently one of the most important causes of mortality in developing countries. High resistance to available antimalarial drugs has been reported frequently, thus it is crucial to focus on the discovery of new antimalarial drugs. Artemisinin, an effective antimalarial medication, is isolated from various *Artemisia* species. To identify the *Artemisia* species producing high quantity of artemisinin, eight species of *Artemisia* were screened with the genetic sequence characterized amplified region (SCAR) marker for higher quantity of artemisinin. The DNA band corresponding to SCAR marker was cloned into pGEM®-T Easy vector and sequenced. The content of artemisinin in tested species was also measured using high-performance liquid chromatography (HPLC) assay. The primers designed for high-artemisinin SCAR marker could amplify a specific band of approximately 1000 bp which was present in two *Artemisia annua* and *Artemisia absinthium* species. These SCAR marker sequences for two selected species were submitted into the GenBank databases under KC337116 and KC465952 accession numbers. HPLC analysis indicated that two selected *Artemisia* species, genetically recognized as high-artemisinin yielding plants, had higher artemisinin content in comparison to other examined species. Therefore, in this study, we propose developed SCAR marker as a complementary tool for confidently detection of high-artemisinin content in *Artemisia* species.

Keywords: *Artemisia absinthium*; *Artemisia annua*; Artemisinin; SCAR markers

INTERODUCTION

Malaria is one of the most important infectious diseases affecting approximately 300 to 500 million people annually worldwide (1) leading to the death of about 2 million people every year (2). Since *Plasmodium flaciparum* parasite is becoming resistant to multidrug therapy (3), it is important to find new potential sources of antimalarial drugs. Artemisinin is an effective antimalarial drug (4) which, along with its derivates, have been treated over two million patients with no serious reported adverse effects (5). This compound is a secondary metabolite extracted from the leaves of *A. annua* L., a traditional herb which has been used for many centuries in the Chinese traditional medicine (6). The genus *Artemisia* is one of the large stone in the Asteraceae family with more than 800 species spreading all over the world (7). It has recently been reported that there are approximately 34 Artemisia species in Iran (8). Chemical structure of artemisinin suggests that antimalarial activity of this compound is due to an endoperoxide bridge (9) which is cleaved by free iron leading to a toxic free radical intermediate (10). Current researches have also indicated that artemisinin possesses anticancer (11), anti-schistosomiasis (12), and antiviral activities (13). However, due to the low concentration of artemisinin in *Artemisia* species, this metabolite is very expensive and hardly available for patients (14). Therefore, many efforts such as organ culture (15), metabolic engineering (16), hormone-containing media (17), use of *Agrobacterium rhizogenes* (18), and elicitors in the culture

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medium (19) have so far been made to increase the yielding of artemisinin.

Production of artemisinin by metabolite engineering seems to be an important direction in future but it is limited by complexity and high cost of synthesis (20). Because of these limitations, it would be interesting to identify which species of *Artemisia* with genetically high-artemisinin yielding. Sequence characterized amplified region (SCAR) markers are developed with a pair of extended sequence of a random amplified polymorphic DNA (RAPD) primer with specific sequence of approximately 20 bases. Compared with universal primers, specific primers for special regions decrease results sensitivity to reaction conditions and make more reproducible by increasing the specificity (20). The SCAR markers have been successfully derived from RAPD fragments in *Lettuca* (21). In this study, a previously reported SCAR marker linked to high artemisinin region was used to detect species with higher content of artemisinin among eight *Artemisia* species examined. This attempt could be a useful step for artemisinin pathway engineering or using effective elicitors in selected species as plants capable of producing higher yield of artemisinin.

MATERIALS AND METHODS

Plant materials and DNA extraction

The seeds of eight species of *Artemisia* including *A. annua*, *A. campestris, A. diffusa*, *A. spicigeria, A. scoparia, A. absinthium, A. sieberi,* and *A. vulgaris* were obtained from the Iranian Biological Resource Center. These species were grown in the greenhouse under 16 h photo period/8 h dark at 3000 lux intensity light and 24 ±2°C temperature. Total genomic DNA was extracted from fresh leaves of green house-grown plants according to modified method described by Kump and Javornik (21) using CTAB extraction buffer. The DNA extraction samples with high quality were selected visually by agarose gel electrophoresis and used in polymerase chain reaction (PCR)-mediated DNA amplification.

HPLC measurement of artemisinin

Artemisinin content was determined by high-performance liquid chromatography (HPLC). Briefly, 100 mg of dried unopened flower buds were powdered, 20 ml of petroleum ether added and extracted in an ultrasonic bath for 30 min (22). The supernatant was evaporated to dryness in a fume hood and the residue was dissolved in 5 ml acetonitrile and filtered with a 0.45 μM filter. Subsequently, the samples were analyzed using HPLC (Agilent, Eclipse plus C18 analytical column 4 × 125 mm) with a mobile phase of acetonitrile:acetic acid 0.01% (50:50, v/v) at a flow rate of 1 ml/min, and artemisinin was detected at 210 nm. The artemisinin standard solutions (5, 7, 10, 15, 20 and 25 μg/ml) were prepared and used to construct a standard curve for artemisinin. Artemisinin content in mg/g dry weight of flowers were determined using constructed calibration curve considering sample volumes and dilution factors and following equation (23).

(Artemisinin amont (mg)/Sampling volume) (Total volume/Sampling weight) × 1000

SCAR amplification and sequencing of PCR product

Eight species of *Artemisia* with different artemisinin contents were analyzed by SCAR marker described by Paran and Michelmore (24). Two sequence specific primers for high-artemisinin SCAR marker were used and could amplify a specific band of approximately 1000bp. Based on reliable amplification reported in Zhang and coworkers study (25), we used the same primers for SCAR marker amplification in our study. The sequence of forward primer with 18 bases length and reverse primer with 22 bases length were HA15F (5′-TTCCGAACCCAGCAGGGG -3') and HA15R: (5′-TTCCGAACCCGTACATACTATC-3'), respectively. The PCR program was 3 min at 94 °C, followed by 35 cycles of 30 s at 94 °C, 30 s at 58 °C, and 2 min at 72 °C, with a final extension of 4 min at 72 °C. The target PCR products were purified from the agarose gel by the glass milk technique and cloned into *pGEM®-T Easy* vector (Promega Corporation, Madison, WI, Germany) and transformed into competent *Escherichia coli* DH5α cells and positive colons were then sequenced. Characterizing the sequence of these bands was performed by Seqlab (Sequence Laboratories Göttingen, Germany). Similarity

comparison and confirmation of DNA sequences was accomplished using NCBI Basic local alignment search tool (BLAST) (26).

RESULTS

Artemisinin content in budding stage

The amount of artemisinin in the eight species at budding stage is shown in (Fig. 1). The highest amount of artemisinin was observed in two species including *A. annua* (0.46% of total dry weight) and *A. absinthium* (0.38% of total dry weight), respectively.

Cloning and sequencing of specific SCAR bands

The desired SCAR marker amplified only in *A. annua* and *A. absinthium* species, while this band was not observed in other tested *Artemisia* species (Fig. 2). The selected band cloned into *pGEM®-T Easy* vector and *Escherichia coli* DH5α as the host cell. After subsequent screening the recombinant bacteria colons were selected and sequenced (data not shown). The result of sequencing indicated high similarity to original SCAR marker of high artemisinin-yielding species in NCBI. Therefore, two isolated sequences were submitted in GenBank under KC337116 and KC465952 accession numbers (26). The BLAST results showed that KC337116 and KC465952 sequences has considerable DNA homology with the *A. annua* high-yielding SCAR marker which previously submitted in the GenBank® database by Zhang and colleagues (25). However, some substitutions, mainly cytosine (C) to thymine (T), were observed at different sequence similarity levels (Fig. 3).

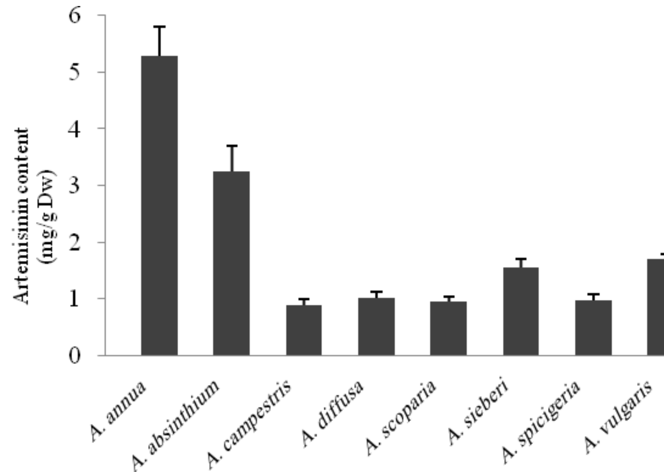

Fig. 1. Artemisinin content in budding stage (mg/g dry weight) in eight species of *Artemisia.* Artemisinin content (mg/g) is defined as the amount of artemisinin/dry weight of plant material.

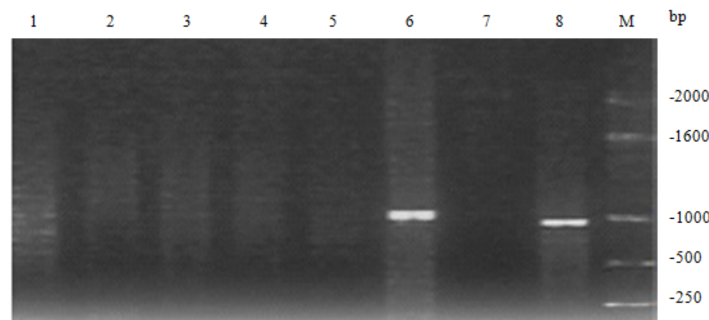

Fig. 2. Agarose gel electrophoresis (1%) of PCR products showing sequence characterized amplified region marker amplified in *Artemisia annua* and *Artemisia absinthium,* M; molecular weight marker, 2000bp. 1; *Artemisia vulgaris*, 2; *Artemisia sieberi*, 3; *Artemisia spicigeria*, 4; *Artemisia diffusa*, 5; *Artemisia campestris*, 6; *Artemisia absinthium*, 7; *Artemisia scoparia*, 8; *Artemisia annua.*

Fig. 3. Multiple alignment of high artemisinin sequences belonging to the *A. anuua* (KC337116) and *A. absinthium* (KC465952) with GenBank accession number, EU131677 for high artemisinin content reported first from *A. annua*. Deletions in sequences compared to EMBL-EBI International Nucleotide Sequence Data Library accession, EU131677, are indicated by a dash and sequences differences are boxed.

DISCUSSION

As artemisinin is one of the most effective antimalarial compounds and its content is very low in plant, it is important to screen for species having high content of artemisinin. In this regard, SCAR-based method is an efficient technique to screen high artemisinin-yielding species. The objective of the present study was to develop DNA-based markers to identify and distinguish *Artemisia* species with high artemisinin content. Our results of SCAR marker detection on eight *Artemisia* species indicated that both *A. annua* and *A. absinthium* species amplified high artemisinin relating to SCAR marker and were genetically selected as high artemisinin producer species.

Various methods such as chemo-profiling have been reported to determine high-artemisinin yielding species. However, these techniques are still having limitations including chemical complexity and lack of reliable markers (27). Therefore, new complementary methods for correct identification of high-artemisinin yielding species are required.

We scanned available genomic sequences of *A. annua* and *A. absinthium* to identify the presence of regions with high homology. BLAST searches using GenBank indicated that both species were highly homologous to a region in amorpha-4, 11-diene synthase (ADS) promoter sequence. The ADS promoter sequence of *A. annua* showed high coverage

with the ADS promoter sequence of *A. absinthium.* While, the coverage of ADS promoter sequence of *A. annua* with other *Artemisia* species (such as *A. scoparia* and *A. vulgaris*) was low. According to this result we can conclude that the specific primers for high-artemisinin related SCAR marker amplify a region in ADS promoter sequence.

ADS is a key enzyme of artemisinin production which promote early steps in artemisinin biosynthesis pathway (28). The association of high-artemisinin related SCAR marker with ADS promoter is compatible with previous reports defining this gene as a key regulatory gene in artemisinin biosynthesis (29). Several studies also reported a relationship between the increasing of ADS expression and the amount of artemisinin. ADS catalyzes the conversion of farnesyl diphosphate to amorpha-4,11-diene which has been suggested as the first specific precursor of artemisinin (30). In addition, Lulu and coworkers showed that under UV light, heat and cold shock conditions, ADS expression level up-regulated and subsequently increased the amount of artemisinin (31). Moreover, Pu and colleagues indicated that salicylic acid treatment increased the expression of ADS and this overexpression may increase artemisinin content (22). Therefore, it can be concluded that ADS has an important regulatory role in the biosynthesis of artemisinin.

Dried *Artemisia* parts have been studied in many previous studies (32). In the current study artemisinin analysis was also carried out on dried buds. There is a positive correlation between plant developmental stages and artemisinin yield (33). The highest artemisinin concentration in *A. annua* has been reported in leaves and flowers during full bloom stage (34). Artemisinin content increases obviously in the full-flower stage and correlates closely to trichome changes in developmental stages (35). One study showed that artemisinin reached the highest content before the flower bud was first observed (36). The flower bud stage, first flowering stage and full-bloom stage had significantly positive correlation with the artemisinin content in *A. annua*. One analysis showed differences in artemisinin content among *Artemisia* species. The highest artemisinin concentration was detected in leaves (0.44 ± 0.03%) and flowers (0.42 ± 0.03%) of *A. annua.* The *Artemisia* species at the flowering stage showed high levels of artemisinin (32).

We found that high-artemisinin related SCAR marker with approximately 1000 base pair length consistently was amplified in two species of *Artemisia* including *A. annua* and *A. absinthium* and was absent in the other species of *Artemisia*. Interestingly, the artemisinin amount in two selected species was higher than that of other species. The artemisinin content in high-yielding species was more than 3.8 mg/g total dry weight but less than 2 mg/g total dry weight in other low-yielding artemisinin species that is in accordance with previous studies (37). The presence and absence of the high-artemisinin SCAR band may be correlated with key regulatory function responsible for the biosynthesis of artemisinin.

CONCLUSION

In conclusion, we propose the developed SCAR markers that could be considered as complementary tools to distinguish high-artemisinin producer species. We found that high-artemisinin producing species, *A. annua* and *A. absinthium*, amplified SCAR marker noticeably. The use of high artemisinin related genetic markers could greatly reduce the time, labor, land usage, and other costs associated with breeding of this trait, while facilitating the screening of a large number of progeny.

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