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Research paper

Expression of artemisinin biosynthesis and trichome formation genes in five *Artemisia* species

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ABSTRACT

Artemisinin, a sesquiterpene lactone produced by some *Artemisia* species, is an efficacious anti-malarial drug, effective against cancer, hepatitis, and schistosomiasis. *A. annua* is a main source of artemisinin while other *Artemisia* species produce less artemisinin content. The aim of the current study was to identify the limiting factor of artemisinin biosynthesis in studied *Artemisia* species, compared to *A. annua*. The specialized 10-celled biseriate glandular trichomes on the leaves, stems, and inflorescences of some *Artemisia* species are as a site of artemisinin synthesis. The leaves of five *Artemisia* species, having different artemisinin contents were assessed in terms of the glandular trichomes density, and area per leaf, and the expression of artemisinin biosynthesis genes and two genes (*Aa-TTG1* and *Aa-TFAR1*) involved in trichome formation. This study identified one novel plant sources of artemisinin (*A. deserti*, 5.30 mg g^{-1} DW) that statistically performed as well as *A. annua* of Iran (6.27 mg g^{-1} DW), but inferior to *A. annua* cv. Anamed (14.50 mg g^{-1} DW) at the flowering stage. *A. deserti* had the highest trichome area per leaf area accompanied with a high expression of *Aa-ADS*, *Aa-AlDH1*, *Aa-CYP71AV1*, *Aa-TTG1,* and *Aa-TFAR1* genes. *A. persica* with low artemisinin content had a high density of glandular trichome, high expression of *TTG1* and *TFAR1*, but low expression of artemisinin biosynthetic genes. *A. khorassanica* with no artemisinin content had a very low density of glandular trichome and gene expression. The artemisinin content of *A. deserti* is significantly as same as *A. annua* of Iran and inferior to *A. annua* cv. Anamed despite having the highest glandular trichome area per leaf, and high relative expression of *Aa-ADS*, *Aa-ALDH1*, *Aa-CYP71AV1, Aa-TTG1,* and *Aa-TFAR1*. We suggest that it is related to the preferential oxidation of artemisinic aldehyde to artemisinic acid than the reduction of the artemisinic aldehyde to dihydroartemisinic aldehyde, due to the very high expression of *Aa-ALDH1* and *Aa-CYP71AV1*, and the low expression of *Aa-DBR2*. It is possible to develop high artemisinin producer plant by overexpression of *Aa-DBR2* in *A. deserti*. It is concluded that there is a relationship between the enhancement of artemisinin content and increased expression of some genes.

1. Introduction

Malaria is a global health problem which is the main reason of disease and death in humans for over a century (Xiao et al., 2016; Muangphrom et al., 2016). Artemisinin, a sesquiterpene lactone, an efficacious anti-malarial drug and effective against a number of cancers and viral diseases (Efferth et al., 2009), is produced by some *Artemisia* species (Duke et al., 1994; Willcox et al., 2004; Arab et al., 2006; Hsu, 2006; Zia et al., 2007; Mannan et al., 2010; Ranjbar et al., 2015). Tu was awarded her Nobel Prize in Physiology or Medicine in 2015 for the discovery of this effective antimalarial compound as a head of a scientific group in 1967–1969. *Artemisia* L. is a genus of small herbs and shrubs, belonging to an important family Asteraceae. It has over 500 species which are mainly found in Asia, Europe, and North America (Bora and Sharma, 2011) and Iran has 35 species of the genus (Abad et al., 2012). *Artemisia* species inhabit in all provinces of Iran, some of those are limited to the special area (Naghavi et al., 2014), and therefore the science of sesquiterpene biosynthesis in *Artemisia* species is substantial for natural products research in the near future. In Flora Iranica, Podlech, (1986) classified *Artemisia* genus into three subgenera,

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Artemisia, *Dracunculus*, and *Seriphidium*. Duke et al. (1994) reported that artemisinin is produced solely in glandular trichomes of *A. annua* L. They evaluated the normal biotype of *A. annua* with both filamentous and glandular trichomes, and a biotype with only filamentous trichomes, observed that just in the presence of glandular trichomes, artemisinin was detected. Then, it was reported that only three *Artemisia* species, including *A. annua*, *A. apiacea*, and *A. lanceolata* produced artemisinin (Willcox et al., 2004). Recent studies have reported that artemisinin is also produced in many other *Artemisia* species, *e.g. A. afftangutica*, *A. absinthium*, *A. bushriences*, *A. cina*, *A. dracunculus*, *A. dubia*, *A. indica*, *A. japonica/em*, *A. moorcroftiana*, *A. parviflora*, *A. roxburghiana*, *A. sieberi*, *A. vulgaris*, *A. campestris*, *A. diffusa*, *A. scoparia*, and *A. sieberi* (Arab et al., 2006; Hsu, 2006; Zia et al., 2007; Mannan et al., 2010; Ranjbar et al., 2015). All of those species produced less artemisinin contents than *A. annua.* Glandular secretory trichomes as a site of artemisinin synthesis are very important. The morphology of its structure can vary highly with tissue and species (Wagner, 1991). Nevertheless, biseriate and capitate glandular trichomes are common in certain genera of Asteraceae (Fahn, 1988), such as *Artemisia* (Kelsey and Shafizadeh, 1980; Slone and Kelsey, 1985; Cappelletti et al., 1986; Ascensão and Pais, 1987). Bi-seriate 10-celled glandular trichomes have been reported on both leaf surfaces of *A. nova* (Kelsey and Shafizadeh, 1980), in floral stalks of *A. tridentata* (Slone and Kelsey, 1985), on both leaf surfaces and on the ovary surfaces of *A. umbeliformis* (Cappelletti et al., 1986), on the adaxial leaf surface of *A. campestris* ssp. maritima (Ascensão and Pais, 1987), in the leaf (Duke and Paul, 1993) and flower (Ferreira and Janick, 1995) of *A. annua*, and in the different parts of *A. nitida* Bertol (Corsi and Nencioni, 1995). However, the exudate accumulation capacity may be related to gland size. Two putative transcription factors including transparent testa glabra1 (*Aa-TTG1*) and enhancer of glabra3 (*Aa-GL3*) (Liu et al., 2009), a cuticular wax biosynthesis gene, trichome-specific fatty acyl-CoA reductase (*Aa-TFAR1*) (Maes et al., 2011), a transcription factor of AP2/ERF superfamily, trichome and artemisinin regulator 1 (*Aa-TAR1*) (Tan et al., 2015), and *AaMYB1* (Matías-Hernández et al., 2017), involved in glandular secretory trichome development and artemisinin biosynthesis were identified. *AaMYB1* plays a role in trichome initiation and trichome branching. It may contribute to improve artemisinin production either by downregulating its competitive pathway, or by upregulating pathway such as the GA metabolism pathway that is indirectly useful (Matías-Hernández et al., 2017). Ranjbar et al. (2015) studied the artemisinin biosynthetic pathway (*Aa-ADS*, *Aa-CYP71AV1*, *Aa-ALDH1*, *Aa-DBR2,* and *Aa-RED1*; Fig. 1) in eight *Artemisia* species, where *A. absinthium* showed a higher expression level of both genes *Aa-ALDH1* and *Aa-CYP71AV1* compared to *A. annua* at all developmental stages. Komori et al. (2013) were unable to detect the expression of *Aa-ADS* in *A. afra* and *A. absinthium*, but they reported that *Aa-CYP71AV1* expressed in both species. Moreover, Muangphrom et al. (2014) detected the expression of *Aa-DBR2* in *A. absinthium*. The enzymes coding by these genes showed similar activities to those coded by *Aa-CYP71AV1* and *Aa-DBR2* in *A. annua* (Komori et al., 2013; Muangphrom et al., 2014). In any *Artemisia* species other than *A. annua*, there are no published studies on the genes involved in trichome formation. It is noteworthy that *A. annua* is still the main source of artemisinin and the productivity of artemisinin in the wide-type of *A. annua* is very low and inadequate to cover the demand of all patients (Xiao et al., 2016). A promising method for overcoming the natural barriers of production is genetic manipulation of an organism (Naghavi et al., 2014). Metabolic engineering is a potent approach to increase the range of bioactive compounds, but none of the metabolic engineering methods of *A. annua* for commercialization of artemisinin has been successful (Tang et al., 2014; Yuan et al., 2015). Lacking genetic evidence of biosynthesis pathway has hampered efforts of metabolic engineering for the high production of artemisinin (Xie et al., 2016). Glandular secretory trichomes of *A. annua* possess all urgent elements, such as pathway enzymes genes and transcription factors and oil environment that are essential in artemisinin biosynthesis (Xiao et al., 2016). The knowledge of factors affecting trichome density and morphology, whole biosynthesis pathway and regulatory mechanisms controlling the start and the flux of the pathway, can be resulted in a successful metabolic engineering. In the current study, we tried to detect the limiting factor of artemisinin biosynthesis in *Artemisia* species other than *A. annua*. It was aimed to determine the artemisinin contents of 17 *Artemisia* species and select species with high, medium, low, and no artemisinin contents and then evaluate them in the view of glandular trichomes, and the expression of artemisinin biosynthesis genes and two genes (*TTG1* and *TFAR1*), involved in trichome formation at the flowering stage.

2. Materials and methods

Seeds of 16 *Artemisia* species were collected from different parts of Iran (Table 1). Plants were propagated and their seeds were collected and cultured in Iranian Biological Resource Center. Furthermore, *A. annua* cv. Anamed as a high artemisinin cultivar, and *A. annua* of Iran were included as controls. For primary screening, the leaves of 16 *Artemisia* species and *A. annua* cv. Anamed and *A. annua* of Iran were sampled at the vegetative stage for determining artemisinin content. Then, *A. annua* cv. Anamed and *A. annua* of Iran as controls and five *Artemisia* species having high, low, and no artemisinin contents were selected and their leaves were assessed in the view of artemisinin content, density, and area of glandular trichomes, using fluorescent microscopy and scanning electron microscopy (SEM), respectively at the flowering stage. Half of each leaf was cut and mixed for RNA extraction and expression analyses, and another half was considered for artemisinin measurement. Thence based on artemisinin content, and the morphology of glandular secretory trichome in the previous step, five *Artemisia* species, including *A. annua* L. of Iran (S1) as a control species, *A. khorassanica* Podlech. (S2), and *A. persica* Boiss. (S3) as endemic species of Iran having no and low artemisinin contents, respectively, *A. deserti* Krasch. (S4) with high artemisinin content, and *A.*

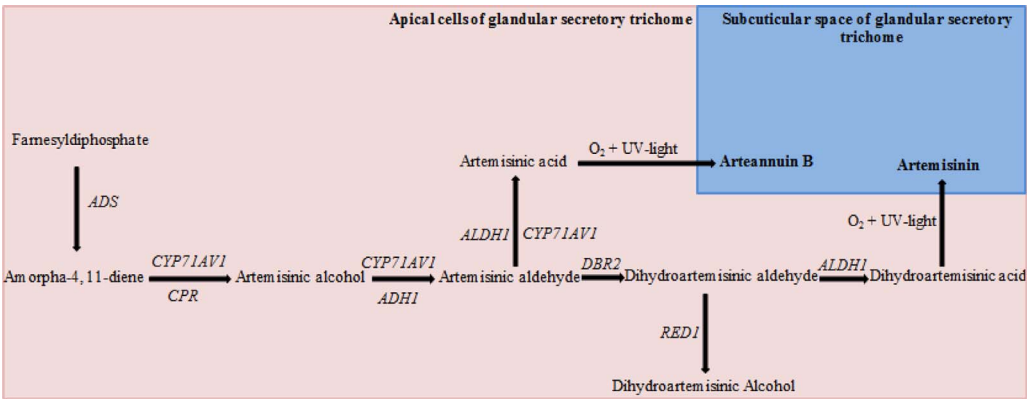

Fig. 1. Summary of artemisinin biosynthesis pathway. Aa-ADS: amorpha-4,11-diene synthase, Aa-CYP71AV1: amorphadiene-12-hydroxylase, Aa-CPR: cytochrome P450 reductase, Aa-ADH1: alcohol dehydrogenase 1, Aa-ALDH1: aldehyde dehydrogenase 1, Aa-DBR2: artemisinic aldehyde Δ11(13) reductase, Aa-RED1: dihydroartemisinic aldehyde reductase.