Isolation and Identification of Novel Genes Involved in Artemisinin Production from Flowers of *Artemisia annua* Using Suppression Subtractive Hybridization and Metabolite Analysis

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- Qinghaosu
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- Asteraceae

Abstract

Malaria is a global health problem that threatens 300–500 million people and kills more than one million people annually. Artemisinin is highly effective against multidrug-resistant *Plasmodium falciparum* and it has been widely used as part of the artemisinin-based combination therapies against malaria. To elucidate the biosynthetic pathway of artemisinin and to clone related genes in *Artemisia annua*, differentially expressed genes between blooming flowers and flower buds were isolated and characterized by a combined approach of suppression subtractive hybridization (SSH) and metabolite analysis. A total of 350 cDNA clones from a subtractive cDNA library were randomly picked, sequenced and analyzed and 253 high-quality sequences were obtained. BLASTX comparisons indicated that about 9.9% of the clones encoded enzymes involved in isoprenoid (including artemisinin) biosynthesis. The expression of 4 gene transcripts involved in artemisinin biosynthesis was examined by RT-PCR and the results confirmed the higher expression of these transcripts in blooming flowers than in flower buds. In addition, 2 putative transcript factors transparenta testa glabra 1 (TTG1) and ENHANCER OF GLABRA3 (GL3), which promote trichome initiation, were presented in the library. Finally, this study demonstrated that the increase of expression level of the putative *TTG1* gene correlated with the improvement of glandular trichome density and artemisinin production in *A. annua* leaves. The subtractive cDNA library described in the present study provides important candidate genes for future research in order to increase the artemisinin content in *A. annua*.

Supporting information available online at http://www.thieme-connect.de/ejournals/toc/plantamedica

Introduction

Today, malaria is one of the world's most devastating parasitic diseases. There are at least 300 million acute cases of malaria each year globally, resulting in more than a million deaths [1]. Disease control is hampered by the occurrence of multidrug-resistant strains of the malaria parasite *Plasmodium falciparum* [2]. Artemisinin, a sesquiterpene lactone endoperoxide extracted from *Artemisia annua* L. (commonly known as sweet wormwood), is highly effective against *P. falciparum* and the World Health Organization recommends its use as part of the artemisinin-based combination therapies [2]. As artemisinin cannot be synthesized chemically in an economically feasible way, *A. annua* is the only practical source of this valuable drug [3]. Unfortunately, *A. annua* contains only very small amounts of artemisinin ranging from 0.001 to 1.54% of dry weight [3].

In order to increase the artemisinin content in *A. annua* and to maintain a reliable and low cost supply of artemisinin, a number of biochemical and molecular biological studies about artemisinin biosynthesis have been carried out [4]. Bouwmeester et al. firstly reported the partial purification of natural amorpha-4,11,-diene synthase (ADS) protein from *A. annua* [5], which is responsible for the cyclization of farmesyl diphosphate (FPP) to amorpha-4,11,-diene, the first specific precursor of artemisinin. The corresponding gene *ADS* has been identified and cloned by several groups independently [6,7], and then Keasling and his collaborators produced amorpha-4,11,-diene in an *E. coli* strain engineered with the yeast mevalonate pathway and an *ADS* gene [8,9]. Bertea and his coworkers demonstrated that *A. an-*

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nua leaf microsomes converted amorphadiene to artemisinic alcohol in the presence of NADPH [10]. Teoh et al. [11] have currently isolated a cDNA clone encoding a cytochrome P450 monooxygenase CYP71AV1 from the *A. annua* trichome library. When amorpha-4,11-diene is supplemented as a substrate, artemisinic alcohol is synthesized in an NADPH-dependent manner [11]. Their experiments have also suggested that CYP71AV1 is a multifunctional enzyme that oxidizes artemisinic alcohol to generate artemisinic acid via artemisinic aldehyde intermediates [11]. Ro et al. [12] also obtained a full-length cDNA of CYP71AV1 from *A. annua* and co-expressed with ADS in *Saccharomyces cerevisiae*, resulting in the production of artemisinic acid [12]. More recently, Zhang and his collaborators cloned the artemisinic aldehyde Δ11(13) double bond reductase (DBR) gene *Dbr2* from *A. annu*a flower buds [13], and the gene showed more specific reaction activity for artemisinic aldehyde over artemisinic alcohol and acid, as well as arteannuin B and artemisitene, which indicates that at least some artemisinin is made via dihydroartemisinic aldehyde and subsequently dihydroartemsinic acid. Although the characterization of *Dbr2* provides important support for the "early" Δ11(13) reduction in artemisinin biosynthesis, the final steps in artemisinin biosynthesis remain controversial with regard to the identification of either artemisinic or dihydroartemisinic acids as the later precursor [13]. On the other hand, the exact regulation mechanism of artemisinin biosynthesis is still not fully understood [4]. Functional genomic approaches to study artemisinin biosynthesis in *A. annua* have been carried out in several laboratories aiming to discover a large number of genes encoding structural proteins or regulatory factors involved in the metabolism of artemisinin and other secondary metabolites [11, 14]. However, these expressed sequence tags (ESTs) from the above laboratories have not yet been made available to the public. Although, Lindquist and his coworkers (http://www.ncbi.nlm.nih.gov/dbEST/) have submitted a large batch of EST obtained from *A. annua* leaf to Expressed Sequence Tags Database, the identification of these genes is very difficult, expensive and time-consuming.

Ferreira et al. reported that artemisinin content in inflorescences increased rapidly at the *A. annua* flower development stage and it was 4 to 11 times higher than that at pre-flowering time [15], suggesting that artemisinin biosynthesis and related genes in *A. annua* flowers may vary during flower development. Suppression subtractive hybridization (SSH) is an attractive method for cloning enriched differentially expressed genes through amplification of target cDNA fragments (differentially expressed genes) and simultaneously suppression of non-target DNA amplification [17]. This approach has been successfully applied to reveal biosynthetic pathways and the enzymes involved in pathways of plant secondary metabolite production [16]. The present study reported isolation of differentially expressed genes between blooming flower and flower bud of *A. annua* using SSH. The differently expressed genes do not necessarily have to be responsible for the enzymes involved in the biosynthetic pathway, but they can also be involved in the mechanism influencing the amount of produced artemisinin, such as formation of glandular trichomes (biosynthetic site for artemisinin) located on the surface of leaves.

Materials and Methods

Plant material and treatment

Detailed descriptions of *Artemisia annua* L. (Asteraceae) and its treatment are provided in the Supporting Information (**Text 1**, Supporting Information).

Extraction and analysis of artemisinin

Extraction of artemisinin from plant material was performed according to the process described by Liu et al. [18]. The extract solution was analyzed by gas chromatography with electron-capture detection (GC-ECD) according to the method described by Liu and his colleagues [18] with a slight modification (**Text 2**, Supporting Information).

Subtractive cDNA library construction

Subtractive cDNA library was constructed with cDNA fragments generated from the mRNA of blooming flower and flower bud as tester and driver, respectively (**Text 3**, Supporting Information).

EST sequencing and analysis

EST sequencing was finished by Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. (**Text 4**, Supporting Information). Sequences analysis was performed using the BLASTX search algorithm (**Text 4**, Supporting Information). The functional categories of ESTs are based on those used for the annotation of the *Arabidopsis thaliana* genome [19].

RT-PCR analysis of expression of artemisinin biosynthesis related genes

The mRNA expression patterns of 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR), farnesyl diphosphate synthase (FPS), ADS and CYP71AV1 were examined by semiquantative RT-PCR (**Text 5**, Supporting Information).

Treatment of jasmonic acid on leaves

Jasmonic acid (JA) solution was sprayed to *A. annua* leaf (**Text 6**, Supporting Information).

Determination of trichome density and artemisnin concentration

Methods for determination of trichome density and artemisinin concentration in *A. annua* leaf after JA treatment are provided in Supporting Information (**Text 7**, Supporting Information) according to document [20].

RT-PCR analysis of TTG1 expression in leaves

TTG1 expression patterns in *A. annua* leaf after JA treatment were examined by RT-PCR (**Text 8**, Supporting Information).

Supporting information

Detailed descriptions of materials and methods, pictures of glandular trichomes on leaves are available as Supporting Information.

Results and Discussion

The concentration of artemisinin in blooming flowers was about 3 times higher than that in blooming flowers in terms of dry weight (**Fig. 1 A** and **B**). The value was less than that previously reported [17], which was obtained from analysis of inflorescen-

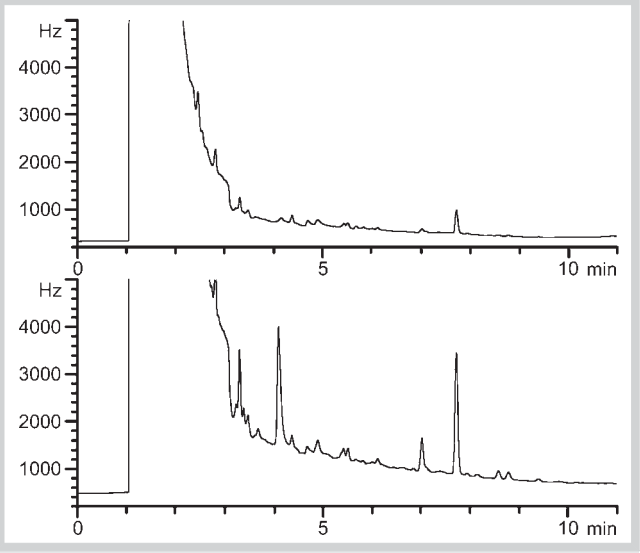

Fig. 1 Gas chromatogram of *A. annua* flower extract. Samples are prepared from flower buds (**A**) and blooming flower (**B**).

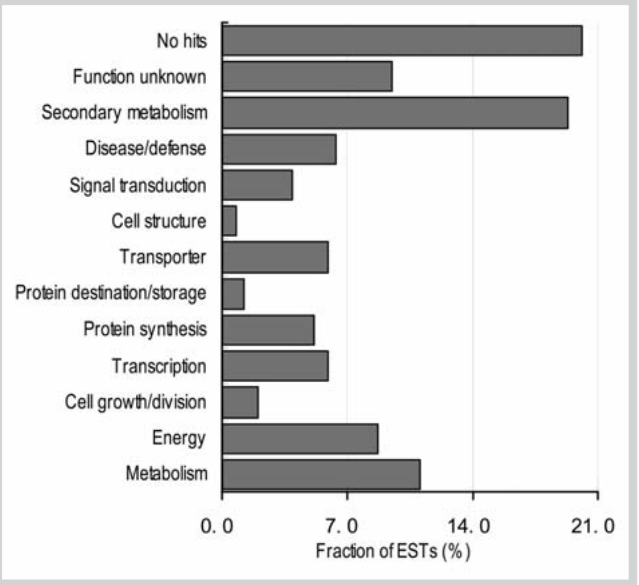

Fig. 2 Distribution of the putative functions of differentially expressed gene transcripts in *A. annua* blooming subtractive library. Classification was performed according to the functional catalogues established for *Arabidopsis thaliana* using BLASTX search results against proteins present in GenBank.

ces. This discrepancy was likely due to the fact that different genotype plants were used in the two studies. Furthermore, we also compared the total amount of artemisinin in a single flower at different development stages. Significant differences were observed, and the total amount of artemisinin showed the highest level at full bloom stage in terms of the single flower, almost 5 times more than the value obtained at the pre-flowering stage. These results implicated that the biosynthesis of artemisinin may be enhanced in blooming flowers through upregulating the expression of related genes, which encouraged us to isolate the different expressed genes involved in artemisinin biosynthesis by SSH.

More than 900 recombinant clones were isolated from the forward subtraction. Of those, cDNAs from 350 randomly picked white clones were sequenced and analyzed. After removal of vector, poor quality, and poly(A) sequences, 253 useable sequences with inserts longer than 100 bp were obtained and 121 non-repeated sequences were submitted to GenBank dbEST (http://www.ncbi.nlm.nih.gov/projects/dbEST) with accession numbers from GE471409 to GE471528, and GE471530. BLASTX analysis using the GenBank nonredundant (nr) protein database revealed that 178 (70.3%) deduced amino acid sequences showed significant homology to proteins with known functions and the rest (29.7%) showed no significant similarity to any known proteins in public databases. The subtractive ESTs were categorized into 13 putative functional groups (**Fig. 2**) based on the functional catalogues established for *A. thaliana*, including metabolism, secondary metabolism, energy, cell growth/division, transcription, protein synthesis, protein destination/storage, transport, cell structure, signal transduction, disease/defense, unknown protein and no hits [19].

The largest group of genes with known function was metabolism-related genes and approximately 19% of the transcripts were included in this category. About 3.2% of the clones encoded enzymes involved in flavonoid biosynthesis, which is comparable with that in the *A. annua* leaf glandular trichome cDNA library established by Bertea et al. [14], with 4% of clones belonging to enzymes involved in flavonoid biosynthesis. The occurrence of flavonoid biosynthesis related genes indicated that the biosynthesis of flavonoid in flowers might vary during flower growth, which agrees with the recent study described by Baraldi and his collaborators, who demonstrated that the level of total flavonoids in inflorescences at full bloom stage was higher than that at pre-flowering stage [21]. Up to 9.9% of the clones appear to be involved in the isoprenoid path, which is larger than that calculated from the leaf gland cDNA libraries in which only 8% of the ESTs were estimated to be involved in isoprenoid biosynthesis [14]. This was likely because that subtractive library was devoid of nontarget genes after subtractive hybridization [15], which also confirmed that SSH is a powerful tool to clone interesting genes. Among this portion, 13 clones representing 7 genes were involved in the biosynthetic pathway of artemisinin. Previous studies revealed that biosynthesis of artemisinin involved two biosynthesis stages [4]. The first stage is responsible for the production of FPP, via two independent biosynthetic pathways: the cytosolic mevalonate pathway and the plastidic methylerythritol phosphate pathway [22]. In this stage, four genes have been identified in *A. annua*, which encode 3-hydroxy-3-methylglutaryl coenzyme A reductase, FPS, 1-deoxy-D-xylulose 5-phosphate synthase and DXR [4]. In our study, ESTs shown similarity to *FFPS, DXR*, acetoacetyl CoA thiolase (*ACAT*), mevalonate kinase (*MK*) were found and this is the first time to obtain the genes encoding for *ACAT* and *MK* in *A. annua*. The second stage is the post-FPP biosynthetic pathway, which is not yet completely elucidated. In this stage, three genes have been reported, including *ADS, CYP71AV1* and more recently *Dbr2*. Both *ADS* and *CYP71AV1* were represented in the subtractive library, while *Dbr2* was not found in this study. However, we found 2 ESTs showing high identity with plant double-bond reductase, which have the ability to transform α,β-unsaturated aldehydes to saturated aldehydes [23]. Besides, five ESTs in this library showed significant sequence similarity with carotenoid cleavage dioxygenase 2 (*CCD2*) from *Lactuca sativa* (GenBank accession no. AB120112.1). High ex-

pression of the *CCD* transcript, coupled with high accumulation of artemisinin, suggested that this gene might contribute to artemisinin production.

The group of ESTs putatively assigned to the photosynthesis enzymes was also abundant (about 8.7%). In this category, chlorophyll a/b binding protein (9 ESTs) was the highest expressed single cDNA, which was in accordance with the result obtained from the *A. annua* glandular trichome library [14]. These results may be explained by the hypothesis proposed by Wallaart, who thought that chlorophyll and the oxygen-generating system in *A. annua* were a potential source of singlet oxygen production that was an essential molecule for the conversion of dihydroartemisic acid to artemisinin [24].

Another group of ESTs of high abundance was represented by genes related to transcription, which play a major role in regulation of crucial cellular processes such as cell growth and differentiation. Previous genetic analyses have identified that four transcription factors, including GLABROUS1 (GL1), TTG1 and ENHANCER OF GLABRA3 (GL3), are involved in promoting trichome initiation [25], which is strongly coupled to the production of terpenoids (including artemisinin) in *A. annua* plant [20]. ESTs that showed high identity to *TTG1* and *GL3* were found in the subtractive library, and this study reported genes related to trichome initiation in *A. annua* for the first time.

Lipid transfer proteins (LTPs) are widely believed to be involved in the transport of lipophilic molecules, such as essential oils, between different cell compartments and they were found in high abundance in many glandular trichome cDNA libraries from aromatic plants [26]. Recently, Bertea et al. also reported 10% of the total ESTs in *A. annua* trichome library representing *LTP*s [14]. However, only about 4% of the clones in our subtractive library showed significant similarity to LTPs. This might be attributed to the fact that differentially expressed genes with low transcript abundance were enriched in the process of SSH and thereby that with particularly high transcript abundance were relatively decreased.

About 29.7% of ESTs could not be assigned any putative function since they showed no similarity to any entry in the databases (20.2%) or because they showed similarity to unknown genes from other species (9.5%). Because our knowledge of the biosynthetic pathways involved in the production of artemisinin and other isoprenoids is incomplete, these ESTs with homology to uncharacterized transcripts or with no identifiable homology and therefore representing novel genes, are candidates for these unknown genes.

To verify if the gene expressions corresponding to ESTs generated by SSH were differentially expressed and to evaluate the success of the subtractions, the expression patterns of 4 critical artemisinin biosynthetic genes (*DXR, FPS, ADS*, and *CYP71AV1*) were examined by RT-PCR analysis in both blooming flower and buds. RT-PCR analysis (**Fig. 3**) indicated that transcripts of *A. annua* homologues of *ADS* and *CYP71AV1* were very low in flower buds, in which low artemisinin content was present. However, the expression level of *ADS* was significantly increased in blooming flowers and that of *CYP71AV1* gene was also upregulated. Another significantly elevated expression was observed on the *DXR* gene, which was also highly expressed in flower buds. The *FPS* gene is expressed in flower buds in a moderate level as compared with the other 3 genes and its expression level was slightly increased at full flower stage. The expression patterns of *DXR, FPS, ADS*, and *CYP71AV1* were consistent with the artemisinin biosynthetic profile in flowers, which agrees with the result obtain from transgenic *A. annua* plants by Yang et al., who reported that overexpression of *ADS* and *CYP71AV1* led to overproduction of artemisinin [27]. The results of semiquantitative RT-PCR analysis indicate that the SSH method is suitable for the isolation of cDNA molecules for secondary metabolism-related genes that are preferentially expressed in blooming flowers of *A. annua*.

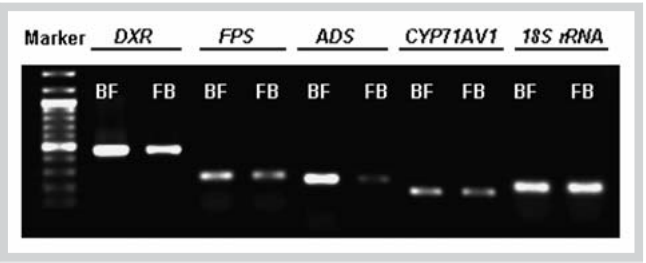

Fig. 3 Differential expression analysis of four genes between blooming flower (BF) and flower bud (FB) by semiquantitative RT-PCR. Amplification of *18S rRNA* gene was used to ensure that equal amounts of templates were added to each RT-PCR reaction.

In order to confirm that the newly isolated *A. annua* ESTs in the present study were related to artemisinin production, the correlation of the granular trichomes density with the expression of the putative *TTG1* gene induced by JA was investigated. JA has been shown to increase trichome density on newly formed leaves of *A. thaliana* [28]. Recently, Boughton and his coworkers [29] reported that methyl jasmonate caused significant increases in glandular trichome production of tomato leaves. In this study, JA also caused a significant increase in the densities of glandular trichomes on new leaves expanding after treatment. Trichome densities were significantly higher (Student's t-test, $p < 0.05$) on JA plants than on control plants at 7, 14, and 21 d after treatment

Table 1 Effect of JA treatment on glandular trichome density and artemisinin production.

Time point (D)	Mean trichome density[a] (no./mm² ± SD)			Mean artemisinin content[b] (μg/mg ± SD)		
	Control	JA-treated	P value[c]	Control	JA-treated	P value[c]
3	28.3 ± 1.3	30.7 ± 1.5	0.473	2.23 ± 0.10	2.36 ± 0.11	0.397
7	31.1 ± 1.6	46.3 ± 1.8	< 0.05	2.70 ± 0.09	3.85 ± 0.19	< 0.05
14	41.7 ± 1.9	86.6 ± 3.7	< 0.05	3.65 ± 0.13	6.18 ± 0.29	< 0.05
21	45.3 ± 1.7	73.8 ± 3.9	< 0.05	3.93 ± 0.12	5.57 ± 0.25	< 0.05
28	51.9 ± 2.1	62.7 ± 3.3	0.215	4.37 ± 0.15	4.86 ± 0.23	0.138

JA = Jasmonic acid; [a] Mean density of glandular trichomes on upper surface of new leaves; N = 7 plants per treatment. One leaf examined per plant. Plants sampled only once; [b] Mean content of artemisinin in new leaves. N = 7 plants per treatment. Content determined from the same whole leaf for trichome density examining; [c] P value shown for Student's t-test

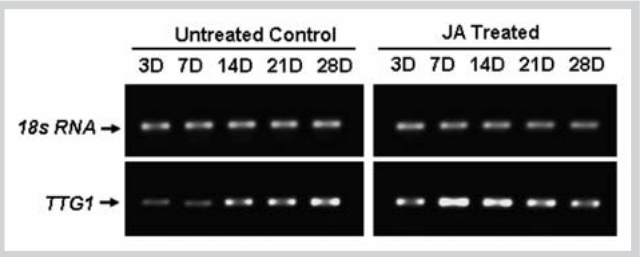

Fig. 4 RT-PCR analysis of *TTG1* expression in new *A. annua* leaves expanding after JA (jasmonic acid) treatment. RT-PCRs were carried out from the total RNA isolated from JA-treated or untreated samples at 3, 7, 14, 21, and 28 d after treatment, respectively. The *18S rRNA* was used as a control to show the normalization of the amount of templates in PCR reactions.

(**Table 1**). The highest density of glandular trichomes was present on leaves produced 14 d after JA treatment, which was approximately two times of that of control (**Fig. 1SA** and **B**, Supporting Information). Similarly, a significantly enhanced artemisinin production was found at 7, 14, or 21 d after treatment. The concentration of artemisinin increased to about 200% of the control at 14 d (**Table 1**). A significant correlation ($R^2 = 0.941$) between artemisinin level and glandular trichome density of *A. annua* was observed, which agrees with the previous results [17, 20, 30]. The relationship between glandular density and artemisinin accumulation has been widely studied based on the distribution analysis of artemisinin and glandular trichomes in different tissue, part, genotype or growth stage of *A. annua* [17, 20, 30]. However, the direct evidence supporting involvement of enhanced glandular trichome density in the improvement of artemisinin concentration has not been documented. Our results directly demonstrated that the increase of glandular trichomes on *A. annua* leaves induced a higher production of artemisinin. Furthermore, the expression level of the putative *TTG1* gene was significantly induced by JA at 3, 7, 14 d after treatment and it reached the highest level at 7 d (**Fig. 4**). The JA-induced overexpression of the putative *TTG1* gene was coupled with the overproduction of glandular trichomes and artemisinin, suggesting that the putative *TTG1* gene was potentially influencing artemisinin biosynthesis. In addition, the consequence of *TTG1* expression analysis also showed that the induction effect of JA on putative *TTG1* gene expression appeared and disappeared more early than that on trichome density and artemisinin content, which implicated that the overexpression of putative *TTG1* might directly or indirectly upregulate the formation of glandular trichomes and artemisinin production in *A. annua*. These results also showed that the *TTG1* represented a potential target gene for genetic modification of *A. annua* aimed to improve artemisinin production.

In summary, this study has reported higher levels of artemisinin and artemisinin biosynthesis-related genes expression in blooming flowers than in flower buds. A large number of genes with potential roles in biosynthetic pathway of artemisinin and other secondary metabolites in *A. annua* were cloned. Among of them, a putative transcript factor TTG1 was characterized by overexpression assay, and the result showed the *TTG1* gene might be involved in the regulation of artemisinin biosynthesis via promoting the formation of glandular trichomes. The remaining candidate genes still need to be further identified by RNA interference technology or heterologous expression assay.

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