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Research article

Molecular cloning and characterization of a flavanone 3-Hydroxylase gene from *Artemisia annua* L.

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ABSTRACT

Flavonoids were found to synergize anti-malaria and anti-cancer compounds in *Artemisia annua*, a very important economic crop in China. In order to discover the regulation mechanism of flavonoids in *Artemisia annua*, the full length cDNA of flavanone 3-hydroxylase (F3H) were isolated from *Artemisia annua* for the first time by using RACE (rapid amplification of cDNA ends). The completed open read frame of *AaF3H* was 1095 bp and it encoded a 364-amino acid protein with a predicted molecular mass of 41.18 kDa and a pI of 5.67. The recombinant protein of AaF3H was expressed in *E. coli* BL21(DE3) as His-tagged protein, purified by Ni-NTA agrose affinity chromatography, and functionally characterized *in vitro*. The results showed that the His-tagged protein (AaF3H) catalyzed naringenin to dihydrokaempferol in the present of Fe^{2+}. The *K*m for naringenin was 218.03 μM. The optimum pH for AaF3H reaction was determined to be pH 8.5, and the optimum temperature was determined to be 35 °C. The *AaF3H* transcripts were found to be accumulated in the cultivar with higher level of flavonoids than that with lower level of flavonoids, which implied that *AaF3H* was a potential target for regulation of flavonoids biosynthesis in *Artemisia annua* through metabolic engineering.

1. Introduction

Artemisia annua L. (*A. annua*) is Chinese traditional medicinal plant, which has been used to treat fever, inflammation and malaria for a long time in China (Liu et al., 1992; Udaykumar 2014), and now it is growing in many European countries and North America (Dhingra et al., 1999). *A. annua* contains hydroxylated flavonoids and polymethoxylated flavonoids such as eupatin, cirsilineol, chrysoplenetin, chrysosplenol D, casticin, and artemetin (Bhakuni et al., 2001). Flavonoids from *A. annua* were reported to have biological activities, such as anti-oxidation, anti-cancer and anti-malaria (Iqbal et al., 2012; Kim et al., 2014, 2015). In addition, *A. annua* flavonoids were found to synergize anti-malaria and anti-cancer compounds (Weathers et al., 2014), especially artemisinin, an endoperoxide sesquiterpene lactone isolated from *A. annua* leaves that is widely used to treat malaria, one of the world's most important parasitic diseases, affecting approximately 300–500 million people worldwide and causing more than one million deaths per year (Salako, 1998). Recently, more and more research indicate that *A. annua* flavonoids inhibit the activity of CYP450 enzymes responsible for altering the absorption and metabolism of artemisinin in the body, while it improve the immunomodulatory activity in subjects afflicted with parasitic and chronic diseases (Cherng et al., 2008). Therefore, it is very important to improve the content of flavonoids through engineering the biosynthetic pathway of flavonoids in *A. annua*.

The flavonoids, a large group of plant secondary metabolites consist of a 15 carbon atom phenylpropanoid core. Two aromatic rings (2 × 6 carbons) are joined by a heterocyclic ring (3 carbons). Therefore the flavonoid structure is also referred to as C6-C3-C6 (Harborne and Williams, 2000). The flavonoid biosynthetic

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pathway has been extensively studied in plants by a combination of genetic and labeling studies (Quattrocchio et al., 2006). Hydroxylation of (2*S*)-flavanone to (2*R*,3*R*)-dihydroflavonol, which are catalyzed by a (2*S*)-flavanone 3-hydroxylase (F3H), is a key step in the biosynthesis of flavonols, anthocyanin and catechins. The activity of F3H enzyme was first reported in flowers of *Matthiola incana* (Forkmann et al., 1980), while the first successful purification and characterization of this enzyme was performed on a red-flowering mutant *Petunia hybrida* (Britsch and Grisebach, 1986). After that numerous amounts of F3H genes have been cloned and characterized from many plants, including *Saussurea medusa* (Jin et al., 2005), *Ginkgo biloba* (Shen et al., 2006), *Scutellaria viscidula* (Lei et al., 2010). However, no gene involved in flavonoids biosynthetic pathway from in *A. annua* has been identified. Recently, we obtained a red-stem *A. annua* cultivar containing higher level of anthocyanin in comparison with the wild-type, which allowed us to isolate several flavonoids related gene fragments. In the present work, we described the full-length cDNA cloning and identification of a gene encoding a (2*S*)-flavanone 3-hydroxylase (AaF3H) from *A. annua*.

2. Results and discussion

2.1. Candidate gene selection, full-length cloning and sequence analysis

A differently expressed cDNA library was constructed between two *A. annua* cultivars, in one of which the total anthocyanin level (aerial part) was 12.55 ± 9.78 mg/g dried weight, about 2-fold higher than that in another one (6.36 ± 0.56 mg/g). From the library, 67 non-repeated sequences were isolated by using cDNA-AFLP. Among them, an EST of 126 bp was found to show a 77% identity to F3H from *Arabidopsis thaliana*. In order to further identify the putative F3H, a full-length cDNA of 1866 bp long was obtained by SMART-RACE-PCR. The AaF3H ORF was 1095 bp and encodes a 364-amino acid protein with a predicted molecular mass of 41.18 kDa and a pI of 5.67.

Subsequently, a BLASTP search of Genbank with the predicted amino acid protein sequence of AaF3H was performed and results showed that the deduced AaF3H shared 67%, 76, 77, and 78% of identity at the amino acid level with other plant F3Hs from *Ginkgo biloba*, *Arabidopsis thaliana*, *Camellia sinensis* and *Citrus maxima* (Fig. 1), respectively. As shown in Fig. 1, a highly conserved ferrous iron ligation motif $HXDX_{55}H$ (HTDPGTITLLLQDQVGGLQATRDGGKTWITVEPIEGAFVVNLGDHGYYLSNGRFKNADH) and a 2-oxoglutarate (2-ODD) binding motif RXS (RLS) were found in AaF3H, which implicated that this deduced protein represented a 2-oxoglutarate-dependent dioxygenase. In addition, five conserved motifs commonly contained in plant 2-oxoglutarate-dependent dioxygenase were present in AaF3H (Fig. 1). In motif 2 and motif 3, three strictly conserved prolines ($Pro_{148,\ 204,207}$) were found (as shown in Fig. 1), which were proposed to play important roles on the folding process of the protein.

2.2. Phylogenetic analysis

In order to investigate the evolutionary relationships among AaF3H and other 2-ODD proteins involved in the biosynthesis of flavonoids in plants, the phylogenetic tree was constructed by using neighbor-jointing method. The results (Fig. 2) showed that AaF3H and F3Hs from dicotyledons, such as *Canarium album, Litchi chinensis, Gossypium barbadense, Solanum pinnatisectum, Arabidopsis thaliana* and *Ipomoea batatas*, formed a cluster. While F3Hs from monocotyledon, including *Narcissus tazetta, Dioscorea alata* and *Zea mays*, formed another cluster far from AaF3H cluster. At the same time, all of investigated FLSs and ANSs from different species shared a same cluster, correspondingly, which was coincident with the report by Prescott and John (Prescott and John, 1996).

2.3. Characterization of recombinant AaF3H

To verify the hydroxylation catalytic activity of AaF3H, the CDS of AaF3H was cloned into the expression vector pET28a(+), and over-expressed in *E. coli* BL21(DE3) cells as N-terminal His-tag function protein. Different culture and induction conditions were tested resulted in a high level of soluble protein (Fig. 3). The recombinant AaF3H protein was extracted and purified by Ni-NTA affinity column chromatography (shown as Fig. 3). From 50 mL of induced culture, 9.31 mg/mL of purified recombinant protein was obtained (Fig. 3). Subsequently, the purified AaF3H protein was assayed for flavanone 3-hydroxylase activity with (±)-naringenin as substrate, and 2-oxoglutrate, $FeSO_4$ as cofactors. Analysis of the reaction products by HPLC (Fig. 4b) and LC-MS (Fig. 4c&d) indicated that dihydrokaempferol was produced from naringenin with the catalysis of AaF3H and both of cofactor 2-oxoglutrate and Fe^{2+} were required for the activity of AaF3H (data not shown). The negative control assay, in which the AaF3H was boiled for 10 min at 95 °C, did not yield significant amount of dihydrokaempferol or other products (Fig. 4a). The stereo selectivity of the enzyme AaF3H was not investigated due to the lack of stereo-specific substrates. However, we observed that more than 50% of conversion of racemic mixtures used in the assays could not be achieved under all of tested conditions, which might implicated that AaF3H showed a stereo selectivity to substrate, agreeing with previous reported for F3H which catalyses the stereo-specific (3*R*)-oxidation of (2*S*)- but not (2*R*)-naringenin (Britsch and Grisebach, 1986).

2.4. Kinetic parameter

To further characterize catalytic properties of AaF3H, the reaction products were quantified at different time. And the results (data not shown) suggested that the recombinant AaF3H showed linear catalytic activity from 5 to 25 min. The optimum pH for the recombinant protein was determined to be 8.5 (Fig. 5a). The catalytic activity of recombinant AaF3H was sharply decreased when the pH value was less than 8.5. Only about 50% of activity of recombinant AaF3H was remained at pH 7, and no activity was detected at pH 6. Relatively, the temperature did not significantly affect catalytic activity of recombinant AaF3H at the temperature range from 28 to 37 °C although the maximum activity was observed at 35 °C (Fig. 5b), which implicated that the enzyme of AaF3H was thermal stability to some degree. The kinetic parameters (Fig. 5c) of the AaF3H catalyzed reactions were quantified and yield a Km of 218.03 μM for naringenin.

2.5. Semi-quantitative RT-PCR analysis of AaF3H gene expression level

In order to investigate the relationship between anthocyanin content and *AaF3H* gene expression level, a semi-quantitative RT-PCR was employed to compare *AaF3H* gene expression in a high-anthocyanin level cultivar with that in a low-anthocyanin level cultivar by using 18s rRNA as reference gene. PCRs were performed for 24, 26 and 28 cycles, respectively. No significant electrophoresis band was obtained when the cycle number was 24 (Fig. 6). When the cycle number was more than 26, high-anthocyanin level cultivar showed stronger electrophoresis band than low-anthocyanin level cultivar did (as shown in Fig. 6). The results suggested that *AaF3H* transcripts were much more abundant in the high-anthocyanin level cultivar of *A. annua* compared to low-