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Cloning and Sequence Analysis of an Acetyl-CoA *C*-Acetyltransferase Gene (*AACT*) from *Chamaemelum nobile*

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Abstract

Acetyl-CoA C-acetyltransferase (*AACT*) is one of key genes in the cytosolic mevalonate (MVA) biosynthetic pathway which regulate the terpenoid biosynthesis. To analyse the function of *AACT* gene in terpenoid biosynthesis in *Chamaemelum nobile*, we cloned a AACT gene (*CnAACT*, GenBank accession number MF662816) from *C. nobile* using RT-PCR method. The full-length cDNA of *CnAACT* gene is 1727bp which contains an open reading frame (ORF) of 1224bp, encoding a 408 amino-acid protein. The theoretical molecular weight and pI of the *CnAACT* are 4.83kDa and 6.25, respectively. Multi-alignment comparison analysis showed the protein sequence of CnAACT had high similarity with AACT proteins from other plants. Furthermore, several conserved domain of AACT protein were found in CnAACT, suggesting *CnAACT* is one of *AACT* family members. The cloning and characterization of *CnAACT* provided basic data for further studying the function of *CnAACT* in the sesquiterpene biosynthesis in *C. nobile*.

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Keywords

Acetyl-CoA C-acetyl transferase
Chamaemelum nobile
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Introduction

Chamomile is one of the most ancient medicinal herbs known to mankind. It is a member of the Asteraceae/Compositae family and is represented by two common varieties, *German Chamomile* (*Chamomilla recutita*) and *Roman Chamomile* (*Chamaemelum nobile*) (Srivastava et al., 2010). *C. nobile* is a herbaceous plant which belongs to Matricaria. The essential oil of *C. nobile* has significant antiinflammatory (Jellinek, 1984; Tubaro et al., 1984; Lal et al., 1993), antiseptic (Musselman,1996), antiphlogistic (Isaac, 1979; Jakovlev et al.,1979) and spasmolytic (Maschi et al., 2008) properties and is therefore used widely in pharmaceutical, perfume, cosmetics, aromatherapy, and food industries (Lal et al.,1993). However, the flowers of *C. nobile* only contains 1-2% of the volatile oil (Peña et al., 2006), which cannot meet the market demand. The primary constitutes of the volatile oil of *C. nobile* are terpenoids. Thus, it has important significance to enhance the content of terpenoids of *C. nobile* by genetic engineering.

Terpenoid is known as the largest class of plant secondary metabolites and play an important role in the process of plant growth and development (Holstein and Hohl, 2004). In Nature, terpenoids have essential roles, such as those involved in protein prenylation (e.g. RAS prenylation), plant hormonal regulation (e.g. gibberellin, brassinosteroids, cytokinin and abscisic acid), photosynthetic light harvesting (e.g. carotenoid and chlorophyll), and respiratory electron transport (e.g. ubiquinone) (Berndt et al., 2011; Chappell, 1995; Umehara et al., 2008). Terpenoids often function as

toxins, growth inhibitors or deterrents to protect the producers from competing plants or herbivores (Gershenzon and Dudareva, 2007). Terpenoids also have been extensively exploited by humans for their beneficial functions as pharmaceuticals (e.g. paclitaxel and artemisinin), flavours and fragrance compounds (e.g. menthol, patchoulol and nootkatone) (Bohlmann and Keeling, 2008). However, the amount of terpenoids in the biosome is very low, the amount of plants in the biosome is often only one millionth of the level. Therefore, how to improve plant terpenoid production has been a popular research direction (Ajikumar et al., 2008). The synthesis of MVA pathway was proposed by Chaykin et al. (1958) and Lynen et al. (1958) for the first time. This pathway is mainly located in the fine cytoplasm, also known as cytoplasmic pathways, with three acetyl Co A as a raw material through a series of enzymatic reaction to produce isopentenyl pyrophosphate (IPP), part of IPP isomerized to form dimethylallyl pyrophosphate (DMAPP).IPP and DMAPP form geranyl-pyrophosphate (GPP)(Guo et al., 2012). GPP is combined with one IPP unit to produce farnesyl pyrophosphate (FPP), which is a precursor of sesquiterpene. FPP eventually forms sesquiterpene through heterogeneous, cyclization and complexation. MVA is currently considered to be the main pathway of sesquiterpene biosynthesis.

AACT, also known as acetoacetyl-CoA thiolase (Thiolase II), is an important starting molecule for biosynthesis of various metabolites. At the initial step of the MVA pathway, AACT catalyzes the biological Claisen condensation of two molecules of acetyl-CoA to form acetoacetyl-CoA, which is essential in producing terpenoid and isoprenoid compounds such as sterols, carotenoids and growth regulators. At present, the study of *AACT* genes in plants is less, what we do know is that *AACT* is found in *Elaeis guineensis* Jacq, *Ganoderma lucidum* and *Bacopa monnieri* (Rahman and Samian et al., 2014; Fang et al., 2013; Vishwakarma et al., 2012). However, the *AACT* gene has not been reported in *C. nobile* so far. In view of the fact that AACT is the key enzyme in terpenoids synthesis pathway, the *CnAACT* gene was cloned by RT-PCR and the bioinformatics analysis of the gene was carried out in the present study. The findings of this study provide a reliable theoretical basis for revealing the mechanism of *CnAACT* gene regulation on the biosynthesis of sesquiterpenoid compounds of *C. nobile*.

Materials and methods

Plant material

The leaves of *C. nobile* were collected from botanical garden in Yangtze University, China, and reserved in a —80°C refrigerator after frozen by liquid nitrogen.

Major reagents

MiniBEST Plant RNA Extraction kit, PrimeScript™1st Strand cDNA Synthesis Kit, Agarose Gel DNA purification Kit Ver.4.0, dNTP, Taq DNA polymerase RNase, pMD18-T vector were purchased from Takara Company (Dalian, China). In this experiment, both the primers synthesis and DNA sequencing were completed by Shanghai Sangon Biotechnology Company, in China.

Cloning of *CnAACT*

Total RNA was isolated from frozen leaves of *C. nobile* using MiniBEST Plant RNA Extraction kit. The extracted RNA was reverse transcribed into cDNA using PrimeScript™1st Strand cDNA Synthesis Kit. The Specific PrimersC1 and C2 (Table 1) were designed based on the *AACT* unigene sequence of *C. nobile* transcriptome data. The *AACT* gene was amplified by reverse transcription polymerase chain reaction (RT-PCR) using cDNA obtained by RNA reverse transcription. The PCR reaction system was 1 μL upstream of the primer (10 umol • L^{-1}), Exuq polymerase (5U • μL^{-1}) 0.5μL, 10 × PCR buffer 2.5μL, PCR Enhancer 4μL, dNTP (2.5 mmol • L^{-1}) 2μL, total cDNA (1μg • μl^{-1}) 1μL, ddH_2O make up 25μL. The reaction procedure is: 94°C pre-denaturation 3 min; 94°C denaturation 30s, 64.5°C annealing 30 s, 72°C extension 90s, a total of 35cycles; finally at 72°C for 10min.The amplified products were detected by 1% gel electrophoresis and purified using Agarose Gel DNA purification Kit Ver.4.0. The purified product was cloned into the pMD18-T vector, then transformed into *Escherichia coli* DH5α. Positive clones were selected and sent to Shanghai Sangon Biotechnology Company for sequencing.

Table 1. Primer sequences used in this study.

Primers	Race(5'-3')
Upstream primers of *CnAACT* gene (C1)	CGAAGCTCACACACACTACCTGTAAAC
Downstream primers of *CnAACT* gene (C2)	CCTCAAGTTTTTCACCACCGACG

Bioinformatic analysis

CnAACT gene sequences were translated into amino acid sequences by using DNAMAN software. The open reading frame (ORF) of the *CnAACT* gene was predicted by using Vector NTI 11.5.Protein homology searches were performed by using the bioinformatics software on NCBI website (http://www.ncbi.nlm.nih.gov/BLAST/). Multiple sequence alignment was performed with the software Vector NTI11.5 program. Phylogenetic tree of AACT proteins was constructed with neighbor-joining method using Clustal X2.0 and MEGA5 (Larkin et al., 2007).

Results and discussion

Cloning and sequence analysis of *CnAACT*

A pair of specific primers was designed according to the *AACT* unigene sequence of *C. nobile* transcriptome data, the cDNA sequence of 1727bp was amplified by RT-PCR. The comparison analysis shows that the cDNA sequence is highly homologous to the *AACT* sequences of other plants, it indicated that the cloned cDNA sequence was the *AACT* gene of *C. nobile*. The cDNA sequence was designated as *CnAACT* and GenBank accession number wasMF662816. The full-length cDNA of *CnAACT* is 1727bp. and contained a1224bp ORF which encoded408amino acids (Fig. 1). ExPASy online (http://web.expasy.org/ compute_pi/) analysis results displayed that the theoretical molecular weight and isoelectric point (pI) of the CnAACT protein were 4.83kDa and 6.25, respectively.

Characterization of CnAACT protein

The multiple sequence alignment of AACT protein sequences among different plants using software Vector NTI11.5 showed that CnAACT is highly homologous to the AACT protein sequences of other plants, further sequencing analysis showed that the protein sequence of CnAACT keep a strong conservation during the molecular evolution (Fig. 2), different plant *AACT* genes have similar conserved domains. As shown in Table 2, the protein sequence of CnAACT had high identity with other AACT proteins, it showed 96%, 94%, 91%, 84%, 83%, 83%, 83%, 83%,83%,83%similarity to AACT proteins from *Helianthus annuus*, *Taraxacum kok-saghyz, Taraxacum brevicorniculatum, Ziziphus jujuba, Phlomis umbrosa, Nelumbo nucifera, Populus trichocarpa, Populus euphratica, Camellia oleifera* and *Camellia chekiangoleosa,* respectively, it was further verified that *CnAACT* is one of members of the *AACT* gene family of *C. nobile*.

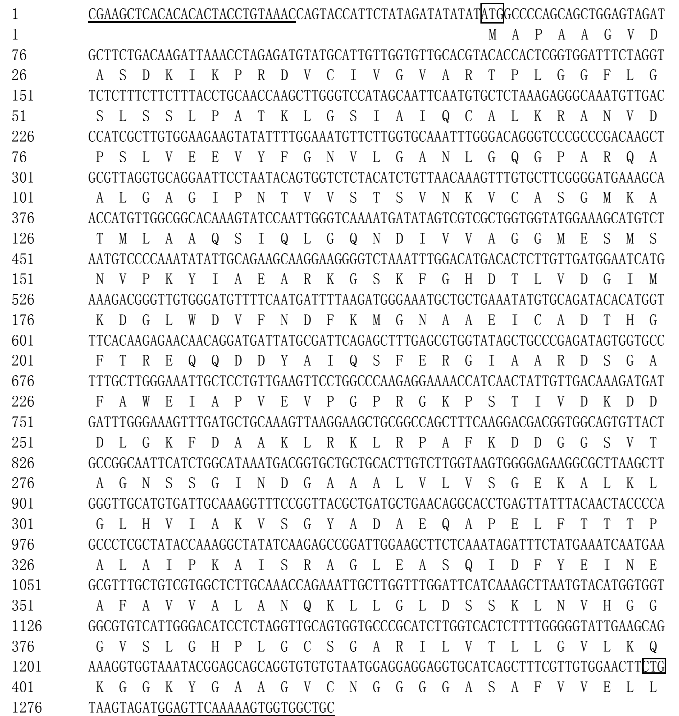

Fig. 1: The Nucleotide sequence and deduced amino acid sequences of the full-length cDNA of *CnAACT*. The initial codon and the stop codon are highlighted in square box.

Table 2. Protein sequence of CnAACT similarity to AACTs of other plant species.

Species	Accession No. in GenBank	Identity/%
Helianthus annuus	OTG07344.1	96
Taraxacum kok-saghyz	AMB19691.1	94
Taraxacum brevicorniculatum	ARB18354.1	91
Ziziphus jujuba	XP_015880842.1	84
Phlomis umbrosa	APU50933.1	83
Nelumbo nucifera	XP_010252788.1	83
Populus trichocarpa	XP_002320528.1	83
Populus euphratica	XP_011008068.1	83
Camellia oleifera	ADD10719.1	83
Camellia chekiangoleosa	AGH32909.1	83

Phylogenetic analysis of CnAACT

To investigate the evolutionary relationships among AACT proteins, a phylogenetic tree was constructed based on the deduced amino acid sequences of predicted CnAACT and AACT proteins from other plant species (Fig. 3). AACT phylogenetic tree is divided into two branches of monocotyledons and dicotyledons. Among them, monocotyledons composed by Poaceae and Arecaceae, and dicotyledons composed by Salicaceae, Leguminosae, Solanaceae, Rosaceae, Euphorbiaceae, Malvaceae and Asteraceae. *C. nobile* belonged to Asteraceae of dicotyledons in the branch of dicotyledons and monocotyledons. *C. nobile* has the closest relationship with *Asteraceae*, probably because it belong to the *Asteraceae*. *C. nobile* also has a genetic relationship with other dicotyledons and monocotyledons. This reflects the evolutionary conservation and evolutionary diversity of plant *AACT* genes, which is consistent with the morphological classification of plants.

Fig. 2: Multiple sequence alignment of AACT proteins.The completely identical amino acids are indicated with red foreground and yellow background, the conservative amino acids are indicated with yellow foreground and cyan background, the block of similar amino acids are indicated with blue foreground and yellow background,the weakly similiar amino acids are indicated with gray foreground and white background, the non-similiar amino acids are indicated with black foreground and white background.

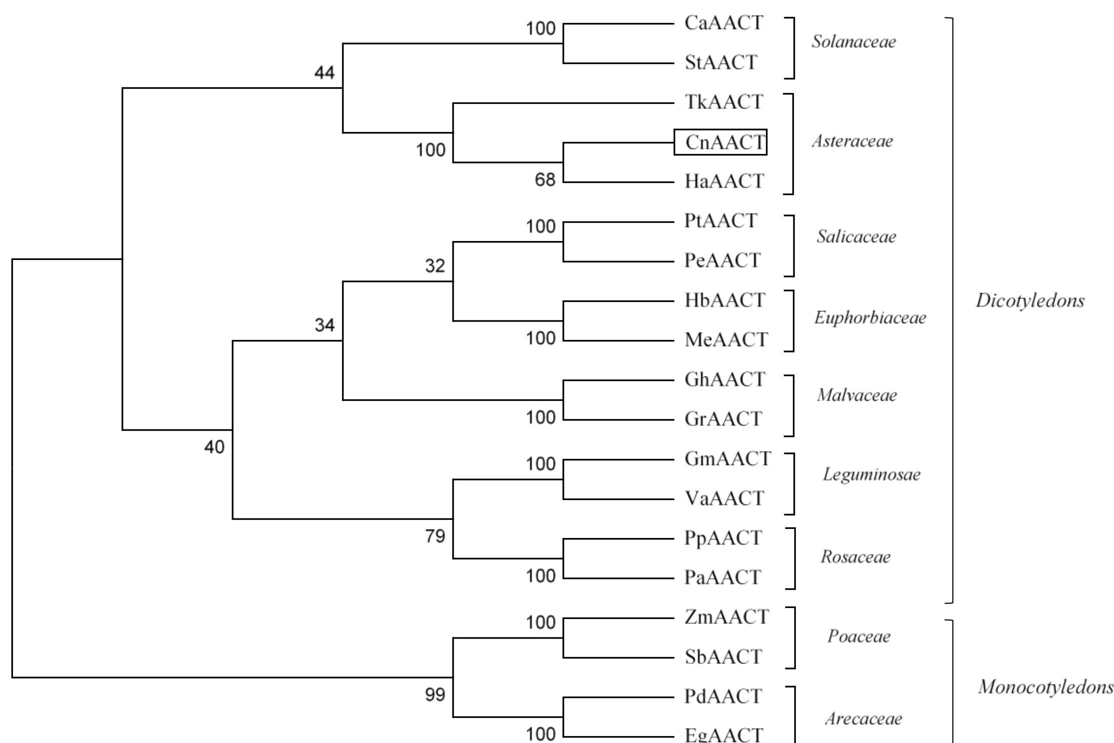

Fig. 3: Phylogenetic tree of AACT using Neighbor-Joining method. The number shown at each branch indicated the bootstrap values (%).
HaAACT: *Helianthus annuus*,OTG07344.1TkAACT: *Taraxacum kok-saghyz*, AMB19691.1
PtAACT: *Populus trichocarpa*, XP_002320528.1PeAACT:*Populus euphratica*, XP_011008068.1
GmAACT: *Glycine max*, XP_003545555.1VaAACT:*Vigna angularis*, XP_017429942.1
CaAACT: *Capsicum annuum*, XP_016580453.1StAACT:*Solanum tuberosum*, XP_015169006.1
PpAACT: *Prunus persica*, XP_007215468.1PaAACT:*Prunus avium*, XP_021805887.1
HbAACT: *Hevea brasiliensis*, XP_021656809.1MeAACT:*Manihot esculenta*, XP_021629494.1
GhAACT: *Gossypium hirsutum*, XP_016696139.1ZmAACT:*Zea mays*,NP_001266315.2
GrAACT: *Gossypium raimondii*, XP_012491016.1SbAACT:*Sorghum bicolor*, XP_021321918.1
PdAACT: *Phoenix dactylifera*, XP_008811276.1EgAACT:*Elaeis guineensis*, XP_010942603.1
CnAACT: *Chamaemelum nobile*, MF662816

Conclusion

AACT catalyzes the formation of acetoacetyl-CoA by transferring an acetyl group from one acetyl-CoA molecule to another (Clinkenbeard et al., 1973). Because this is the first step of many biosynthetic pathways, AACT plays a fundamental role in carbon skeleton assembly patterns in many biological systems, including the synthesis of steroid hormones, cholesterol, terpenoid and ketone bodies (Modis and Wierenga, 2000; Kursula et al., 2002). In this study, we have successfully isolated and cloned the *CnAACT* gene from *C. nobile*. The full-length cDNA of this gene is 1727bp and the ORF of 408 amino acids is 1224 bp. Multiple sequence alignment indicated that *CnAACT* was highly homologous to other *AACT* genes isolated from other plants. In plants, there are two distinct forms of thiolases detected, each Thiolase I or 3-ketoacyl-CoA thiolase (KAT) and AACT, or Thiolase II (Heath and Rock, 2002). AACT is specific for the thiolysis of acetoacetyl-CoA, and it is involved in polyb-hydroxybutyrate synthesis in certain bacteria and isoprenoid biogenesis in eukaryotes (Vollack and Bach, 1996). *C. nobile* AACT protein as an important starting molecule for biosynthesis of various metabolites has important research value. At present, *AACT* gene is reported in plant research less, only in the *Elaeis guineensis* Jacq, *Ganoderma lucidum*, *Bacopa monnieri* and *C. nobile* have never been reported. In this study, the *CnAACT* gene was cloned by genetic engineering and sequenced. It was helpful to understand the biosynthetic pathway of *C. nobile* and its

regulatory mechanism, and to provide candidate genes for *C. nobile* terrestrial metabolic engineering.

Conflict of interest statement

Authors declare that they have no conflict of interest.

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