Cloning and Identification of an ACO Gene Involved in Ethylene Production Pathway from Ginkgo biloba

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ABSTRACT

1-Aminocyclopropane-1-carboxylic acid (ACC) oxidase (ACO), is one of the core enzymes in the ethylene biosynthesis pathway, which catalyzes the final step in ethylene biosynthesis converting ACC to ethylene, cyanide, CO₂, dehydroascorbate and water with inputs of Fe(II), ascorbic, bicarbonate and oxygen. In the present study, a 1288 bp cDNA sequence fragment of the ACO gene (designated as GbACO) was isolated from ginkgo (Ginkgo biloba) leaves by using reverse transcriptase-polymerase chain reaction (RT-PCR) with two PCR primers designed according to the ginkgo transcriptome data. This cDNA sequence contained a 990bp open reading frame (ORF), which encoding a 329 amino acid sequence showed high similarity to other plant ACO proteins. The secondary structure prediction indicated that GbACO protein comprised of α-helix (40.43%), β-turn (10.33%), extended strand (13.07%), and random coil (36.17%). Phylogenetic tree analysis revealed that GbACO clustered with the ACO in the Gymnospermae clade. The isolation and characterization analysis of GbACO is helpful to understand the biosynthesis of ethylene in G. biloba at the molecular level and also provides some theoretical support for improving the ethylene production.

Keywords
1-Amino Cyclopropane-1-carboxylic acid oxidase (ACO), Ginkgo biloba, Gene cloning, Bioinformatics analysis.

Introduction

Ginkgo biloba is a living fossil from the Cenozoic Quaternary glacial period. It is a precious tree species in China and has been extensively studied given its important medical and ornamental value. It has a long juvenile phase and spends 15 to 20 years in the flowering transition, and ethylene has played a very important role in its life cycle. As we all know, ethylene, one of the most important plant hormones, involves in the various aspects of plant growth and development, such as seed dormancy and germination, cell elongation, root growth and nodulation, shoot and leaf formation, flower and fruit development, sexual development, plant defense mechanism, and so on (Abeles et al., 1992; Wang et al., 2002; Chaves and Mello-Farias, 2006; Argueso et al., 2007; Lin et al., 2009). Although ethylene is plays an essential role for proper plant growth, development, and survival, it may also be deleterious to plants in some instances. Ethylene is biologically active in trace amounts, and the effects of this hormone are commercially important (Yang and Hoffman, 1984). Lieberman and Mapson (1964), Murr and Yang (1975), Adams and Yang (1979) made major contributions to our
understanding of ethylene biosynthesis. A simplified overview of the pathway is presented in figure 1. The number of sequenced lower plant genomes has increased extensively in the past few years, providing opportunities to trace the evolutionary origins of biosynthetic and signaling pathways of plant hormones (Ross and Reid, 2010; Ju et al., 2015). The ethylene biosynthetic pathway, which includes three key enzymes reactions, has been well-documented in higher plants. First, the biosynthesis of ethylene begins with the conversion of the amino acid methionine to S-adenosyl-methionine (SAM) via enzyme SAM synthase (Yang and Hoffman, 1984; Zarembinski and Theologis, 1994). Second, the rate-limiting step catalyzed by 1-aminocyclopropane-1-carboxylic acid (ACC) synthase (ACS) involves the cyclization of SAM to ACC, and 5’-methylthioadenosine (MTA) is produced by the ACS to utilize the synthesis of new methionine by the activated methyl cycle. Finally, ACC oxidase (ACO) catalyzes the oxygen-dependent conversion of ACC to ethylene (Yang and Hoffman, 1984; Liu et al., 1999; Choudhury et al., 2008).

Increased ethylene levels in plants when exposed to various types of stress including chilling, heat, nutrient deprivation, anaerobic life, wounding, and pathogen infection with increased damage to plant growth and health has been reported (Stearms and Glick, 2003). There is considerable commercial interest in modifying the amount of ethylene produced under ripening, senescent or stress conditions and thereby creating plants with more robust and/or desirable traits (Handa et al., 2010). The level of ethylene in plants is determined by the activity of two enzymes that catalyze the two final reaction steps in the biosynthetic pathway: ACS and ACO gene. Both ACS and ACO are encoded by multigene families that are differentially regulated by diverse developmental and environmental inputs (Barry et al., 1996; Chen and McManus, 2006), the majority of the regulatory mechanisms of ethylene biosynthesis at the level of ACC production by ACC. However, there are additional regulatory mechanisms. Under conditions of high ethylene production, the pathway can also be regulated at the level of conversion of ACC into ethylene by ACO (Vanderstraeten and Van Der Straeten, 2017). ACO enzyme belongs to the family of oxidoreductases which utilize Fe (II) as a cofactor and 2-oxoglutarases (2OG) as co-substrate, although ACO uses ascorbate as a co-substrate (Prescott, 1993; Ryle and Hanusinger, 2002; Zhang et al., 2004; Mirica and Klinman, 2008). ACO needs bicarbonate as an activator and catalyzes the oxidation of ACC to give ethylene, CO₂, and HCN. It has been revealed that ACO forms a complex with Fe (II) and its active site contains a single Fe (II) matched with three residues (Zhang et al., 2004; Jafari et al., 2013). In its sequence, two distinct domains can be distinguished: an N-terminal located highly conservative non-heme dioxygenase DIOX_N region and a C-terminal located 2OG-Fe II_Oxy region.

The practical implication of ethylene biosynthesis regulation for plant improvement has supported the continuous basic research on dissecting the structure of genes encoding ethylene biosynthetic enzymes, their differential expression patterns, and mechanisms underlying their transcriptional activity (Izabela et al., 2013). ACO has been cloned and characterized in many plant species such as peach, potato, strawberry, white clover, plum, Arabidopsis, rice, apple, pine, pear (Ruperti et al., 2001; Nie et al., 2002; Trainotti et al., 2005; Chen and McManus, 2006; Fernandez-Otero et al., 2006; Babula et al., 2006; Quyang et al., 2007; Binnie and McManus, 2009; Yuan et al., 2010; Qi et al., 2015). Here, we identified the GbACO gene originated from the G. biloba leaves that promoted the growth of
elongation followed by incubation at 72°C for 10 min. The PCR products were separated on a 1% (w/v) agarose gel, then the target DNA (with the expected size) was excised from the gel and purified using TaKaRa Agarose Gel DNA purification Kit Ver.4.0. The purified products were ligated into the pMD19-T plasmid vector (TaKaRa), and then cloned into the Escherichia coli DH5α followed by sequencing.

Materials and Methods

Plant materials

The 15-years-old *G. biloba* plants were grown in the Ginkgo Garden of Yangtze University (Jingzhou, China). Ginkgo leaves were immediately frozen in liquid nitrogen at the time of collection and then stored at -80°C for further use. Both primers synthesis and DNA sequencing were performed by Shanghai Sangon Biotechnology Company, China.

Cloning of *ACO* homologous from *G. biloba*

Total RNA was extracted from frozen ginkgo leaves using the TaKaRa MiniBEST Plant RNA Extraction kit (Dalian, China). First strand cDNA was synthesized using TaKaRa PrimeScript™ 1st Strand cDNA Synthesis Kit according to the manufacturer’s instruction. A set of primers GbACO-up (5'-CTCATAACAATAATAGAGATTACTTTC-TCA-3’) and GbACO-dn (5'-TTAAGATTTGAACTAAAGTAGATTGT-3’) were designed based on the previous ginkgo transcriptome data (CL1905Contig1). Each 25 µl reaction volume contained 0.5 µg cDNA as the template; 1.5 mM MgCl₂; 10×Taq buffer; 0.4 µM of each primer; 0.25 mM dNTPs; and 0.25U Taq polymerase. The reactions were initiated at 94°C (4 min), followed by 35 cycles of denaturing at 94°C (1 min), annealing at 53.5°C(50 s), and elongation at 72°C (1 min), and ended with

Bioinformatics and molecular evolution analyses

The obtained nucleotide sequence from sequencing was analyzed and translated using Vector NTI 11.5 program, and the predictions for pI and molecular mass were using the pI/MW tool at Expasy (http://web.expasy.org/compute_pi/).

Secondary structure was predicted by SOPMA (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=NPSA/npsa_sopma.html). The deduced protein sequence was searched for homologous proteins using the BLAST-Basic Local Alignment Search Tool (http://www.ncbi.nlm.nih.gov/BLAST/).

Sequences with the highest similarities were downloaded and multiple alignments were performed using Vector NTI 11.5 program, and the phylogenetic tree was constructed using Clustal X2 and MEGA 6.0 software.

Results and Discussion

Cloning and characterization of *GbACO*

In order to characterize the full-length *GbACO* DNA, total RNA was isolated from ginkgo leaves. cDNA was reverse transcribed from RNA, and then, the corresponding *GbACO* DNA amplification was performed using the primers (GbACO-up and GbACO-dn), designed based on previous transcriptome data (CL1905Contig1). A single PCR fragment of the expected size was
obtained, purified, ligated into the pMD19-T vector, and then cloned into the E. coli DH5α, finally sequenced. Sequencing results showed that the full-length cDNA of GbACO was 1288 bp and contained a 990bp ORF, which encoding a 329-amino-acid protein (Figure 2). The molecular weight and isoelectric point of the deduced polypeptide were 37.2 kDa and 5.20, respectively.

Characterization of the deduced GbACO protein

The comparison of the amino acid sequence of GbACO with other plant ACO proteins showed high sequence similarity. The GbACO protein showed 64%, 62%, 61%, 60%, 60%, 59%, 60%, 63%, and 61% similarity to the counterparts of Picea sitchensis (ABF20552.1), Pseudotsuga menziesii (ABF20554.1), Elaeis guineensis (XP_010905322.1), Phoenix dactylifera (XP_008805862.1), Nelumbo nucifera (XP_010269058.1), Ficus carica (AKJ87578.1), Cucumis melo (XP_008444197.1), Pinus pinaster (CBL95267.1), and Ricinus communis (XP_002520313.1), respectively. Multiple sequence alignments showed that the peptide is a member of ACO proteins, with DIOX_N (non-haem dioxygenase in morphine synthesis N-terminal), and 2OG-Fe II_Oxy (2-oxoglutarate (2OG) and Fe (II)-dependent oxygenase) domains (Figure 3). As we all know, ACO is a member of Fe (II)-dependent family of oxidases, which requires ascorbate as a co-substrate and Fe (II) as a cofactor for its enzymatic activity (McGarvey and Christoffersen, 1992). All motifs for binding of the cofactor (His-Xaa-Asp-Xaa-His) and the co-substrate (Arg-Xaa-Ser) that are highly conserved among all members of Fe (II) ascorbate family of dioxygenases were also well conserved in GbACO and other plant ACO proteins (Figure. 3). The Fe2OG dioxygenases catalyze a variety of electron oxidations, including hydroxylations, desaturations, and oxidative ring closures (Costas et al., 2004). Although the sequence homology among these enzymes is not high, all their active sites contain a single ferrous ion bound in a tridentate ligand arrangement referred to as a “2-His-1-carboxylate facial triad” (Mirica and Klinman, 2008). Secondary structure analysis of GbACO protein by SOMPA revealed that GbACO consists of α-helix (40.43%), β-turn (10.33%), extended strand (13.07%), and random coil (36.17%).

**Figure. 1** Ethylene biosynthesis pathway. The enzymes catalyzing each step are shown above the arrows. The methionine is converted to S-adenosyl-methionine (SAM) via enzyme SAM synthase (SAMS). SAM is subsequently converted to ACC by ACC synthase (ACS). MTA finally, ACC is converted to ethylene by ACC oxidase (ACO). (Yang and Hoffman, 1984; Vanderstraeten and Van Der Straeten, 2017)
Figure 2 cDNA sequence of *GbACO* and the translated amino acid sequence. The red box is the start codon, and the asterisk represents the termination codon.

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1  ctcataacataataatagagattacttttcatttttcattttttgaatactctttgtgtgtgtgtgtcttctagtt
76  attataaataatggctatccttctgcttctttttattttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttt
**Figure 3** Alignment of the deduced GbACO amino acid sequence with other ACO proteins. The species names are shown as following: GbACO, *G. biloba*; EgACO, *E. guineensis*; PdACO, *P. dactylifera*; CmACO, *C. melo*; RrACO, *R. communis*; NnACO, *N. nucifera*; FaACO, *F. carica*; PmACO, *P. menziesii*; PpACO, *P. pinaster*; PsACO, *P. sitchensis*. Green and red boxes are represented DIOX_N and 2OG species names are shown as following Figure 3.
Figure 4 Phylogenetic relationships between GbACO and other plant ACO proteins. The phylogenetic tree was constructed by use of Clustal X2 and MEGA 6.0 software with the neighbor-joining method based on the amino acid sequences multiple alignments. The species names are listed as following: PtACO, Populus tremula × Populus tremuloides; PtrACO, P. trichocarpa; RcACO, R. communis; JcACO, Jatropha curcas; PxACO, Pyrus × bretschneideri; FvACO, Fragaria vesca subsp. Vesca; PmuACO, Prunus mume; GhACO, Gossypium hirsutum; GrACO, G. raimondii; GmACO, Glycine max; VrACO, Vigna radiata var. radiate; AiACO, Arachis ipaensis; MtACO, Medicago truncatula; CsACO, C. sativus; CmACO, C. melo; MnACO, Morus notabilis; FaACO, F. carica; NsACO, Nicotiana sylvestris; CanACO, Capsicum annuum; StACO, Solanum tuberosum; BnACO, Brassica napus; BrACO, B. rapa; EgACO, E. guineensis; PdACO, P. dactylifera; ZmACO, Zea mays; SbACO, Sorghum bicolor; GbACO, G. biloba; PsACO, P. sitchensis; PpACO, P. pinaster; PmACO, P. mume.

Molecular evolution analysis

In order to better understanding the molecular evolution relationship of GbACO protein, a phylogenetic tree was constructed from an alignment of the deduced amino acid sequence from the ginkgo ACO with other plant ACO proteins via the neighbor-joining method with the Clustal X2 and MEGA 6.0 software. As shown in figure 4, GbACO was classified into the Gymnospermae branch; all the species showing such a high degree of homology belonged to the Gymnospermae. Thus, GbACO protein has its closest relationship to Gymnosperms, which form a separate cluster on the tree, while another cluster consists of the ACO proteins from Angiosperms, including eleven families (Salicaeae, Euphorbiaceae, Rosaceae, Malvaceae, Leguminosae, Cucurbitaceae, Moraceae, Solanaceae, Cruciferae, Palmaceae, Monocotyledonae).
Moraceae, Solanaceae, Cruciferae, Palmaceae and Monocotyledonae). Taken together, phylogenetic analysis suggests that GbACO has a common evolutionary origin with other plant ACO proteins based on the conserved sequence and sequence characteristics.

**Conclusion and perspectives**

ACO catalyzes the final step in the biosynthesis of the plant signaling molecule ethylene that is involved in the regulation of array of biological processes in plants, including seed dormancy and germination, root growth, shoot and leaf formation, flower and fruit development, different organs senescence, plant defense mechanisms, and a number of interactions with other plant hormones. Here, an ACO homologue, GbACO was successfully isolated and characterized for the first time from ginkgo. Multiple sequence alignments indicated that GbACO had a high similarity to other plant ACO proteins. Phylogenetic tree analysis showed that GbACO keeps a strong conservation during the molecular evolution. All of these results not only contribute to understand the ethylene biosynthesis in Gymnosperms but also promote researches of the role of ethylene in the regulation of ginkgo growth and development.

Nowadays, the number of sequenced plant genomes has increased extensively in the past few years, providing opportunities to probe the evolutionary origins of biosynthetic and signaling pathways of plant hormones. There is a commercial interest in increasing the final products through increasing the expression level of key genes in the biosynthesis pathway. Even though ACO genes have been isolated from a lot of species, the biochemical properties, in vivo stability, and/or physiological functions of the gene products are in many cases not fully understood. Thus, further study is needed to verify the role played by different ACO genes in the regulation of ethylene production. A better understanding of both structure and function of genes involved in the ethylene biosynthesis, and also the mechanisms responsible for their activity during different developmental processes of plants. All this has a profound practical importance as it develops the tools for ginkgo improvement.

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