Cloning and expression of the 1-aminocyclopropane-1-carboxylic oxidase gene from *Agrostis stolonifera*

G.Z. Xiao\(^1\)\(^2\), L.J. Li\(^1\), K. Teng\(^1\), Y.H. Chao\(^1\) and L.B. Han\(^1\)

\(^1\)Turfgrass Research Institute, College of Forestry, Beijing Forestry University, Beijing, China
\(^2\)College of Horticulture and Garden, Yangtze University, Jingzhou, China

Corresponding authors: Y.H. Chao / L.B. Han
E-mail: chaoyuehui@163.com / hanliebao@163.com

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**ABSTRACT.** A gene encoding 1-aminocyclopropane-1-carboxylic oxidase (ACO), which catalyzes the terminal step in ethylene biosynthesis, was isolated from *Agrostis stolonifera*. The *AsACO* gene is composed of 975 bp, encoding 324 amino acids. Three exons interspersed by two introns form *AsACO* gDNA. A BLAST search of the nucleotide sequence revealed a high level of similarity (79-91%) between *AsACO* and *ACO* genes of other plants. A phylogenetic tree was constructed via BLAST in the NCBI, and revealed the highest homology with wheat *TaACO*. The calculated molecular mass and predicted isoelectric point of *AsACO* were 36.25 and 4.89 kDa, respectively. Analysis of subcellular localization revealed that *AsACO* is located in the nucleus and cytoplasm. The Fe(II)-binding cofactors and cosubstrate were identified, pertaining to the *ACO* family. The expression patterns of *AsACO* were determined by quantitative real
time PCR. *AsACO* expression was highest in the stem, and was strongly up-regulated in response to ethephon, methyl jasmonate, salicylic acid, and cold temperature, but down-regulated in response to drought and NaCl treatment. The protein encoded by *AsACO* exhibited ACC oxidase activity *in vitro*. Taken together, these findings suggest that *AsACO* contains domains common to the *ACO* family, and is induced in response to exogenous hormones. Conversely, some abiotic stress conditions can inhibit *AsACO* expression.

**Key words:** *AsACO, Agrostis stolonifera; Ethylene biosynthesis; Abiotic stress*

**INTRODUCTION**

Ethylene is one of the most important gaseous phytohormones in plants, and is involved in many physiological processes. Signaling regulated by ethylene is involved in plant growth, including seed germination, flower and leaf formation, sexual development, fruit ripening, and senescence (Bleecker and Kende, 2000). Ethylene biosynthesis has been demonstrated to play a crucial role not only in plant development, but also in stress tolerance (Yang and Hoffman, 1984; Binnie and McManus, 2009). Therefore, studies have investigated the mechanisms of ethylene production and manipulated the amount of ethylene produced, thus increasing plant vigor and stress tolerance (Ski and Ska, 2005; Han et al., 2011). The results of studies on rice have shown that ethylene application can improve drought-resistant traits (Wan et al., 2011). It has been elucidated that 1-aminocyclopropane-1-carboxylate synthase (ACS) functions in the first step of ethylene biosynthesis and can enhance drought tolerance and inhibit drought-induced senescence in maize (Young et al., 2004). It has also been reported that selected strains of *Ocimum sanctum* containing ACC deaminase tolerate waterlogging stress by reducing ethylene generation (Barnawal et al., 2012). ACS catalyzes the conversion of S-adenosylmethionine (AdoMet) to ACC. Then, 1-aminocyclopropane-1-carboxylic acid oxidase (*ACO*) converts ACC to ethylene in the ethylene biosynthesis pathway (Adams and Yang, 1979). By regulating ethylene biosynthesis in advanced plant species; the *ACO* gene may play an auxiliary or major role in this process. (Ruduš et al., 2013).

ACO is classified as a member of a non-heme oxygenase family, in which ferrous is indispensable and 2-oxoglutarate (2OG) serves as a cosubstrate (Mirica and Klinman 2008). Using bicarbonate as an activator, ACO catalyzes the oxidation of ACC, producing ethylene, CO₂, and hydrogen cyanide (Zhang et al., 2004). Using molecular cloning and heterologous expression, *ACO* can be isolated to homogeneity. ACO is encoded by a divergent multigene family, and modulation of *ACO* expression differs under different environmental conditions; however, the mechanism of *ACO* activity requires further study (Kende and Zeevaart, 1997).

Much research has been performed to isolate and utilize the *ACO* gene in dicotyledons, such as apple (Binnie and McManus, 2009), melon (Lasserre et al., 1996), white clover (Chen and McManus, 2006), and the model organism *Arabidopsis thaliana* (Babula et al., 2006). However, limited studies have been performed using monocotyledonous plants such as *Agrostis stolonifera*, to explore the mechanisms controlling ethylene biosynthesis.

*A. stolonifera* is a very common cool-season grass used extensively for golf courses and residential lawns (Zhang, et al., 2003). However, the stoloniferous growth habit of *A.*
stolonifera can result in excessive thatch formation, engendering poor rooting, reduced water uptake, and pathogen infection. The purpose of the present study was to determine how hormones and NaCl stress affect expression of AsACO by isolating and cloning a full-length cDNA encoding ACO from A. stolonifera leaf. qRT-PCR techniques were used to examine AsACO expression in different parts of the plant and to determine the response to ethephon (Et), methyl jasmonate (MeJa), salicylic acid (SA), abscisic acid (ABA), and NaCl stress. Furthermore, the subcellular location and expression patterns were studied by transient expression. This study on the AsACO gene may lay a theoretical foundation for future studies on ethylene regulation and transgenic breeding.

MATERIAL AND METHODS

Plant materials

The A. stolonifera cultivar Penncross and Nicotiana benthamiana seeds, preserved in the laboratory, were planted in mixed aggregate general-purpose plant growth medium, including coarse perlite, vermiculite, and peat in a 1:1:1 ratio. Plants were grown in a growth chamber, and treated under the following conditions: average day/night temperature of 22°C ± 1/16 ± 1°C, relative humidity 55-60%, 14-h photoperiod of 120 mmol∙m^{-2}∙s^{-1} par, and were fertilized weekly with half-strength Hoagland’s (Hoagland and Arnon, 1950) solution.

Isolation of AsACO

Total RNA was extracted from 3-month-old Penncross leaves using the Trizol (Tiangen Biotech, Beijing, China) method. Genomic DNA was removed by RNase-free DNAse (Tiangen Biotech, Beijing, China) prior to reverse transcription. The reverse transcription reaction involved preheating at 37°C for 30 min in a PCR machine, followed by then performed at 85°C for 5 s. cDNA concentration was measured by spectrophotometric measurement. cDNA concentration was measured by spectrophotometric measurement. Hordeum vulgare ACO2a (GenBank: JX046058), Triticum aestivum ACO (GenBank: KF900070), Oryza sativa ACO (GenBank: AF049889), and Zea mays ACO (GenBank: NM_001136755) were used at the test standards. The common conserved sequence was identified and degenerate primers were designed accordingly (Table 1).

<table>
<thead>
<tr>
<th>Name of primer</th>
<th>Sequence (5'-3')</th>
<th>Purpose of primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>5'RACE</td>
<td>TCATCACTTGCCTGTAGTGGTCG</td>
<td>5'RACE-PCR</td>
</tr>
<tr>
<td>A4cO-gDNA</td>
<td>F: ATACCGAGAGGCGGTAGGAGAAGA</td>
<td>RT-PCR of A4cO-gDNA</td>
</tr>
<tr>
<td>A4cO-gDNA</td>
<td>R: GATCUGACTGTCACTGAGACG</td>
<td>RT-PCR of A4cO-gDNA</td>
</tr>
<tr>
<td>A4cO-RT</td>
<td>F: CAGGGAGGACAGGTTCAAGGAGTG</td>
<td>Real-time PCR analysis of A4cO gene</td>
</tr>
<tr>
<td>3302Y-AsACO</td>
<td>F: cacgggggactcttgaccatttagTTGGCGACTGCAGCAGCTG</td>
<td>Analysis of AsACO protein</td>
</tr>
<tr>
<td>3302SP6-AsACO</td>
<td>F: gacactatagaacagaccaccATGGCGACTGCAGCAGCTG</td>
<td>Sp6-3xFlag vector of AsACO</td>
</tr>
</tbody>
</table>

Table 1. Genetic cloning of AsACO and primer sequence.
Utilizing the sequence as a template, \textit{AsACO} was cloned by 5’- and 3’-RACE to obtain the full coding sequence (CDS) according to the manufacturer instructions (SMARTer RACE 5’- and 3’- Kit, TaKaRa, Dalian, China) at an annealing temperature of 57°C. PCR products were cloned using a pMD19-T vector (TaKaRa) and subsequently sequenced at the Beijing Genomics Institute (Beijing, China).

Amplification of the full-length \textit{AsACO}-cDNA was performed using \textit{AsACO}-F and \textit{AsACO}-R, obtained using primers and cDNA as templates. \textit{AsACO}-gDNA was obtained by utilizing DNA as a template, \textit{AsACO}-F, and \textit{AsACO}-R were used as primers. Next, the intron-exon structure of \textit{AsACO} was determined.

**Bioinformatic and sequence analysis**

Comparison of homologous sequences was performed using the DNAMAN software (v. 8.0). Homologue similarity was analyzed using BLAST in the NCBI database. An unrooted phylogenetic tree was generated by the neighbor-joining method using MEGA v. 5.0. One-thousand bootstrap replicates were used to ensure stability of the tree branch. The isoelectric points (pI) and molecular weights (MW) were predicted using the compute pI/MW tool (http://web.expasy.org/compute_pi/). Underlying sites and the existence of signal peptide cleavage sites were identified by SignalP 4.1 Server (http://www.cbs.dtu.dk/services/SignalP/) (Petersen et al., 2011). Subcellular localization of \textit{AsACO} was determined and its level of expression was identified by analyzing the protein sequence through ProtComp v. 9.0 (http://www.softberry.com).

**Analysis of \textit{AsACO} gene induction**

Gene expression was analyzed in 3-month-old seedlings, which were cultured in a growth chamber and then anatomized into root, stem, new leaf, and old leaf. To examine the effects of plant hormones and abiotic stress on the expression of \textit{AsACO}, 3-month-old seedlings were treated with 10 µM Et, 10 µM MeJA, 10 µM ABA (Teng et al. 2016), 50 mM SA, cold temperature (4°C), drought (30% PEG6000), and 200 mM NaCl. Expression of the \textit{AsACO} gene in the shoot was measured at selected points over a 24-h period (0, 1, 3, 6, 12, and 24 h). \textit{AsACO}-RT and \textit{AsActin}-RT were used as primers (Table 1). Each qRT-PCR was performed in a 96-block-RT-PCR system (CFX Connect, BIO-RAD, Hercules, CA, USA) with an initial 12.5 µL UltraSYBR Mixture (CoWin, Beijing, China) in a final 25-µL volume, under the following conditions: 15 s denaturation at 94°C and 1 min annealing at 68°C for 40 cycles. The endogenous gene \textit{AsActin} (GenBank: JX644005.1) was used in the comparative $C_{\text{T}}(2^{-\Delta\Delta C_{T}})$ method, in which $\Delta G_{T} = C_{\text{Target gene}} - C_{\text{Reference gene}}$, allowing the relative quantification of gene expression. This equation was improved through the comparative $\Delta\Delta C_{T}$ method (Chao et al., 2009). Four independent biological repeats were conducted for each treatment to ensure data accuracy.

**Subcellular localization of \textit{AsACO}**

To generate the CaMV35S:\textit{AsACO}-YFP construct, the \textit{AsACO} CDS was amplified using a pair of designed 3302Y-\textit{AsACO} primers (Table 1), and then subcloned into a \textit{Bal}I site in CaMV35S on a 3302Y vector (Figure 1a), which was then introduced into EHA105
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agrobacteria using the CaCl₂ freeze-thaw method. EHA105 agrobacterium-mediated transformation of *N. benthamiana* (Yang et al., 2000) was followed by 48-h incubation in the dark. The yellow fluorescent protein (YFP) signal and chlorophyll from leaf epidermal cells were examined under a laser confocal microscope (Leica SP-5; Leica, Mannheim, Germany).

**Figure 1.** Vector construction of *AsACO* for transient expression and AsACO for protein expression. **a.** vector construction of *AsACO* for transient expression, **b.** vector construction of AsACO for protein expression.

**AsACO** protein expression and western blot

To express AsACO protein and generate the SP6:AsACO-Flag construct, the *AsACO* CDS was amplified using a pair of designed 3302SP6-AsACO primers (Table 1), and then subcloned into NcoI sites of CaMV35S in a 3302SP6-Flag vector (Figure 1b). The SP6-AsACO-Flag CDS was amplified using a pair of designed SP6-AsACO primers (Table 1). The amplified PCR product was introduced into a TNT® SP6 High-Yield Wheat Germ Protein Expression System (Promega Corporation, Madison, WI, USA) following the manufacturer specifications.

The synthesized protein was subjected to Tris-Glycine SDS-PAGE (Sigma Aldrich, St. Louis, MO, USA) using a 5-12% gradient gel. Briefly, electrophoresis was performed for 20 min at 60 V and for 35 min at 200 V, following which samples were transferred to pure nitrocellulose blotting membrane (Pall Life Sciences, Ann Arbor, MI, USA) for 20 min at 25 V. The blot was blocked with 5% non-fat dry milk (NFDM) in 1X TBS (pH 7.4) at 37°C for 1 h and washed three times with 1X TBS (pH 7.4) buffer at 5-min intervals. The blot was incubated with monoclonal anti-FLAG M1 primary antibody (Sigma Aldrich) at 10 mg/mL in 1X TBS (pH 7.4) in 5% NFDM solution followed by 1:250 goat anti-mouse IgG peroxidase secondary antibody (Sigma Aldrich) in 5% NFDM solution. The blots were incubated for 1 h at 37°C in each antibody solution after which the blots were washed with 1X TBS (pH 7.4) three times for 5 min each (Alkanaimsh et al., 2016).

**RESULTS**

**Isolation of the *AsACO* gene and bioinformatics analysis**

*AsACO* cDNA (GenBank: KU359229) contained 975 bp and encoded a 324-amino acid AsACO protein (Figure 2). The *AsACO* gDNA (GenBank: KU921687) sequence contains
three exons (Figure 3a). After comparison of the protein sequence, N-terminal DIOX-N (non-phaem dioxygenase in morphine synthesis N-terminal) and 2OG-FeII-Oxy [2-oxo-glutarate (2OG) and Fe(II)-dependent oxygenase] (Figure 3b) were identified. Aligning the amino acid sequences of AsACO with those of TaACO (GenBank: AHJ14562.1), HvACO2a (GenBank: AFO63016.1), and ObACO (GenBank: XP_006647976.1) identified sequences characteristic of the ACO family. The conserved domains of different species were identical by confirming Fe(II)-binding cofactors (His-Xaa-Asp-Xaa-His) and cosubstrate-binding motifs (Arg-Xaa-Ser) (Figure 3c).

Figure 2. AsACO cDNA sequence from Agrostis stolonifera and the translated amino acid sequence. cDNA was obtained from mRNA extracted from the leaf.

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The theoretical pI was 4.89 and the calculated MW was 36.25 kDa. No signal peptide was predicted by the SignalP 4.1 Server.

Phylogenic analysis

A phylogenetic tree was used to show the relatedness of the ACO genes based on data from GenBank. Comparison of the different isoforms revealed the highest identity with TaACO of wheat (99% identity). The deduced amino acid sequences also indicated a high degree of homology between ACO (Figure 3d) of other species, such as the GenBank accession Mos of AsACO are expressed as the following: ObACO: XP_006647976.1; TaACO: AHJ14562.1; HvACO2a: AFO63016.1; HvACO2c: AFO63018.1; OsACO2: AAC05507.1; ZmACO: NP_001130227.1; PpACO: BAB32502.1; SaACO: ABM74187.1; CyACO1: BAF36562.1; SoACO: ACH99202.1; and SiACO: XP_004956979.1.

Pattern of AsACO expression

qRT-PCR was performed to determine how AsACO expression varies in different
plant tissues, in response to plant hormones, and abiotic stress (Figure 4). High expression levels were observed in stems, and other parts, such as the roots, new leaves, and old leaves were also examined (Figure 4a). The content of \textit{AsACO} increased rapidly within the first hour of Et exposure, peaking 1.5-fold higher than the amount in the control. Conversely, after 1 h, expression was inhibited (Figure 4b). Notably, the \textit{AsACO} expression began to increase at 3 h and peaked with a 4.6-fold enhancement 6 h after induction with MeJa (Figure 4c). Transcription of \textit{AsACO} was induced slowly within 3 h, but then decreased after 12 h in response to SA treatment (Figure 4d). When exposed to ABA, \textit{AsACO} expression had no regularity. The expression level of \textit{AsACO} was highest around 3 h, whereas expression was suppressed at other time points (Figure 4e). \textit{AsACO} expression was augmented from 3 h, when a peak 2.3-fold increase was observed, to 6 h in response to cold stress (Figure 4f). However, \textit{AsACO} expression was inhibited in response to drought (Figure 4g). The expression of \textit{AsACO} was only 0.11-fold that of the control at 6 h, and was thus significantly suppressed in response to salinity stress (Figure 4h).

Together, these results suggest that various plant hormones can induce \textit{AsACO} expression, particularly MeJa. In addition, the responsiveness of \textit{AsACO} to cold stress was observed.

**Subcellular localization of \textit{AsACO}**

Use of the ProtComp v. 9.0 program integral prediction of protein was localized in cytoplasm with score 8.3. Transient expression of \textit{AsACO} with YFP, and set YFP without \textit{AsACO} as a control, with virus 35S serving as drive into \textit{N. benthamiana}. Under UV light, YFP fluorescence was observed in the whole cell (Figure 5a), while the \textit{AsACO} - YFP fusion protein was localized to the nucleus and cytoplasm but not in the chloroplast, which was observed when the images were merged (Figure 5b).
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Western blot analysis

To confirm expression of the AsACO protein, we expressed a Flag-tag fusion protein in a wheat germ protein expression system. Recombinant protein expression was confirmed by western blotting using samples from the wheat germ protein expression system and an anti-Flag antibody. The molecular mass of the recombinant protein was 41.33 kDa (Figure 5c). The results of the western blot demonstrated that AsACO exhibits ACC oxidase activity in vitro.

DISCUSSION

External signals as well as growth signals can contribute to the regulation of ethylene production (Wang et al., 2002). Studies have been performed to determine how ACO functions in the regulation of ethylene production in many dicotyledons such as tomato (Hamilton et al., 1990) and papaya (López-Gómez et al., 2009); however, studies on monocotyledons are limited. Therefore, the present study aimed to investigate this gene from A. stolonifera.

After isolation and sequence comparison, cDNA of the AsACO gene was found to be 975-bp long, encoding 324 amino acids, with a calculated molecular mass of 36.25 kDa. The fact that a polypeptide with a length of 300-300 amino acids and a mRNA sequence of 918-1002 bp, constitutes a ACO protein of 35-40 kDa (Alexander and Grierson, 2002) supports our finding. The AsACO protein contains three exons. However, the ACO gene is usually composed of four exons, with three interspersed introns. Located near the downstream part of the 5'-UTR, exon 1 has a varying length of ~100 bp, along with ~200 bp for exon 2, and ~300 bp for exon 3. Exon 4, consisting of ~300 bp, is adjacent to the 3'-UTR (Ruduś et al., 2013). A three-exon structure was also observed in A. thaliana AtACO1, Dianthus caryophyllus DcACO, and Z. mays AmACO35 (Ruduś et al., 2013).

The Fe(II)-binding cofactors (His-Xaa-Asp-Xaa-His) and cosubstrate (Arg-Xaa-Ser) were highly conserved in the ACO family domain. ACO sequences of species such as melon and petunia were also found to have the same amino acids conserved across all members of the

Figure 5. Subcellular distribution of AsACO in the leaf of Nicotiana benthamiana and AsACO protein detection by western blot. 35S::YFP (control) (a) and 35S::AsACO-YFP (b) were transiently expressed in leaf of N. benthamiana, and western blot of Flag-fused AsACO (c), probed with an anti-Flag antibody.

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Fe/ascorbate-dependent dioxygenase family (Tang et al., 1993; Zarembinski and Theologis, 1994). Phylogenetic analysis revealed that AsACO has the highest identity to TaACO, which also belongs to monocotyledons.

As for the AsACO expression level, several studies have shown that ACO is up-regulated under various abiotic stresses including temperature, cutting, and oxygen deprivation, and biotic stress caused by pathology. (Moeder et al., 2002; Nie et al., 2002; Pan and Lou, 2008). Ethylene levels have been demonstrated to be increased in response to ethephon (Liu and Reid 1992). After treated by MeJa, the peak of ethylene releasing occurred in advance (Han et al., 2011). As expected, the level of ethylene increased dramatically by 1 h in response to ethephon and at around 6 h in response to MeJa treatment. However, it declined sharply afterwards when the exogenous stresses were reduced. Previous studies have reported that SA can inhibit ethylene production by blocking the conversion from 1-aminocyclopropane-1-carboxylic acid (Leslie and Romani, 1986). Interestingly, expression of the ACO gene was up-regulated, suggesting that ethylene production was accelerated. Further investigation is needed to support this mechanism. Exogenous ABA treatment induced the expression of both ACS and ACO genes, stimulating ethylene production (Zhang et al., 2009). Studies investigating abiotic treatments showed that cold stress induced the up-regulation of ACO gene expression, and the results of the present study were consistent with those findings. However, in our study, expression of AsACO was found to be severely inhibited by drought and NaCl stress, which was consistent with the response of TaACO under drought and NaCl treatment (Chen et al., 2014). This could be explained by salt-stress inducing an increase in ACC expression, which was higher in salt-tolerant, whereas a decrease in ACC conversion to ethylene, suggesting a reduction in ACC oxidase activity (Lutts et al., 1996). Analysis of subcellular localization suggested that AsACO is localized in the nucleus and cytoplasm, which is consistent with that observed for TaACO (Chen et al., 2014). Western blot analysis was performed in a wheat germ protein expression system using anti-Flag raised against the gene product of AsACO. The molecular mass of the protein was similar to that of MDACO3 (Binnie and McManus, 2009).

In conclusion, the AsACO gene is composed of three exons interspersed by two introns and has highest homology to TaACO. The amino acid sequence includes two conserved domains DIOX-N and 2OG-FeII-Oxy; which are consistent with the general characteristics of the ACO family. The expression of AsACO was up-regulated in response to exogenous hormone treatment, including Et, MeJa, SA, and cold, and down-regulated in response to drought and NaCl treatment; however, the effect of ABA on AsACO expression was not obvious. The protein encoded by the AsACO gene exhibited ACC oxidase activity in vitro. This study provides a foundation for further exploration of how AsACO functions in the process of ethylene biosynthesis in A. stolonifera.

Conflicts of interest

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

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