Genetics and Molecular Research

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

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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. (Ruduś 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, CO_2, 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° ± 1/16 ± 1°C, relative humidity 55-60%, 14-h photoperiod of 120 $mmol \cdot m^{-2} \cdot s^{-1}$ 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 ACO*2a (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 1. Genetic cloning of *AsACO* and primer sequence.

Name of primer	Sequence (5'-3')	Purpose of primer
5'-RACE	TCATCACTTGCCTGTAGTGGTCG	5'RACE-PCR
3'-RACE	CAGGGAGGACAGGTTCAAGGAGTT	3'RACE-PCR
AsACO-cDNA	F: ATAGCGAGAGGGCCGGAGAGAGAGA	RT-PCR of *AsACO* cDNA
	R: GATCGACTGTCACTGATCGATCAGG	
AsACO-gDNA	F: TATAGCGAGAGGGCCGGAGAGAGAG	RT-PCR of *AsACO* gDNA
	R: GATCGACTGTCACTGATCGATCAGG	
AsACO-RT	F: CAGGGAGGACAGGTTCAAGGAGTT	Real time-PCR analysis of *AsACO* gene
	R: GAATTCCTTCATCACTTGCCTGTAG	
AsActin-RT (GenBank: JX644005.1)	F: TTGAACCCAAAAGCCAACAG	Real time-PCR of *Agrostis stolonifera* actin gene used as internal control
	R: CCAGCAAGATCCAAACGAAG	
3302Y-AsACO	F: cacgggggactcttgaccatggtaATGGCGACTGCAGCAGCTG	Subcellular localization vector of *AsACO*
	R: ggtacacgcgtactagtcagatcGGCCGTGGCGATGGGCGCG	
3302SP6-*AsACO*	F: gacactatagaacagaccaccATGGCGACTGCAGCAGCTG	Sp6-3xFlag vector of *AsACO*
	R: tccttgtaatccagatctacGGCCGTGGCGATGGGCGCG	
SP6- *AsACO*	F: CTTGATATTTAGGTGACACTATAGA	Analysis of AsACO protein
	R: TTATATGATAATCATCGCAAGACCG	

Utilizing the sequence as a template, *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 *AsACO*-cDNA was performed using *AsACO*-F and *AsACO*-R, obtained using primers and cDNA as templates. AsACO-gDNA was obtained by utilizing DNA as a template, *AsACO*-F, and *AsACO*-R were used as primers. Next, the intron-exon structure of *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 *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 *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 *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 *AsACO* gene in the shoot was measured at selected points over a 24-h period (0, 1, 3, 6, 12, and 24 h). *AsACO*-RT and 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 *AsActin* (GenBank: JX644005.1) was used in the comparative $C_T(2^{-\Delta CT})$ method, in which $\Delta G_T = C_{T(\text{target gene})} - C_{T(\text{reference gene})}$, 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 *AsACO*

To generate the CaMV35S:*AsACO*-YFP construct, the *AsACO* CDS was amplified using a pair of designed 3302Y-*AsACO* primers (Table 1), and then subcloned into a *Bal*gII site in CaMV35S on a 3302Y vector (Figure 1a), which was then introduced into EHA105

agrobacteria using the $CaCl_2$ freeze-thaw method. EHA105 agrobacterium-meditated 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).

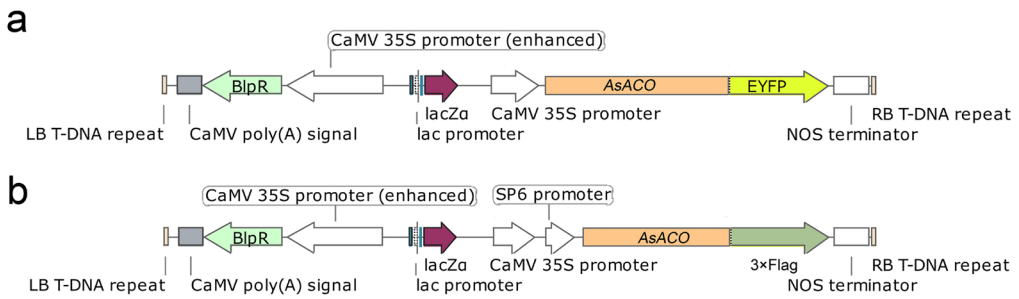

Figure 1. Vector construction of *AsACO* for transient expression and AsACO for protein expression. **a.** vector construction of *AsACO* for transient expression, **b.** vector construction of AsACO for protein expression.

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 *Nco*I 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-haem 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).

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1       ATGGCGACTGCAGCAGCTGCCTCGTTGAGCTTCCCGGTGATCAACATGGAGAAGCTGGAG
1        M  A  T  A  A  A  A  S  L  S  F  P  V  I  N  M  E  K  L  E
61      ACGGAGGAGAGGGGCGCGGCCATGGAGGTCATCCGCGACGCCTGCGAGAACTGGGGCTTC
21       T  E  E  R  G  A  A  M  E  V  I  R  D  A  C  E  N  W  G  F
121     TTCGAGCTTCTGAACCATGGCATCTCGCACGAGCTGATGGACGAGGTTGAGCGGGTGAGC
41       F  E  L  L  N  H  G  I  S  H  E  L  M  D  E  V  E  R  V  S
181     AAGGCGCACTACAAGGACCGCAGGGAGGACAGGTTCAAGGAGTTCGCGGCGAGGACGCTG
61       K  A  H  Y  K  D  R  R  E  D  R  F  K  E  F  A  A  R  T  L
241     GAGGCCGGCGAGAGGGGCGCCGACGTGAAGGACGTGGACTGGGAGAGCACCTTCTTCGTC
81       E  A  G  E  R  G  A  D  V  K  D  V  D  W  E  S  T  F  F  V
301     CGCCACCTCCCCGCCTCCAACCTCGCCGACCTGCCCGACCTCGACGACCACTACAGGCAA
101      R  H  L  P  A  S  N  L  A  D  L  P  D  L  D  D  H  Y  R  Q
361     GTGATGAAGGAATTCGCGTCCGAGATCGAGAAGCTGTCGGAGCGCGTGCTGGACCTTCTG
121      V  M  K  E  F  A  S  E  I  E  K  L  S  E  R  V  L  D  L  L
421     TGCGAGAACCTGGGCCTGGAGAAGGGCTACCTCAAACAAGCCTTCGCGGGTTCCAAGGGC
141      C  E  N  L  G  L  E  K  G  Y  L  K  Q  A  F  A  G  S  K  G
481     AGCCCGACGTTCGGCACCAAGGTGAGCAGCTACCCGCCGTGCCCGCGCCCGGACCTCGTC
161      S  P  T  F  G  T  K  V  S  S  Y  P  P  C  P  R  P  D  L  V
541     GACGGCCTCCGCGCGCACACCGACGCCGGCGGCGTGATCCTGCTGTTCCAGGACGACCAG
181      D  G  L  R  A  H  T  D  A  G  G  V  I  L  L  F  Q  D  D  Q
601     GTGAGCGGGCTGCAGCTCCTGAAAGACGGGGAGTGGGTGGACGTGCCGCCCATGCGCCAC
201      V  S  G  L  Q  L  L  K  D  G  E  W  V  D  V  P  P  M  R  H
661     GCCATCGTCGTCAACATCGGCGACCAGCTGGAGGTGATCACCAACGGGCAGTACAAGAGC
221      A  I  V  V  N  I  G  D  Q  L  E  V  I  T  N  G  Q  Y  K  S
721     GTGCTGCACCGCGTGCTCACCCGCCCCGACGGCAACCGCATGTCCATCGCGTCCTTCTAC
241      V  L  H  R  V  L  T  R  P  D  G  N  R  M  S  I  A  S  F  Y
781     AACCCGGGCGCCGACGCCGTCATCTTTCCGGCACCGGCGCTCGTCGAGGTGGCGGAAGAA
261      N  P  G  A  D  A  V  I  F  P  A  P  A  L  V  E  V  A  E  E
841     GAGGAGGCGGGGGCCGTGTACCCGAGGTTCGTGTTCGAGGACTACATGAACCTGTACGTG
281      E  E  A  G  A  V  Y  P  R  F  V  F  E  D  Y  M  N  L  Y  V
901     CGCCACAAGTTCGAGGCCAAGGAGCCACGCTTCGAGGCCATGAAGTCGGACGCCGCGCCC
301      R  H  K  F  E  A  K  E  P  R  F  E  A  M  K  S  D  A  A  P
961     ATCGCCACGGCCTGA
321      I  A  T  A  *
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Figure 2. *AsACO* cDNA sequence from *Agrostis stolonifera* and the translated amino acid sequence. cDNA was obtained from mRNA extracted from the leaf.

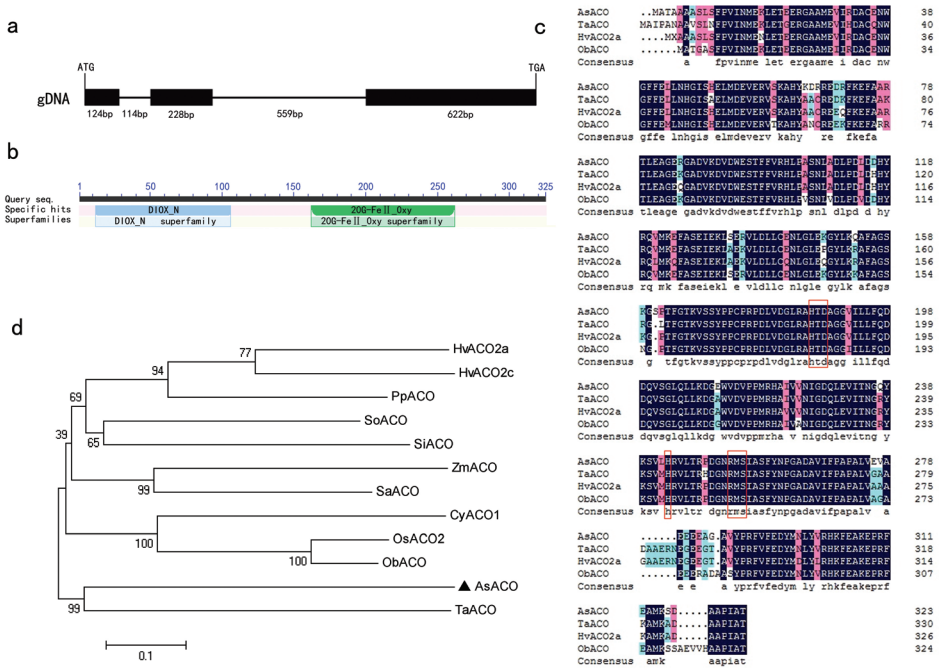

Figure 3. Structure and phylogeny of *AsACO*. **a.** *AsACO* gDNA sequence. Black boxes represent exons while solid lines represent introns. **b.** Predicted protein structure of *AsACO*. DIOX-N: non-haem dioxygenase in morphine synthesis N-terminal; 2OG-Fell_Oxy = 2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase containing domain. **c.** Rectangular structures represent Fe(II)-binding motifs with a dash, cosubstrate-binding motifs with a continuous dash. In the consensus sequence, black shading represents 100% identity, pink shading represents ≥75% identity, blue shading represents ≥50% identity. **d.** Phylogenetic analysis showing homology of the AsACO protein.

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 *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 *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 *AsACO* was induced slowly within 3 h, but then decreased after 12 h in response to SA treatment (Figure 4d). When exposed to ABA, *AsACO* expression had no regularity. The expression level of *AsACO* was highest around 3 h, whereas expression was suppressed at other time points (Figure 4e). *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, *AsACO* expression was inhibited in response to drought (Figure 4g). The expression of *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).

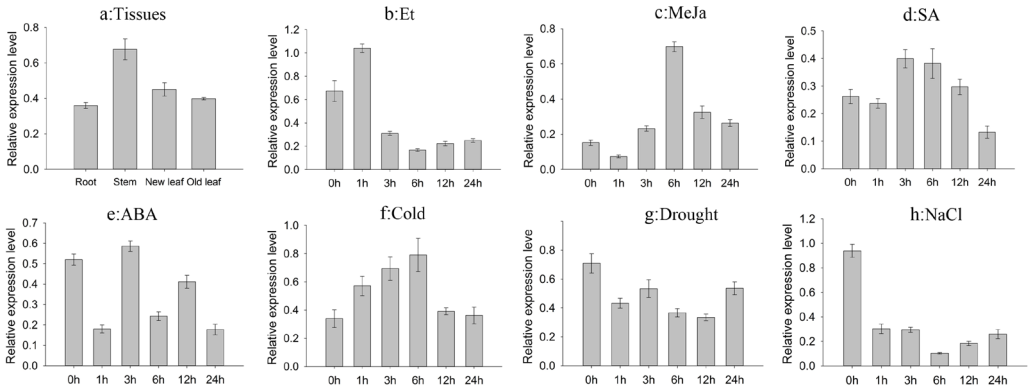

Figure 4. Transcription behavior of *AsACO* under different abiotic stress. **a.** *AsACO* expressed in different parts of plant tissues; **b.** *AsACO* expression is induced by 10 µM ethephon (Et) for 0, 1, 3, 6, 12, 24, and 48 h; **c.** 10 µM methyl jasmonate (MeJa); **d.** 50 mM salicylic acid (SA); **e.** 10 µM abscisic (ABA); **f.** cold; **g.** drought; and (**h.**) 200 mM NaCl. The data represent the means of four replicates. Error bars indicate ± SEM.

Together, these results suggest that various plant hormones can induce *AsACO* expression, particularly MeJA. In addition, the responsiveness of *AsACO* to cold stress was observed.

Subcellular localization of *AsACO*

Use of the ProtComp v. 9.0 program integral prediction of protein was localized in cytoplasm with score 8.3. Transient expression of *AsACO* with YFP, and set YFP without *AsACO* as a control, with virus 35S serving as drive into *N. benthamiana*. Under UV light, YFP fluorescence was observed in the whole cell (Figure 5a), while the *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).

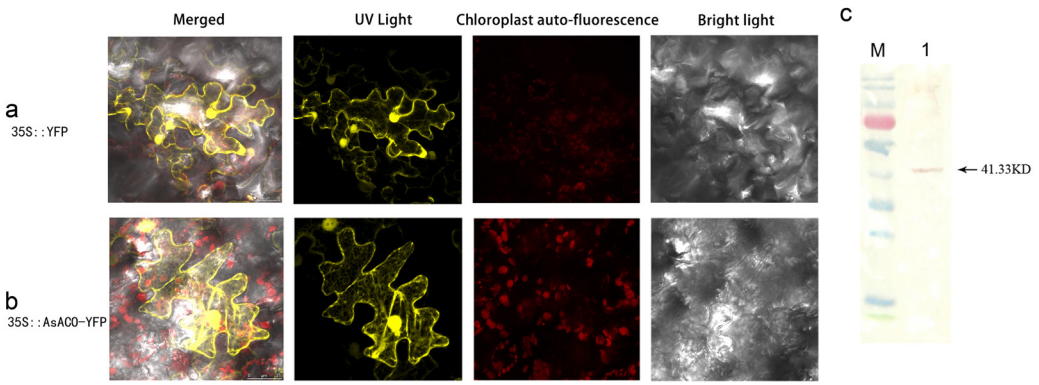

Figure 5. Subcellular distribution of *AsACO* in the leaf of *Nicotiana benthamiana* and AsACO protein detection by western blot. 35S::YFP (control) (**a**) and 35S::AsACO-YFP (**b**) were transiently expressed in leaf of *N. benthamiana*, and western blot of Flag-fused AsACO (**c**), probed with an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

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 AtACO*1, *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 conversed 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

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 conversed 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.

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