Molecules and Cells

# Arabidopsis ACC Oxidase 1 Coordinated by Multiple Signals Mediates Ethylene Biosynthesis and Is Involved in Root Development

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http://dx.doi.org/10.14348/molcells.2018.0092
www.molcells.org

Ethylene regulates numerous aspects of plant growth and development. Multiple external and internal factors coordinate ethylene production in plant tissues. Transcriptional and post-translational regulations of ACC synthases (ACSs), which are key enzymes mediating a rate-limiting step in ethylene biosynthesis have been well characterized. However, the regulation and physiological roles of ACC oxidases (ACOs) that catalyze the final step of ethylene biosynthesis are largely unknown in Arabidopsis. Here, we show that Arabidopsis *ACO1* exhibits a tissue-specific expression pattern that is regulated by multiple signals, and plays roles in the lateral root development in Arabidopsis. Histochemical analysis of the *ACO1* promoter indicated that *ACO1* expression was largely modulated by light and plant hormones in a tissue-specific manner. We demonstrated that point mutations in two E-box motifs on the *ACO1* promoter reduce the light-regulated expression patterns of *ACO1*. The *aco1-1* mutant showed reduced ethylene production in root tips compared to wild-type. In addition, *aco1-1* displayed altered lateral root formation. Our results suggest that Arabidopsis ACO1 integrates various signals into the ethylene biosynthesis that is required for ACO1's intrinsic roles in root physiology.

**Keywords:** ACC oxidase 1, *Arabidopsis thaliana*, ethylene biosynthesis, lateral root, transcriptional regulation

## INTRODUCTION

Ethylene is a gaseous plant hormone that participates in a variety of processes throughout the plant life cycle from seed germination to organ senescence and fruit ripening (Beaudoin et al., 2000; Ghassemian et al., 2000; Li et al., 2013; Maunders et al., 1987; Picton et al., 1993).

In plants, ethylene is biosynthesized from *S*-adenosyl-L-methionine (SAM) through the intermediate 1-aminocyclopropane-1-carboxylic acid (ACC). ACC synthases (ACSs) and ACC oxidases (ACOs) catalyze the conversion of SAM to ACC followed by the conversion of ACC to ethylene, respectively (Yang and Hoffman, 1984). In the ethylene biosynthesis pathway, the conversion of SAM to ACC, which is catalyzed by ACS, is considered as a rate-limiting step. For this reason, studies on the regulation of ethylene biosynthesis by multiple signals, such as MAP kinases and plant hormones, have focused on ACS genes and proteins (Barry et al., 2000;

Received 2 March, 2018; revised 14 June, 2018; accepted 21 August, 2018; published online 1 October, 2018

eISSN: 0219-1032

Chae et al., 2003; Liu and Zhang, 2004; Skottke et al., 2011; Thain et al., 2004; Ye et al., 2015; Yi et al., 1999; Yoon and Kieber, 2013). Arabidopsis ACS family members show diverse affinities for SAM, implying that these enzymes might be optimized to perform different functions in various tissues and cell types (Yamagani et al. 2003).

The conversion of ACC to ethylene catalyzed by ACO is the final regulatory step in ethylene biosynthesis (Alonso and Ecker, 2001; Kende and Zeevaart, 1997; Lasserre et al., 1996; Ruduś et al., 2013; Vriezen et al., 1999). ACO is a member of a large Fe(II)-requiring dioxygenase/oxidase superfamily. However, studies on the function and regulation of ACO have been hindered by the general perception of ACS as the key regulatory enzyme in ethylene biosynthesis, along with an incomplete gene family definition and limited biochemical and physiological studies (Booker and DeLong, 2015). A query for ACO in the TAIR (www.arabidopsis.org) yields 13 loci for Arabidopsis. Among these, ACO4 was the first enzyme predicted to have ACC oxidase catalytic activity (Gómez-Lim et al., 1993), while ACO2 was recognized for its function of counteracting ABA-induced endosperm rupture inhibition during seed germination (Linkies et al., 2009). Although transcript levels for *ACO*s have been analyzed under various conditions (Gómez-Lim et al., 1993; Linkies et al., 2009; Thain et al., 2004; Qin et al., 2007), biochemical, functional, and genetic studies on *ACO*s remain to be conducted.

Here, we investigated tissue-specific expression pattern of *ACO1* using a promoter-GUS reporter system, as well as the biochemical and physiological analysis in the regions where *ACO1* is expressed. We found that the *ACO1* promoter displays a tissue-specific expression pattern that is distinctly modified by different light conditions and treatments with several plant hormones. We demonstrated that the *ACO1* promoter contains two E-box motifs that are indispensable for transcriptional activity and regulation by light signal. Biochemical and physiological studies of the *aco1-1* mutant showed that ACO1 is required for ethylene production and for the control of lateral root development. Our results suggest that ACO1 is an active ethylene biosynthetic enzyme required for normal root development in Arabidopsis and its expression integrates multiple signals into ethylene biosynthesis.

## MATERIALS AND METHODS

### Plant materials, growth conditions, and hormone treatments

*Arabidopsis thaliana* ecotype Col-0 was used as the wild-type in this study. The *aco1-1* (SALK_127963) line was obtained from SALK. T-DNA insertion in *aco1-1* was confirmed by genotyping PCR with *ProACO1-GUS 1154 For* (Supplementary Table S1) and T-DNA left border (5'-CTTTGACGTTGGAGTCCACGTTCTTTAATA-3') primers. Homozygous *aco1-1* was screened by genotyping PCR using *ProACO1-GUS 1154 For* and *ProACO1-GUS 1 Rev* primers (Supplementary Table S1). Seeds were surface-sterilized with ethanol-distilled water (70:30, v/v) for 5 min, washed with distilled water, and stratified at 4℃ for 3 days. Surface-sterilized seeds were planted on 0.8% phytagel (Sigma) containing 0.5× Murashige and Skoog (MS)-salt medium and 1% (w/v) sucrose and grown in the light (120 μmol $m^{-2}$ $s^{-1}$) at 22 ± 1℃ for 16 hr and in the dark at 20 ± 1℃ for 8 h in an environmental growth chamber (Sanyo, Osaka). For etiolated conditions, the plates were wrapped with aluminum foil after exposure to white light for 1 day. Two-week-old seedlings were transferred from agar plates to soil to obtain adult plants. For hormone treatment, seedlings were grown on 1× MS medium containing 0.8% Phytagel for 7 days and then transferred to 1× MS liquid medium for 6 hr. For ethylene treatment, ethylene gas was injected into plates.

### Root length and lateral root measurement

To measure root length and lateral root number, seedlings were incubated on vertical plates. Lateral root number was counted as the total number of emerging and emerged lateral roots under a dissecting microscope (Olympus SZ-PT). Seedlings were photographed to measure root length.

### Preparation of transgenic plants expressing the GUS gene fused to truncated *ACO1* promoters

Genomic DNA was extracted from Col-0 wild-type according to the method described previously (Park et al., 2010). *ACO1* promoter fragments were cloned from genomic DNA by PCR amplification using primer pairs designed to introduce a *Hind*III restriction site into the 5'-end and a *Bam*HI site into the 3'-end of each promoter fragment, respectively (Supplementary Table S1). For the *ProACO1-GUS Δ312* construct, *ProACO1-GUS 1843 For* primer and *ProACO1-GUS Δ312 Rev* primer were used (Supplementary Table S1). *ProACO1-GUS 1843mE2mE4* was generated from *ProACO1-GUS 1843* plasmids by site-directed mutagenesis (Stratagen). The primers used for mutagenesis are shown in Supplementary Table S1. Promoter fragments were cloned into pGEM T easy vector (Promega) and subcloned into pBI121 after confirmation by DNA sequencing. The vector constructs were transformed into *Agrobacterium tumefaciens* strain GV3101::*pMP90* and transformed into Arabidopsis following the method described previously (Clough and Bent, 1998).

### Total RNA isolation, and semi-quantitative and quantitative RT-PCR analysis

Total RNA was extracted from the wild-type, mutant, and chemical-treated seedlings using TRI reagent (Sigma). For semi-quantitative RT-PCR, first-strand cDNA was synthesized using 5 μg of total RNA and M-MLV reverse transcriptase (Promega). PCR was performed in a reaction containing 1 μl cDNA, 0.25 μl Real Taq (RBC, Taiwan), 2.5 μl 2.5 mM dNTP mixture, 2.5 μl 10× buffer (Takara, Japan), and 1 μl of each primer (10 pmol) in a 25 μl reaction. The gene-specific primers for *ACO*s are listed in Table S2. Ten μl samples of the 50 μl PCR products were electrophoresed on 0.8 or 1% agarose gels and stained with ethidium bromide (EtBr). Stained bands were scanned and densitometrically analyzed using the Quantity One program (Bio-Rad). For quantitative real-time PCR (qPCR), total RNA was extracted from 10 DAG long day-grown Col-0 and *aco1-1* using the Spectrum Plant Total RNA kit (Sigma). RevertAid reverse transcriptase

(Thermo Scientific) was used to synthesize complementary DNA from 2 ug total RNA. qPCR was carried out using LightCycler 480 (Roche) and SensiMix SYBR & Fluorescein Kit (Bioline). *ACO1* primers (ACO1 q-Forward primer: 5'-GTGGGGATTCTTCATGGTTGATAATCATGG-3' and ACO1 q-Reverse primer: 5'-CATCGTCTTGCTGAGTTCCTCTGAAATG-3') were used for qPCR. *PP2A* (Forward primer: 5'-TATCGGATGACGATTCTTCGTGCAG-3', Reverse primer: 5'-GCTTGGTCGACTATCGGAATGAGAG-3') was used as an internal control.

### Histochemical GUS staining

Histochemical GUS staining was performed as described previously (Weigel and Glazebrook, 2002). Briefly, samples were incubated in staining solution containing 2 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (Duchefa) in 50 mM $Na_2HPO_4$ buffer, pH 7.2, 2 mM potassium ferrocyanide, 2 mM potassium ferricyanide, 0.2% Triton X-100 at 37℃ overnight after infiltrating under vacuum on ice for 20 min. The samples were dehydrated using 20, 35 and 50% ethanol and fixed with fixative (50% ethanol, 10% acetic acid and 5% formaldehyde). The samples were cleared with 70% ethanol and observed with dissecting (Olympus SZ-PT) and light (Olympus CX21) microscopes.

### GUS activity measurement

To measure GUS activity, plant tissues were ground in liquid nitrogen. Crude plant extracts were resolved in GUS extraction buffer (50 mM $Na_2HPO_4$ buffer, pH 7.0, 10 mM EDTA, pH 8.0, 0.1% SDS, 0.1% Triton X-100, 10 mM β-mercaptoethanol and 25 μg $ml^{-1}$ PMSF). The crude extracts were mixed with a reaction buffer (1 mM 4-methylumbelliferyl-β-D-glucuronide [Duchefa] in GUS extraction buffer). After exactly 10 and 20 min, reactions were stopped by $Na_2CO_3$. Fluorescence was measured with a spectrofluorometer.

### Ethylene production quantification

One hundred root segments (approximately 10 mm from root tip) or root tip segments (approximately 2 mm long) from 5 DAG seedlings were harvested in 25 mL silicon-capped vials containing 200 μL buffer (100 mM MES, pH 6.8 and 1.5 mM chloramphenicol). Vials containing root tissues were incubated at 27 ± 1℃ in the dark with shaking (170 rpm). Ethylene measurement was performed as described previously (Yun et al., 2009). Air samples (1 ml) from these vials were withdrawn with a syringe and injected into a gas chromatograph equipped with a column containing alumina (HP5890 series II; Hewelett Packard, USA, 80/100 Porapak-Q column, oven temperature: 120℃, injector temperature: 150℃, detector temperature: 280℃).

## RESULTS

### Spatio-temporal expression patterns for *ACO1* in Arabidopsis

Given that multiple *ACO* genes are encoded in the Arabidopsis genome, we speculated that each ACO member might have specific function(s) in various tissues, as is the case with ACS family members (Tsuchisaka el al., 2009). Among the 13 ACOs found in Arabidopsis, only ACO2 and ACO4 have been shown to be functional enzymes (Gómez-Lim et al., 1993; Linkies et al., 2009). Thus, we focused on ACO1 (At2g19590), a close homolog of ACO2 and ACO4, which might be functional in ethylene production.

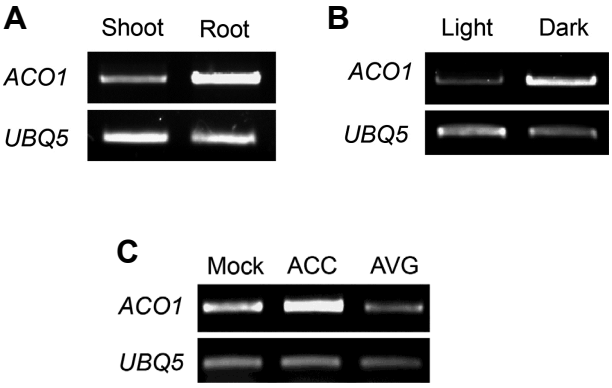


**Fig. 1. *ACO1* gene expression under various conditions.** (A) Comparison of *ACO1* expression in the shoot and root of 5 DAG seedlings. Seedlings were cut into the shoot (cotyledon and hypocotyl) and root by a razor blade. The shoot and root fragments were subjected to total RNA extraction. (B) *ACO1* expression in seedlings grown under different light conditions. Seedlings were grown in the light or in the dark for 5 DAG. (C) Ethylene precursor effect on *ACO1* expression. Five DAG seedlings were treated with 1 μM ACC or 1 μM AVG for 6 hr. Semi-quantitative RT-PCR was performed with total RNA. PCR products were electrophoresed on 1% agarose gels and detected by ethidium bromide (EtBr) staining. *UBQ5* was used to normalize the expression level.

RT-PCR analysis indicated that *ACO1* was more highly expressed in roots compared to shoots in Arabidopsis seedlings grown under light (Fig. 1A). In addition, comparison of *ACO1* transcript levels between light- and dark-grown seedlings showed that *ACO1* mRNA was more highly accumulated in dark-grown seedlings compared to light-grown seedlings (Fig. 1B). As ACOs catalyze ethylene production using ACC as a substrate, we examined whether the ACC level might affect *ACO1* expression. RT-PCR analysis indicated that exogenous ACC application to 5 days after germination (DAG) seedlings promoted *ACO1* expression whereas treatment with an ACS inhibitor, aminoethoxyvinylglycine (AVG), inhibited its expression (Fig. 1C). The ACC and AVG treatment results indicated that *ACO1* expression was positively regulated by ACC level in plant cells. Taken together, these results suggest that *ACO1* expression is tissue-specific, and is regulated by light as well as the ethylene precursor, ACC.

To further explore the physiological and functional relevance of ACO1 in Arabidopsis, we generated transgenic Arabidopsis plant expressing the *β*-glucuronidase (*GUS*) gene driven by the 1,843 bp *ACO1* promoter. Using *ProACO1-GUS 1843* transgenic plants, the spatio-temporal expression of *ACO1* was further analyzed. Consistent with the

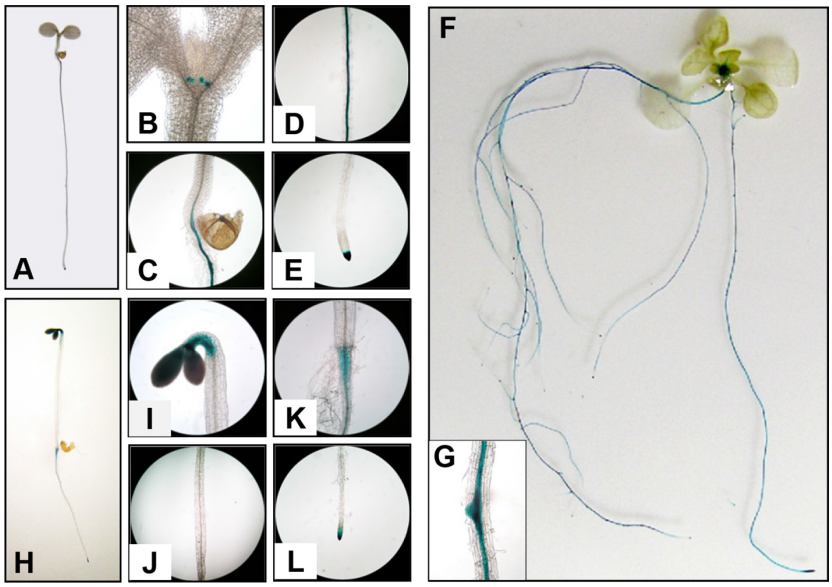


**Fig. 2. Spatio-temporal expression pattern for *ACO1*.** (A-G) *ACO1* promoter tissue-specific activity under light conditions. Images show whole plant (A), shoot apex (B), hypocotyl-root junction (C), root maturation zone (D), and root tip (E) for 3 DAG *ProACO1-GUS 1843* seedlings. (F) *GUS* expression pattern for 12 DAG *ProACO1-GUS 1843* plants. (G) Insert, *ACO1* expression in lateral root primordium from 5 DAG *ProACO1-GUS 1843* seedlings. (H-L) Tissue-specific activity for the *ACO1* promoter under dark conditions. Images show whole plant (H), cotyledon and apical hypocotyl (I), basal hypocotyl (J), hypocotyl-root junction (K) and root tip (L) from 4 DAG dark-grown *ProACO1-GUS 1843* seedlings.

RT-PCR results for *ACO1*, in 3 DAG seedlings grown under light, GUS activities were predominant in roots (Figs. 2A-2E). On the other hand, *ACO1* expression in aerial parts was restricted to the stipule (Fig. 2B). In the root, *ACO1* was expressed in vascular tissue from the hypocotyl-root junction to the maturation zone and root tip, but not in the elongation zone (Figs. 2C-2E). The expression pattern of *ACO1* in the root was consistent with previously reported *in silico* gene expression analysis (Dugardeyn et al., 2008). In 12 DAG seedlings, GUS activity was detected only in the rosette leaf basal region but was ubiquitously observed in roots (Fig. 2F). Additionally, enhanced *ACO1* expression was detected at the lateral root primordial formation sites (Fig. 2G). When *ProACO1-GUS 1843* was grown in the dark, strong GUS activities were detected in the cotyledon and the concave side of the apical hook (Figs. 2H and 2I) but were not detected in other hypocotyl regions (Figs. 2I and 2J). Furthermore, *ACO1* in etiolated roots was expressed in the hypocotyl-root junction and root tip, which was similar to the pattern observed in light-grown seedlings (Figs. 2K and 2L). Collectively, these results indicated that *ACO1* is predominantly expressed in roots and its expression is strictly regulated by light. In adult plants, *GUS* expression was detected in apical primary and lateral inflorescence stems, the petiole, the basal pedicel, and the leaf vein in addition to the whole root (Supplementary Fig. S1). No *ACO1* promoter activity was detected in flowers, silique, or rosette leaf (Supplementary Fig. S1).

## Tissue-specific regulation of *ACO1* expression by external and internal stimuli in Arabidopsis

We further investigated the effect of external and internal stimuli on *ACO1* promoter activity. For this purpose, we first examined whether light could regulate *ACO1* expression. Consistent with prominent *ACO1* expression in the dark, *ACO1* expression in the hypocotyl and the hypocotyl-root junction of light-grown seedling was greatly increased by

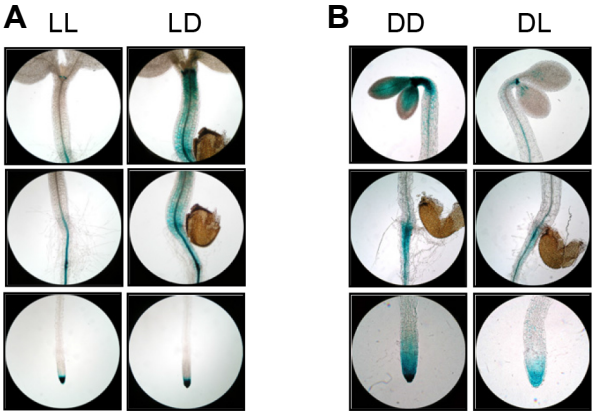


**Fig. 3. Light effect on *ACO1* promoter activity.** (A) *GUS* expression in light-grown *ProACO1-GUS 1843* seedlings before (LL) and after (LD) transition to dark. (B) *GUS* expression in dark-grown *ProACO1-GUS 1843* seedlings before (DD) and after (DL) transition to light. Transitions between dark and light were performed for 6 hr. Three or more seedlings were used for GUS staining and showed similar staining patterns.

dark treatment for 6 hours, although it was not altered in roots and cotyledons (Fig. 3A). In contrast, the dark to light transition strongly reduced *ACO1* expression (Fig. 3B), suggesting that *ACO1* expression was strongly regulated by light signals.

As multiple plant hormones regulate ethylene production (Chae et al., 2003; Yi et al., 1999; Yun et al, 2009), we examined whether plant hormones alter *ACO1* promoter activity. Thus, we treated *ProACO1-GUS 1843* seedlings with brassinosteroid (BR), ethylene, auxin, ACC, and gibberellin (GA). Brassinolide (BL), the most bioactive BR, and ethylene enhanced *ACO1* promoter activity in roots and the hypocotyl

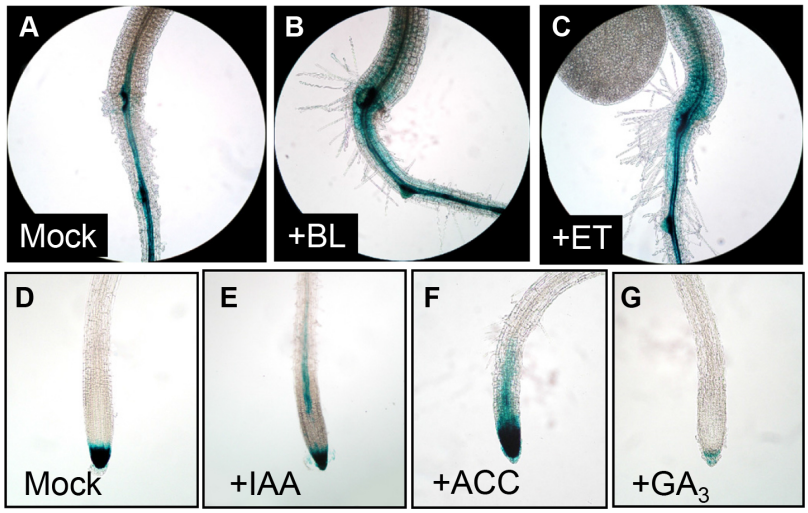


**Fig. 4. *ACO1* promoter tissue-specific regulation by various plant hormones.** (A-C) Hypocotyl-root junction in *ProACO1-GUS 1843* seedlings. (D-G) Roots from *ProACO1-GUS 1843* seedlings. 5 DAG seedlings were left untreated (A, D) or treated with 100 nM BL (B), 1 ppm ethylene (C), 1 μM IAA (E), 10 μM ACC (F), and 50 μM $GA_3$ (G) for 6 h.

compared to the controls (Figs. 4A-4C). Exogenous application of auxin specifically activated the promoter in the root elongation zone of vascular tissues (Fig. 4E). Consistent with RT-PCR result (Fig. 1C), ACC treatment enhanced promoter activity from the root tip to the elongation zone (Fig. 4F). In contrast, AVG treatment reduced *GUS* expression in root maturation zone but not in the root tip (Supplementary Fig. S2). GA repressed promoter activity in the root tip (Fig. 4G). These results indicated that plant hormone signals regulated *ACO1* expression differently with distinct tissue specificity. Taken together, these findings imply that multiple signals can modulate ethylene production by altering *ACO1* transcription in a tissue-specific manner.

## Determination of the E-box motif on the *ACO1* promoter required for *ACO1* expression

To define the regulatory region required for tissue-specific *ACO1* expression, we analyzed *cis*-regulatory elements in the *ACO1* promoter. This promoter contains four E-box motifs (CANNTG, *E1~E4*) known as BR/light-regulated *cis*-regulatory elements (Fig. 5A; Martínez-García et al., 2000; Sun et al., 2010). Thus, we generated transgenic plants expressing the *GUS* reporter gene driven by four different truncated *ACO1* promoter lengths (*ProACO1-GUS 1154*, *769*, *536*, *and 184*) in which the *cis*-regulatory elements were deleted (Fig. 5A). In addition, transgenic plants possessing the *ProACO1-GUS Δ312* construct were prepared. The *ProACO1-GUS Δ312* plants contained the entire 5'-intergenic regions but 312-bp fragment upstream of the start codon was deleted (Fig. 5A). *ProACO1-GUS 1154* seedlings showed indistinguishable GUS activity compared to the *ProACO1-GUS 1843* (Fig. 5B). However, *ProACO1-GUS 769* seedlings showed significantly reduced GUS activity compared to the *ProACO1-GUS 1843* implying that the region between 1,154 and 769 bp from the start codon was required for *ACO1* promoter activity. Consistently, plants with *ProACO1-GUS 536* or *ProACO1-GUS 184* showed no detectable *GUS* expression (Fig. 5B). *ProACO1-GUS Δ312* showed only weak GUS activity in the maturation region and root tips (Fig. 5B), suggesting that the 312-bp fragment upstream of the start codon was also important for *ACO1* promoter activity. These results suggest that at least two regulatory regions containing *E2* and *E4* motifs are essential for *ACO1* expression. We mutated the E-box motifs (CANNTG to TCTGAA; *mE2mE4*) without creating novel motif(s) when the mutagenized sequences were combined with adjacent sequences. Histochemical and quantitative analysis further demonstrated that *GUS* expression driven by *ProACO1-GUS 1843* was greatly abolished by point mutations in the two E-box motifs (Figs. 5C and 5D). Furthermore, *ProACO1-GUS 1843 mE2mE4* GUS activity was not altered by light-dark transition, suggesting that *E2* and *E4* elements were also involved in light regulation of *ACO1* expression (Fig. 5E).

## ACO1 is required for ethylene production and regulates lateral root development

To address the biochemical and physiological functions of ACO1, an Arabidopsis mutant harboring a T-DNA insertion 279-bp upstream of the *ACO1* start codon was obtained, and a homozygous line was isolated (referred to as *aco1-1*; Fig. 6A). *aco1-1* was the only mutant that was publically available. The T-DNA insertion significantly reduced *ACO1* transcript levels in the mutant (Fig. 6B). The reduced *ACO1* transcript levels in the *aco1-1* mutant are consistent with the histochemical evidence that deletion of the 312-bp fragment upstream of *ACO1* start codon greatly reduced *ACO1* promoter activity (Fig. 5B). Quantitative RT-PCR analysis showed that *ACO1* transcript level in *aco1-1* was decreased by more than 60-fold compared to that in the wild-type (Fig. 6C). When we examined the expression of other *ACO-like* genes in the *aco1-1* mutant, no gene showed a significantly altered expression level (Supplementary Figs. S3 and S4).

Considering that *ACO1* was predominantly expressed in roots, we speculated that ACO1 might be required for ethylene biosynthesis to regulate root developmental and physiological responses. Thus, we measured ethylene production in roots from the wild-type and *aco1-1* mutant

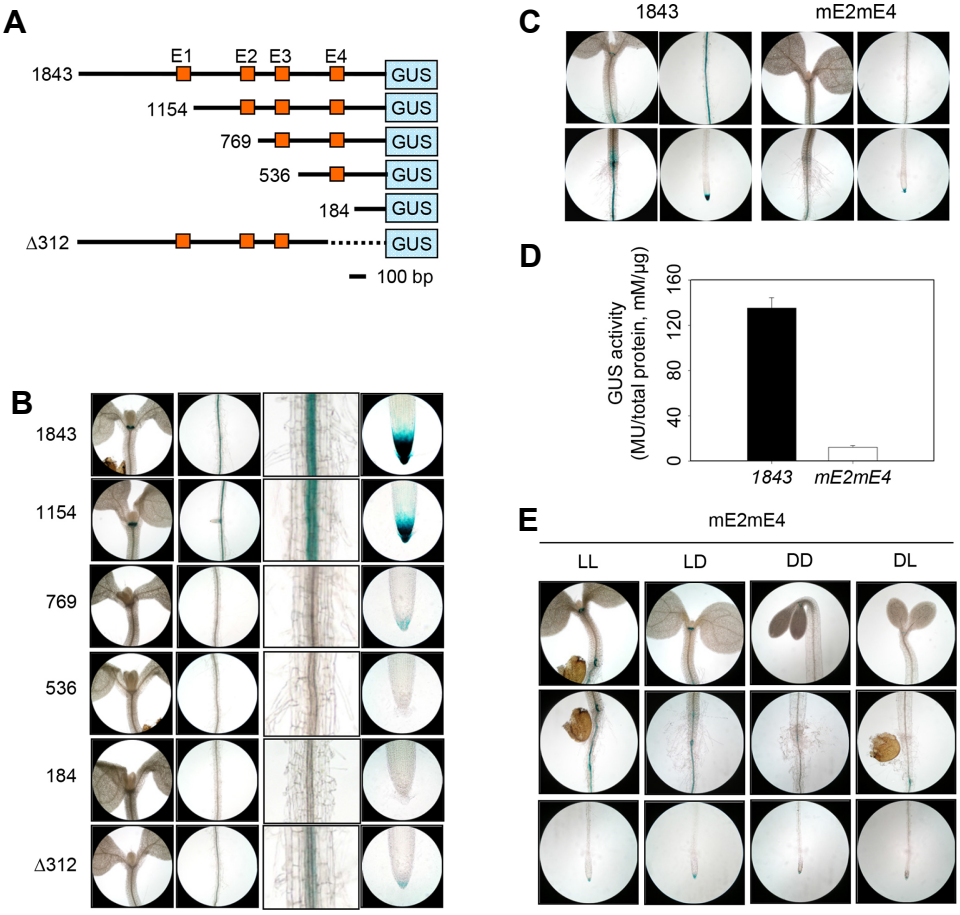


**Fig. 5. Histochemical GUS staining in truncated series from *ProACO1-GUS* transgenic seedlings.** (A) Schematic diagram from truncated *ACO1* promoter-GUS constructs. Numbers represent the number of nucleotides from truncated *ACO1* promoters fused to *GUS* gene (blue box), except for Δ312, which indicates promoter deletion size. Orange boxes indicate localization of E-box motif (*E1~E4*). The bar represents 100 nucleotides in length. (B) *GUS* expression in truncated *ProACO1-GUS* seedlings. Seedlings from *ProACO1-GUS 1843* (1843), *ProACO1-GUS 1154* (1154), *ProACO1-GUS 769* (769), *ProACO1-GUS 536* (536), *ProACO1-GUS 184* (184), and *ProACO1-GUS Δ312* (Δ312) were grown on vertically oriented plates for 5 DAG then subjected to GUS staining. From left to right, each image shows hypocotyl, root maturation zone, magnified root maturation zone, and root tip, respectively. Three or more seedlings were used for GUS staining and showed similar staining patterns. (C) Histochemical analysis of GUS activity in *ProACO1-GUS 1843* and *ProACO1-GUS 1843 mE2mE4* (mE2mE4). GUS staining was repeated with three independent transgenic plants and the representative of similar results are shown. (D) GUS activity quantification in *ProACO1-GUS 1843* and *ProACO1-GUS 1843 mE2mE4* (mE2mE4). Columns indicate mean values from two independent experiments. Error bars show standard deviation. (E) *GUS* expression in *ProACO1-GUS 1843 mE2mE4* (mE2mE4) seedlings under different light conditions; continuous light-grown (LL), light-grown seedlings treated with dark (LD), continuous dark-grown (DD) and dark-grown seedling treated with light (DL).

plants. Ethylene production in root segments from 5 DAG *aco1-1* was not significantly different from that from the wild-type plants (Supplementary Fig. S5A). However, when we analyzed ethylene production in root tips where the expression level was high (Fig. 2E), the *aco1-1* mutant showed greatly reduced ethylene production compared to the wild-type plants (Fig. 6D), suggesting that ACO1 is required for ethylene biosynthesis in Arabidopsis seedling root tips.

To test whether *ACO1* expression in roots contributed to ethylene-mediated root growth inhibition, we measured primary root lengths of *aco1-1* and the wild-type plants. However, we could not detect statistically significant differences in root length (Supplementary Fig. S5B), indicating that ACO1 may not be a major enzyme involved in ethylene-regulated root growth.

Based on *ACO1* tissue-specific expression in root vascular tissue and increased promoter activity at the lateral root primordia formation sites (Figs. 1D-1F), we measured the lateral root number of *aco1-1* and the wild-type. Although the phenotypic differences between the wild-type and *aco1-1* mutant primary root lengths were subtle, the number of lateral roots was significantly higher in *aco1-1* compared to that in the wild-type (Fig. 6E). It is well known that exogenous treatment of ACC negatively impacts on lateral root

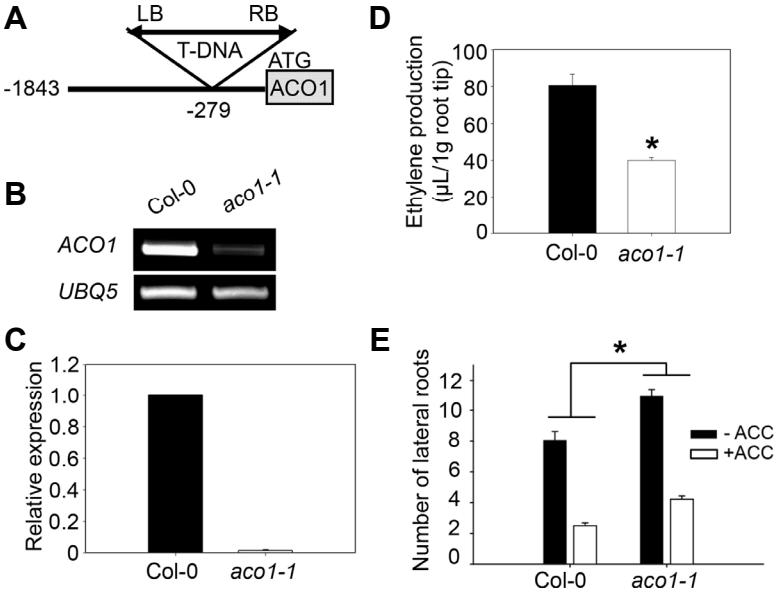


**Fig. 6. Biochemical and physiological phenotypes of *aco1-1*.** (A) T-DNA insertion map. T-DNA was inserted at 279 bp upstream of the start codon in the *ACO1* gene. LB, left border; RB, right border. Numbers represent the number of nucleotides upstream (-) of the start codon. (B) Transcript level for *ACO1* gene in *aco1-1* seedlings. The wild-type and *aco1-1* seedlings were grown for 5 DAG in the light. Semi-quantitative RT-PCR was performed with total RNA extracted from the wild-type and *aco1-1* seedlings. PCR products were detected by EtBr staining. *UBQ5* was used to normalize the expression level. (C) Quantitative real time-PCR was performed with total RNA extracted from 10 DAG seedlings grown in the light. *ACO1* expression level in *aco1-1* was normalized to that of *PP2A* and is shown relative to the expression levels in the wild-type. Two biological repeats along with three technical repeats were performed for quantification of *ACO1* expression level. The error bars indicate the S.D. (n=2). (D) Ethylene production in *aco1-1* root tips. Ethylene production from 2 mm root tip segments was analyzed after 6 hr incubation. Values are expressed as the mean of three biological replications ±S.E. Each biological replicate was performed with two technical repeats; each repeat used 100 root tips weighing approximately 0.001 g. The asterisk indicates significant differences from the wild-type at $P<0.01$ using *t*-test. (E) Effect of ACC treatment on *aco1-1* lateral root formation. Lateral root numbers of 9 DAG seedlings grown on control (-ACC) or 1 μM ACC (+ACC)-containing media were counted under a dissecting microscope. Each column represents the average lateral root number. Error bars represent the S.E. ($n \geq 22$). The asterisks indicate significant difference between the wild-type and *aco1-1* at $P < 0.001$ using *t*-test. Three biological repeats were performed and showed similar results. The average value of the three repeats is shown.

formation (Negi et al., 2008). As ACOs catalyze conversion of ACC to ethylene, we tested whether *aco1-1* reduces the ACC-induced inhibition of lateral root development. The wild-type displayed 68.9% (5.5/8.0) reduction in lateral root formation upon ACC treatment, whereas the *aco1-1* mutant showed 61.5% (6.7/10.9) reduction (Fig. 6E). *aco1-1* grown on ACC-treated media showed increased lateral root number compared to the wild-type on the same media (Fig. 6E), suggesting that *ACO1* expression is partially required for lateral root development control by ACC.

## DISCUSSION

Previous studies suggest that conversion of ACC to ethylene, mediated by ACO, is a critical regulatory step in ethylene biosynthesis (Alonso and Ecker, 2001; Kende and Zeevaart, 1997; Lasserre et al., 1996; Van de Poel et al., 2014; Vriezen et al., 1999). ACC is considered a long-distance transported substance but ethylene has limited transport capacity (Bradford and Yang, 1980). Thus, tissue-specific functions of ethylene should be investigated with respect to corresponding ACO activity in each tissue, rather than ACS-catalyzed ACC biosynthesis. Thus, it has been suggested that determination of *ACO* transcript level in each tissue may serve as an indicator of ethylene content (Ruduś et al., 2013).

It is known that *ACO* genes display distinct expression patterns in various plant organs and at different developmental stages associated with ethylene activity in rice, tomato, and garden cress (Barry et al., 1996; Iwamoto et al., 2010; Linkies et al., 2009). However, tissue-specific expression patterns and regulation of *ACO* genes in Arabidopsis are poorly understood, although *ACO* gene transcription is known to be differentially regulated by various biotic and abiotic stresses, light signaling, and multiple hormones (García et al., 2010; Gòmez-Lim et al., 1993; Mazzella et al., 2005; Qin et al., 2007; Thain et al., 2004).

In this study, we demonstrated the tissue-specific expression of *ACO1* and its physiological role in Arabidopsis. Using transcriptional fusion of the *ACO1* promoter and *GUS* reporter gene, we showed that Arabidopsis *ACO1* exhibits a specific spatio-temporal expression pattern (Fig. 2 and Supplementary Fig. S1). Its transcription appeared to be under the control of an intrinsic signaling network involved in responses to light and hormones (Figs. 3 and 5). Light-to-dark transition strongly increased *ACO1* expression whereas dark-to-light transition reduced *ACO1* promoter activity. Furthermore, we identified two E-box motifs that were essential for *ACO1* promoter activity and its regulation by light signal (Fig. 5). Both BR-regulated BZR1 and light-regulated PIF transcription factors are known to interact with each other and bind E-box motifs in their target genes (Oh et al., 2012). In addition, a recent study demonstrated that auxin-regulated ARF6 transcription factor shares its target genes with BZR1, and their transcriptional activity is interdependent as well (Oh et al., 2014). In this study, we showed that *ACO1* promoter activity was regulated by all three signals in a tissue-specific

manner (Figs. 3 and 4). Mutations in two E-box motifs (*E2* and *E4*) in the *ACO1* promoter compromised its regulatory activity by light, suggesting that *E2* and *E4* are essential *cis*-elements required for *ACO1* expression via binding with PIF and BZR1 (Figs. 5C-5E). Interestingly, the *ACO1* promoter also contains core AuxREs that are the binding sites for ARFs (Supplementary Fig. S6; Ulmasov et al., 1999) and the *ACO1* expression pattern was altered by IAA treatment (Fig. 4E). Therefore, two core AuxREs in the transcriptional regulatory region (1,154 and 769 bp from the ACO1 start codon) might also regulate the promoter activity. Taken together, these results imply that tissue-specific *ACO1* expression might be mediated by combinatorial activation of these transcription factors that coordinate multiple signals.

We isolated the *aco1-1* mutant that had a T-DNA insertion at 279 bp upstream from the ATG codon. *ACO1* expression in *aco1-1* was greatly reduced by the T-DNA mutation that displaced regulatory *cis*-elements on the *ACO1* promoter (Figs. 6A and 6B; Supplementary Fig. S6). The reduced *ACO1* expression in *aco1-1* further supports the importance of *cis*-elements for *ACO1* promoter activity.

Although ACO1 has a conserved $Fe^{2+}$-binding motif that is required for binding to the substrate ACC (Linkies et al., 2009), no evidence has supported that ACO1 is a functional enzyme producing ethylene in Arabidopsis. We showed that ethylene production by the *aco1-1* mutant was greatly reduced in root tips (Fig. 6D). This finding provides an evidence that ACO1 functions as an active ACC oxidase in Arabidopsis roots.

In this study, we demonstrated that *ACO1* expression is required for lateral root development. *aco1-1* displayed enhanced lateral root development (Fig. 6E). Auxin is a key hormone that promotes lateral root formation through direct *LBD* gene activation in the lateral root primordia (Okushima et al., 2007). It is known that ethylene inhibits lateral root formation through promoting *PIN3* and *PIN7* expression, leading to the disruption of local auxin maximum which is required for lateral root development (Lewis et al., 2011). In addition, ACC treatment increases these protein levels in root tips (Lewis et al., 2011). In our study, we showed that *ACO1* was highly expressed in root tips and its expression was enhanced by ACC treatment (Figs. 2E and 4F). Consistently, ethylene production in *aco1-1* root tips was reduced compared to that of the wild-type (Fig. 6D). Thus, our results suggest that *ACO1* expression in root tips might be responsible for the negative regulation of lateral root development by ethylene through alteration of auxin transport.

*aco1-1* was slightly less sensitive to ACC in lateral root formation rather than complete insensitivity (Fig. 6E). In addition, we could not detect significant changes in root ethylene production and in root length (Supplementary Fig. S5). These faint phenotypes might be due to incomplete disruption of the gene expression in the mutant. Notably, *ACO1* is a close homolog of *ACO2-4* (Supplementary Fig. S7). *In silico* analysis showed that these genes have the same expression pattern in the root (Dugardeyn et al., 2008). Thus, the weak phenotypes of *aco1-1* may also be explained by genetic redundancy that compensates the lack of ACO1 activity. We tried to generate a transgenic plant that overexpressed *ACO1* under control of the Cauliflower mosaic virus (CaMV) *35S* promoter. Although over 50 antibiotic-resistant transgenic plants were selected from three independent transformations, we failed to isolate a transgenic plant overexpressing *ACO1*. Therefore, in future study, multiple knockout mutants of this family genes might be helpful to understand their intrinsic functions.

Although we observed specific *ACO1* expression in the aerial stipule, we could not determine its physiological role. Interestingly, expression of other known ethylene biosynthetic genes was also observed in the stipule (Yun et al., 2009), indicating that ethylene plays a physiological role in this organ. Stipules have been identified to be the first major site for high free auxin production during the very early stages of leaf development (Aloni et al., 2003). It is possible that the physiological interaction between auxin and ethylene in the stipule is crucial for regulation of leaf development.

High expression of *ACO1* in dark-grown seedlings might correlate with the triple responses of ethylene in the dark (Fig. 3B). However, the physiological role of *ACO1* expression increased by light-to-dark transition in hypocotyls is unclear (Fig. 3A). Smale *et al.*, reported that ethylene treatment promoted hypocotyl elongation when seedlings were grown under long day conditions (Smale. et al., 1997) In addition, recent study demonstrated that ethylene-induced hypocotyl elongation also occurred in the dark period (Das et al., 2016). Thus, it will be interesting to study whether *ACO1* expression induced by the light-to-dark transition correlates with ethylene-stimulated hypocotyl elongation in the long day-grown seedling.

*Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).*

## ACKNOWLEDGMENTS

This work was supported by grants from the Next-Generation BioGreen 21 Programs (PJ0132082018 to S.K.K), Rural Development Administration. This research was also supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Science and ICT (NRF-2017R1A2B4004274 to T.W.K.).

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