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

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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 ACSs, 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;
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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; Rudu et al., 2013; Vriens 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 ACOs 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 ACOs 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′-CTTTGACAGTGGAGTCCACGTCCTTAAATA-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°C 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°C for 16 hr and in the dark at 20 ± 1°C for 8 hr 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 HindIII restriction site into the 5′-end and a BamHI 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 (Stratagene). The primers used for mutagenesis are shown in Supplementary Table S1. Promoter fragments were cloned into pGEM T easy vector (Promega) and subcloned into pBluescript II 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 ACOs 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...
stopped by Na$_2$CO$_3$. Fluorescence was measured with a
traction buffer). After exactly 10 and 20 min, reactions were
methylumbelliferyl-

were mixed with a reaction buffer (1 mM 4-

mercaptoethanol and 25 μM EDTA). The crude extracts

were subjected to total RNA extraction. (B) ACO1 expres-

sion in seedlings grown under different light conditions. Seed-

lings were grown in the light or in the dark for 5 DAG. (C) Eth-

ylene 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 prod-

ucts were electrophoresed on 1% agarose gels and detected by

fluorescence 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: Hewleett 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 Ara-
bidopsis genome, we speculated that each ACO member
might have specific function(s) in various tissues, as is the
case with ACS family members (Tsuchisaka et 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.

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 posi-
tively 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 rele-
vance 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
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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. 2H and 2I). 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. 2I and 2J). 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, siliques, 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 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...
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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 GA3 (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 ∆312 construct were prepared. The ProACO1-GUS ∆312 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 ∆312 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. 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. 6B). 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.
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

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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 ∆312, 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 ∆312 (∆312) 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).
of ACO transcript level in each tissue may serve as an indicator of ethylene biosynthesis. Thus, it has been suggested that determination of ACO activity in each tissue, rather than ACS-catalyzed ACC synthesis, should be investigated with respect to corresponding biosynthesis (Alonso and Ecker, 2001; Kende and Zeevaart, 1997; Lasserre et al., 1996; Van de Poel et al., 2014; Vriezen et al., 2014). In this study, we demonstrated the 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; Gómez-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.
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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 (Dugard 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 knock-out 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).

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