Molecules and Cells

Regulation of Ethylene Biosynthesis by Phytohormones in Etiolated Rice (*Oryza sativa* L.) Seedlings

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The gaseous hormone ethylene influences many aspects of plant growth, development, and responses to a variety of stresses. The biosynthesis of ethylene is tightly regulated by various internal and external stimuli, and the primary target of the regulation is the enzyme 1-aminocyclopropane-1-carboxylic acid (ACC) synthase (ACS), which catalyzes the rate-limiting step of ethylene biosynthesis. We have previously demonstrated that the regulation of ethylene biosynthesis is a common feature of most of the phytohormones in etiolated Arabidopsis seedlings via the modulation of the protein stability of ACS. Here, we show that various phytohormones also regulate ethylene biosynthesis from etiolated rice seedlings in a similar manner to those in Arabidopsis. Cytokinin, brassinosteroids, and gibberellic acid increase ethylene biosynthesis without changing the transcript levels of neither *OsACS* nor ACC oxidases (*OsACO*), a family of enzymes catalyzing the final step of the ethylene biosynthetic pathway. Likewise, salicylic acid and abscisic acid do not alter the gene expression of *OsACS*, but both hormones downregulate the transcript levels of a subset of *ACO* genes, resulting in a decrease in ethylene biosynthesis. In addition, we show that the treatment of the phytohormones results in distinct etiolated seedling phenotypes, some of which resemble ethylene-responsive phenotypes, while others display ethylene-independent morphologies, indicating a complicated hormone crosstalk in rice. Together, our study brings a new insight into crosstalk between ethylene biosynthesis and other phytohormones, and provides evidence that rice ethylene biosynthesis could be regulated by the post-transcriptional regulation of ACS proteins.

Keywords: ethylene biosynthesis, hormone, OsACO, OsACS, post-transcriptional regulation, rice

INTRODUCTION

Rice is one of the most important food crops, and it feeds more than half of the world's population. While rice has evolved elaborate mechanisms to adapt various stress conditions including hypoxia (Ma et al., 2010), its productivity and sustainability are significantly threatened by many abiotic and biotic stresses such as drought, chilling, submergence, and salinity (Sahi et al., 2006; Shimamoto, 1999). During these stress conditions, ethylene plays a primary role in acclimating plants to unfavorable surroundings (Abeles, 1973; Morgan and Drew, 1997). Many studies have shown that the biosynthesis of ethylene is highly regulated transcriptionally and post-transcriptionally (Argueso et al., 2007; Yoon, 2015). The biosynthesis of ethylene consists of three simple steps, starting with the amino acid methionine in both monocot and dicot plants (Yang and Hoffman, 1984). In the first step, methionine is converted to S-adenosyl methionine

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(SAM), which is subsequently converted to 1-aminocyclopropane-1-carboxylic acid (ACC) by a family of enzymes known as ACC synthases (ACS). Conversion of SAM to ACC by ACS is the first committed and generally rate-limiting step in the pathway. In the last step, ACC is finally converted to ethylene by ACC oxidases (ACO), a member of the oxygenase superfamily member (Bidonde et al., 1998). The Arabidopsis genome contains 12 putative *ACS*-like genes (e.g., *ACS1* to *12*). Among the *ACS* genes, *ACS3* is a pseudogene with a short sequence, and *ACS10* and *ACS12* encode an aminotransferase without the catalytic activity of ACS (Yamagami et al., 2003). The other nine *ACS* genes encode a group of ACS proteins that can be classified into 3 types, named type-1, type-2, and type-3, based on the presence or absence of putative phosphorylation sites at the C-terminal domain of the proteins (Chae and Kieber, 2005). Type-1 ACS proteins contain putative phosphorylation target sites for both mitogen-activated protein kinase (MAPK) and calcium-dependent protein kinase (CDPK). Type-2 ACS proteins have only a putative target site for CDPK, but they also contain a unique regulatory motif called a Target of ETO1 (TOE) at their C-termini. TOE is a binding site for ETHYLENE OVERPRODUCER 1 (ETO1) (Wang et al., 2004), which is a Broad complex/Tramtrack/Bric-a-brac (BTB) domain substrate adaptor for CULLIN-3 E3 (CUL3) ubiquitin ligase. ETO1 and its two paralogs, ETO1-like 1 (EOL1) and EOL2 specifically interact with type-2 ACS proteins via the TOE motif, and this interaction further leads to the degradation of type-2 ACS proteins via the 26S proteasome (Yoshida et al., 2005; 2006).

Compared to Arabidopsis, rice possesses a relatively small number of *ACS* genes. It contains only 5 putative *ACS* genes (*OsACS1* to *5*) which share 50 to 80% of similarity of amino acid sequence among the isoforms (Supplementary Table S1) (Zarembinski and Theologis, 1997). All *OsACS* genes possess seven conserved domains that were found in Arabidopsis and other plant species, including all 11 invariants amino acid residues found in ACS and aminotransferases (Yamagami et al., 2003). A phylogenetic tree of ACS proteins from Arabidopsis and rice and the analysis of ACS protein structure indicate that OsACS proteins also fall into three different types (Figs. 1A and 1B). OsACS2 protein exhibits a similar structure to Arabidopsis type-1 ACS2 and 6 proteins. It has the longest C-terminal domain among OsACS proteins with putative phosphorylation sites for MAPK and CDPK. OsACS1 was closely related to Arabidopsis type-2 ACS, and its C-terminus contains a potential CDPK target site and a putative TOE domain (Yoshida et al., 2006). While type-2 ACS proteins were the dominant form of ACS proteins in Arabidopsis, type-3 ACS is the most abundant ACS proteins in rice, with three isoforms of type-3 ACS. Similar to the Arabidopsis type-3 ACS, type-3 OsACS proteins have a short C-terminal domain and lack of any known regulatory sites. The structural similarity and the conserved regulatory sites found in both ACS proteins from Arabidopsis and rice imply the existence of an evolutionally conserved mechanism that underlies the regulation of ethylene biosynthesis in these two plant species.

Given the diverse roles of ethylene in plant growth and physiological responses to various external and internal signals, ethylene biosynthesis is highly regulated at both transcriptional and post-transcriptional levels (Argueso et al., 2007). Regulation of the transcript levels of *ACS* genes appears to be a key control mechanism of ethylene production during stress conditions (Argueso et al., 2007; Zarembinski and Theologis, 1994). In Arabidopsis, the transcript levels of a subset of *ACS* have been shown to be under the control of biotic and abiotic stresses such as salinity, chilling, submergence, drought, and pathogen invasion (Argueso et al., 2007). Similarly, the transcript levels of *OsACS* genes were also affected by various stimuli, including submergence, herbivore attack, and fungus infection, resulting in altered ethylene

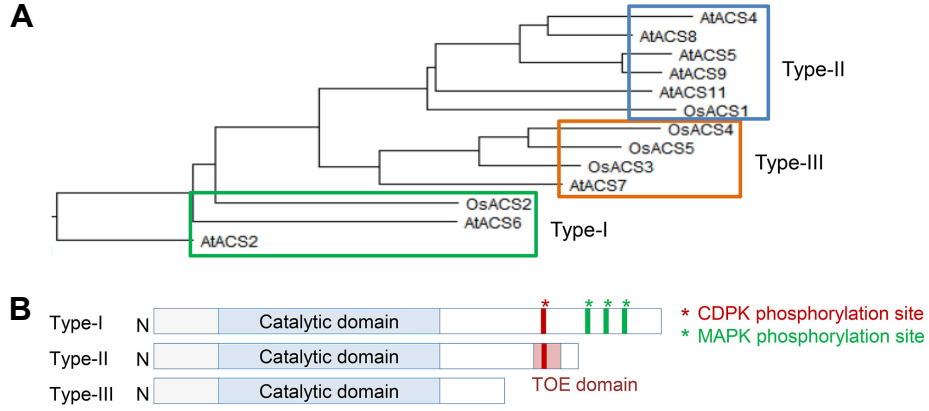

Fig. 1. Phylogenetic tree of ACC synthases from Arabidopsis and rice and cartoon representing the structure of different types of ACS proteins. The amino acid sequences of Arabidopsis and rice ACS proteins were aligned using ClustalW and the phylogenetic tree was generated using DNAstaw software (A). The structure of ACS proteins (B). The conserved catalytic domain is shown with blue rectangle boxes. The target serine residues for calcium-dependent protein kinase (CDPK) and mitogen-activated protein kinase (MAPK) are shown as a red or green asterisk, respectively. Target of ETO1 (TOE) motif in type-2 ACS is highlighted in red box. Type-3 ACS does not contain any regulatory motifs.

production (Hazman et al., 2016; Iwai et al., 2006; Lu et al., 2014). In addition to the transcriptional control of *ACS*, several studies have provided compelling evidence that post-translational modifications, such as phosphorylation and ubiquitination, serve as an important mechanism to regulate the stability of Arabidopsis ACS proteins, thus controlling the levels of ethylene in plants (Chae and Kieber, 2005; Liu and Zhang, 2004; Wang et al., 2004; Yoon and Kieber, 2013).

ACC oxidase (ACO) is another enzyme involved in the final step of ethylene biosynthesis, converting ACC to ethylene (Argueso et al., 2007; Yang and Hoffman, 1984). *ACO* genes have been identified from various plant species, including but not limited to Arabidopsis (Lin et al., 2009), rice (Iwai et al., 2006), petunia (Tang et al., 1993), tomato (Barry et al., 1996), potato (Nie et al., 2002), and apple (Binnie and McManus, 2009). In most plant species investigated, ACO isoforms are encoded by a multigene family of *ACO* genes. For example, Arabidopsis and rice possess six and seven *ACO* genes, respectively (Iwai et al., 2006; Lin et al., 2009). Similar to the *ACS*, the gene expression of *ACO* from different species is also correlated to the rate of ethylene biosynthesis, and the transcript levels of multiple *ACO* genes are under the control of developmental and stress conditions (Gómez-Jiménez et al., 1998; Linkies and Leubner-Metzger, 2012; Matillaa and Matilla-Vázquezb, 2008). It has been shown that *OsACO1* plays a role in the internode elongation of deepwater rice; submergence increases the levels of *OsACO1* mRNA and ACO enzyme activity (Iwamoto et al., 2010; Mekhedov and Kende, 1996). The gene expression of *OsACO2* and *OsACO3* in etiolated rice seedlings is also shown to be differentially regulated by ethylene and auxin (Chae et al., 2000). Additionally, the transcripts of a subset of *OsACO* (*OsACO2* and *OsACO7*) were highly induced upon infection with blast fungus, leading to an increase in ethylene production, which ultimately contributes to the disease resistance of rice to blast fungus (Iwai et al., 2006). Compared to the transcriptional regulation of *OsACO* genes, less is known about the post-transcriptional control of OsACO proteins.

Hormonal crosstalk plays a large role in enabling plants to respond to a given developmental or environmental input with plasticity (Depuydt and Hardtke, 2011). Ethylene interacts with many phytohormones and the resulting crosstalk regulates many important developmental processes in both rice and Arabidopsis. We have recently revealed that several phytohormones regulate ethylene biosynthesis in etiolated Arabidopsis seedlings, indicating that ethylene biosynthesis is a central crosstalk point among phytohormones (Lee et al., 2017). Phytohormones, including cytokinin, GA, BR, IAA, SA, methyl jasmonate (MJ), and ABA control ethylene biosynthesis either through the modulation of the turnover of ACS proteins or the transcript levels of *ACS* genes, or both (Lee et al., 2017). Cytokinin, BR, and GA mainly increase the stability of type-1 and type-2 ACS proteins without changing the transcript levels of *ACS* genes. SA, MJ, and ABA influence ethylene production from etiolated seedlings by affecting both the transcript levels of *ACS* genes and protein turnover rate of type-1 and 2 ACS proteins (Lee et al., 2017). Contrary to the previous known role of auxin in ethylene biosynthesis, IAA promotes ethylene production by regulating both *ACS* transcript levels and protein turnover of ACS proteins (Abeles et al., 1992; Tsuchisaka and Theologis, 2004; Wang et al., 2005; Yang and Hoffman, 1984). Interestingly, unlike type-1 and type-2 ACS, there is no effect on type-3 *ACS7* transcript levels or ACS7 protein turnover by these phytohormones (Lee et al., 2017).

Here we report that various phytohormones control ethylene biosynthesis in rice. We examine the effects of phytohormones in controlling ethylene production in etiolated rice seedlings. We demonstrate that nearly all phytohormone-induced changes in ethylene biosynthesis are not regulated by *ACS* transcript changes in etiolated rice seedlings. However, contrary to the *ACS*, we found that some fractions of the phytohormones affect the transcript levels of *ACO* genes resulting in a decrease in ethylene biosynthesis. In addition, we show that various phytohormones induce distinctive morphological changes in rice seedlings, some of which resemble ethylene-responsive phenotypes, suggesting a possible role of these plant hormones in ethylene biosynthesis regulation. Together, our results provide new insights into the role of phytohormones in ethylene biosynthesis and the hormonal crosstalk between ethylene and other phytohormones in rice etiolated seedlings.

MATERIALS AND METHODS

Plant materials and growth conditions

Rice (*Oryza sativa* L. cv. Nipponbare) seeds were dehusked and sterilized with 30% sodium hypochlorite for 10 min, followed by rinsing with deionized water. The sterilized seeds were subsequently placed onto a sterile sieve, placed in a liquid MS media in a magenta box. The seedlings were grown in a growth chamber at 28℃ in the dark.

Measurements of ethylene production

Ethylene measurements were performed as previously described (Hansen et al., 2009) with a brief modification. Surface-sterilized seeds were germinated in 22-ml gas chromatography vials containing 3 ml MS with 1% sucrose, 1% bactoagar, and with and without indicated concentration of hormones. Following 2 days of dark treatment, the vials were capped and incubated at 28℃ for 3 days in the dark. The accumulated ethylene was measured by a gas chromatography using a Shimadzu GC2010 Plus capillary gas chromatography system with a HS-20 headspace autosampler. All treatments were measured from at least three replicates.

RNA extraction and quantitative RT-PCR

Total RNA was extracted by using a RNeasy Plus kit (Qiagen). cDNA was prepared from the total RNA using SuperScript II reverse transcriptase (Invitrogen) as described by the manufacturer. Quantitative RT-PCR was performed using PowerUP™ SYBR® green Master Mix (Applied Biosystems) and CFX Connect™ Real-Time System (Bio-Rad). Relative expression of *OsACS* or *OsACO* genes were normalized to *OsActin1* as a reference gene and was calculated using $2^{\Delta\Delta CT}$ method. Primers used are listed in Supplementary Table S2.

Analysis of etiolated rice seedling morphology in response to phytohormones

The surface-sterilized wild-type seedlings were grown in liquid MS media in a growth chamber at 28℃ in the dark. 2-day-old germinated rice seedlings were gently transferred onto a sieve that was placed on 1/2 MS solution with or without various phytohormones (10 μM ACC, 10 μM synthetic cytokinin BA, 10 μM BR, 10 μM GA_3, 10 μM IAA, 5 mM SA, 5 μM MJ or 1 μM ABA), and further incubate them at 28℃ in the dark for additional 3 days.

RESULTS

Phytohormones differentially regulate the biosynthesis of ethylene from etiolated rice seedlings

The biosynthesis of ethylene is regulated by many factors, including developmental cues and environmental stimuli, both of which can be integrated into the cells via the action of phytohormones in plants. We have previously demonstrated that most of the phytohormones influence the rate of ethylene biosynthesis in *Arabidopsis thaliana* via the alteration of mRNA levels or protein stability of ethylene biosynthesis components such as 1-aminocyclopropane-1-carboxylic acid (ACC) synthase (ACS) and ACC oxidase (ACO) (Lee et al., 2017). To examine whether phytohormones affect ethylene biosynthesis of rice seedlings in a similar manner to that of Arabidopsis seedlings, we measured ethylene production from wild-type etiolated rice seedlings (*Oryza sativa* L. cv. Nipponbare) grown in the presence of various phytohormones, including cytokinin, BR, GA, IAA, SA, MJ, and ABA (Fig. 2). Cytokinin, BR, IAA, and GA, increased ethylene production in a concentration-dependent manner (Fig. 2). This is consistent with a previous report that these hormones promote ethylene production of Arabidopsis etiolated seedlings over-expressing type-1 or type-2 ACS protein (Lee et al., 2017). By contrast, the growth in the presence of either SA or ABA caused a reduction in the levels of ethylene production from etiolated rice seedlings. These results also agree with that both SA and ABA decrease ethylene production of Arabidopsis seedlings over-expressing ACS proteins (Lee et al., 2017). This is consistent with the observation that SA reduces ethylene production from detached rice leaves resulted from the inhibition of ACC formation and the conversion of ACC to ethylene (Huang et al., 1993). Unlike other phytohormones, MJ appeared not to alter the level of ethylene in etiolated rice seedlings. Together, these results indicate that most phytohormones tested in this study influence the level of ethylene biosynthesis from etiolated rice seedlings, and the alteration of ethylene biosynthesis is a common feature of many phytohormones in both rice and Arabidopsis.

Most of the phytohormones, except IAA, do not alter the transcript levels of *OsACS* in etiolated rice seedlings

The changes in the levels of ethylene production from rice etiolated seedlings in response to the phytohormones indicate that there must be corresponding changes either at the levels of gene expression or protein stability or/and activity of ethylene biosynthesis components. To get further insight into the molecular basis underlying the phytohormone-regulated ethylene biosynthesis in etiolated rice seedlings, we first examined the effects of phytohormones on the transcript levels of different types of rice *ACS* genes, *OsACS1* (type-2), *OsACS2* (type-1), and *OsACS3*, *4*, *5* (type-3) using qRT-PCR (Fig. 3). Most phytohormones did not alter the transcript levels of the majority of *OsACS* genes (Fig. 3). One exception was IAA, which caused a significant increase in the transcript levels of both Os*ACS1* and *OsACS2* compared to its mock treatment. This is in agreement with previous studies showing that auxin induces the transcript levels of Arabidopsis type-1 and type-2 *ACS* (Lee et al., 2017). Interestingly, IAA did not change the transcript levels of *OsACS3*, *OsACS4*, and *OsACS5*, all of which belong to the type-3 ACS family. These results are also consistent with the prior studies showing that Arabidopsis type-3 *ACS7* transcript levels are unchanged by IAA treatment in both etiolated and light-grown Arabidopsis seedlings.

Together, these results showed that most phytohormones, except IAA, do not alter the transcript levels of *ACS* genes in etiolated rice seedlings, suggesting a possible role for the post-transcriptional regulation of ACS proteins in the phytohormone-mediated ethylene biosynthesis in rice.

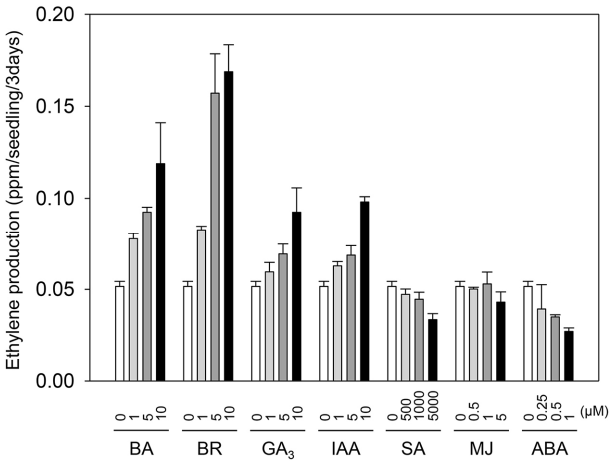

Fig. 2. Ethylene production by 3-day-old etiolated seedlings in response to various phytohormones. Wild-type rice seedlings were grown in MS media with the indicated concentration of phytohormones, capped for 3 days in the dark, and the accumulated levels of ethylene were measured at day 3 using gas chromatography. Error bars indicate SD; $n = 3$.

A subset of the *OsACO* transcript levels are regulated by phytohormones

Unlike the effects of cytokinin, BR, IAA, and GA, treatment of rice etiolated seedlings with SA and ABA led to a reduced ethylene production in a manner dependent on the concentration of the phytohormones used (Fig. 2). Given that the transcript levels of most of the *OsACS* genes were not affected by both SA and ABA, we postulated that the reduction of ethylene biosynthesis in response to SA and ABA could be due to changes in the transcript levels of *ACC oxidases*

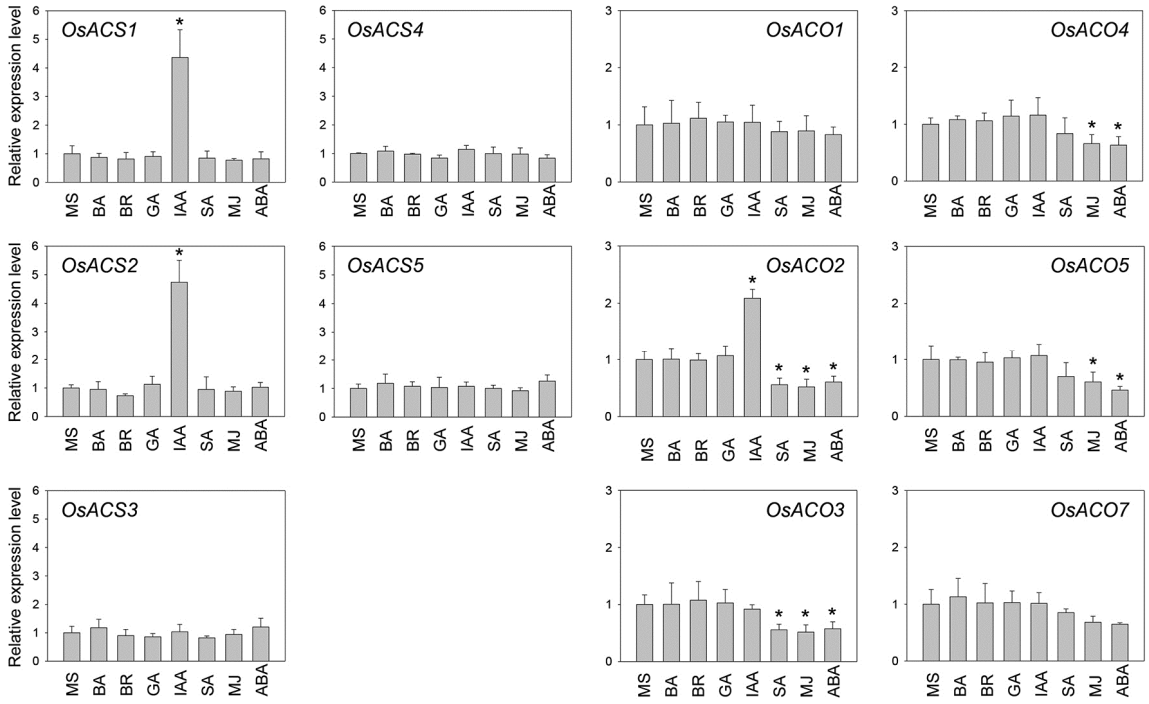

Fig. 3. Phytohormone-induced transcriptional regulation of *OsACS* and *OsACO* genes. Quantitative RT-PCR expression analysis of *OsACS* and *OsACO* genes in rice etiolated seedlings grown in the presence of indicated hormones (10 μM BA, 10 μM BR, 10 μM GA_3, 10 μM IAA, 5 mM SA, 5 μM MeJA or 1 μM ABA) for 3 days. Expression of *OsActin1* was used as an internal control. Error bars represent SD from three biological replicates. $*P < 0.05$, Student's *t*-test.

(*OsACO*). To this end, we determined the transcript levels of six *OsACO* genes from wild-type etiolated rice seedlings treated with various phytohormones (Fig. 3). *OsACO6* was not included in the assay as it is a pseudogene. In general, the treatment of etiolated rice seedlings with ABA reduced the expression of most of the *OsACO*. Likewise, SA treatment also decreased the expression of a subset of the *OsACO* genes, *OsACO2* and *OsACO3*. MJ treatment reduced the transcript levels of *OsACO2*, *OsACO3*, *OsACO4,* and *OsACO5*. Contrary to ABA, SA, and MJ, IAA increased the transcript levels of *OsACO2*, as previously described (Chae et al., 2000). Unlike SA, MJ, and ABA, cytokinin, GA, and BR did not affect the transcript levels of most of the *OsACO*. These results suggest that the reduced transcript levels of *OsACO* in response to ABA, SA, and MJ likely contribute to the decreased ethylene biosynthesis observed in etiolated rice seedlings treated with these hormones. It is still possible that these three hormones may also regulate ACO protein stability and/or activity and ACC conjugation.

Various phytohormones differently influence the growth of etiolated rice seedlings

Dark-grown Arabidopsis seedlings exposed to ethylene produce distinctive ethylene-responsive phenotypes, which are referred as the triple response (Guzman and Ecker, 1990). The triple response of etiolated seedlings includes the formation of an exaggerated apical hook, thickened and shortened hypocotyl, and inhibited root growth. Treatment of dark-grown Arabidopsis seedlings with cytokinin also causes the triple response due to a role of cytokinin in promoting ethylene biosynthesis (Chae et al., 2003). Unlike Arabidopsis, etiolated rice seedlings do not have the typical triple response. Instead, rice seedlings display unique ethylene-responsive phenotypes, namely, an increased growth of the coleoptile and inhibited root growth (Fig. 4A) (Kim et al., 2012; Ma et al., 2010). To determine the effect of the phytohormones in rice seedling growth in the dark, we observed the phenotypes of etiolated rice seedlings after treatment with the phytohormones. Consistent with previous studies, treatment of etiolated rice seedlings with ACC, a direct precursor of ethylene, led to the ethylene responsive phenotypes of elongated coleoptile and shortened root (Fig. 4A). Similar morphological changes were observed in etiolated rice seedlings treated with cytokinin and BR, indicating that these hormones play a role in promoting either ethylene biosynthesis or its signaling. However, IAA, SA, MJ, and ABA only caused the changes in the root growth of etiolated rice seedlings with varying degrees of root growth inhibition (Fig. 4B). The root growth inhibition caused by SA, MJ, and ABA was somewhat moderate compared to those from IAA or ACC-treated seedlings. In contrast, etiolated rice seedlings treated with GA displayed both an elongated coleoptile and root, consistent with the growth-promoting role of this phytohormone in plants. Together, these results revealed that the role of phytohormones in controlling ethylene biosynthesis in etiolated rice seedlings, either positively or negatively, is

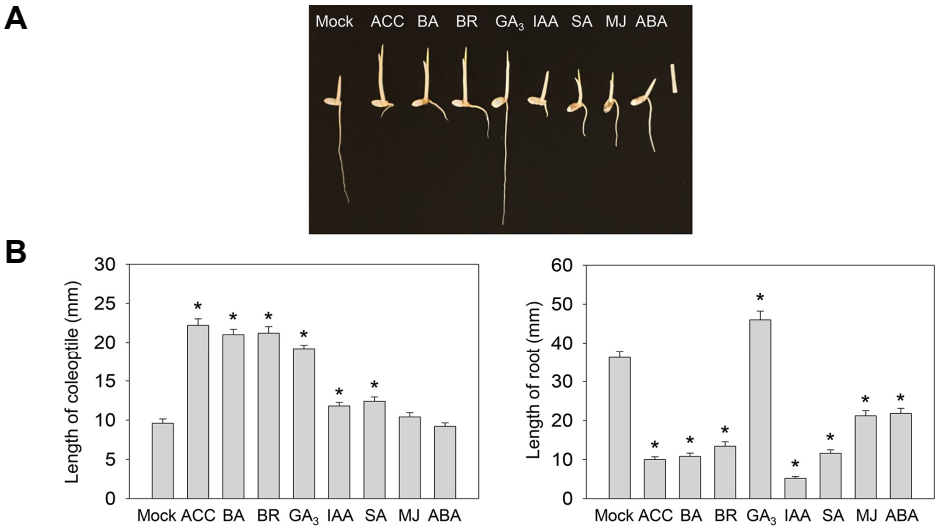

Fig. 4. Morphological analysis of 3-day-old etiolated rice seedlings in the presence of phytohormones. (A) A representative image of wild-type etiolated seedling that were grown on MS media supplemented with or without various phytohormones with the indicated concentration (10 μM ACC, 10 μM BA, 10 μM BR, 10 μM GA_3, 10 μM IAA, 5 mM SA, 5 μM MJ or 1 μM ABA). Scale bar indicates 10 mm. (B) Measurement of the coleoptile (left) or root (right) length of etiolated rice seedlings grown with various phytohormones. Error bars indicate SD; $n = 5$, $*P < 0.005$, Student's *t*-test.

not always comparably reflected in morphological changes, highlighting the complicated nature of phytohormone crosstalk networks in plants.

DISCUSSION

Rice suffers from various biotic and abiotic stresses, resulting in a dramatic loss of yield and productivity. Ethylene plays a pivotal role in adapting the growth of rice in those harsh growth conditions. While the transcriptional regulation of ethylene biosynthesis is relatively well established, not much is known about the post-transcriptional regulation of ethylene biosynthesis in rice to date. However, multiple pieces of evidence support the idea that rice ethylene biosynthesis is in part regulated by the post-transcriptional regulation of ACS proteins, in a manner similar to what is observed in Arabidopsis. Such evidence includes the existence of ETO1-like E3 ubiquitin ligases orthologs in the rice genome and the negative role of OsETOL1 in ethylene biosynthesis regulation (Du et al., 2014). Du et al., showed that OsETOL1 modulates drought and submergence tolerance by down-regulating ethylene biosynthesis. OsETOL1 directly interacts with OsACS2, a homolog of Arabidopsis ACS protein. Moreover, overexpression of OsETOL1 resulted in a reduction in ethylene biosynthesis, which validates the negative role of OsETOL1 in ethylene biosynthesis. Further, rice contains 8 isoforms of 14-3-3 proteins, highly conserved regulatory proteins that regulate diverse physiological processes by phosphorylation-dependent protein-protein interactions (Jaspert et al., 2011). A recent study showed that Arabidopsis 14-3-3 protein acts as a positive regulator of ethylene biosynthesis by enhancing the protein stability of ACS proteins through the interaction with ACS proteins (Yoon and Kieber, 2013). Interestingly, Yao et al, demonstrated that rice 14-3-3 binds to OsACS1 using a yeast two hybrid, but the detailed biochemical analysis of this interaction has not been demonstrated (Yao et al., 2007). Together, these evidences strongly suggest that rice ethylene biosynthesis is likely under the control of the post-transcriptional regulation of ACS proteins.

Compared to the ACS proteins, the post-transcriptional control of ACO has not been clearly demonstrated. In this study, we found that BA, BR, and GA did not affect the transcript levels of all *ACO* genes tested, yet increased ethylene production in etiolated rice seedlings (Fig. 3). These results indicate the possible role of the post-transcriptional control of ACO proteins. Interestingly, a recent study suggested that the turnover of Arabidopsis ACO protein is likely responsible for altering ethylene production in etiolated seedlings. In Arabidopsis, the mutation of Related to Ubiquitin 1 (RUB)-conjugating enzyme (RCE1), an enzyme that catalyzes the covalent modification of RUB1 to SCF-E3 ligase complex, results in an increased activity of ACO, hence elevated ethylene production (Larsen and Cancel, 2004). Despite the widespread knowledge that ACS is the rate-limiting enzyme in ethylene biosynthesis, some evidence supports that ACO could also contribute to sustaining the high levels of ethylene production during some developmental processes such as pollination-mediated senescence and seedling germination (Nadeau et al., 1993; Petruzzelli et al., 2000). During these processes, plants elevate ethylene production by increasing ACO activity which is regulated by either *ACO* expression or the rate of ACO protein turnover, or both. In rice, the role of a subset of *ACO* has been implicated in submergence response and hormone crosstalk. Especially, the expression of *OsACO1* and its enzymatic activity are

greatly increased upon submergence stress to maintain the high level of ethylene production (Iwamoto et al., 2010; Mekhedov and Kende, 1996). This suggests the post-transcriptional control of ACO in addition to the transcriptional regulation of *ACO* gene expression. The interaction between ethylene and other phytohormones including GA during submergence stress may also contribute to an increase in ethylene biosynthesis via the modulation of the ACO activity (Fukao and Bailey-Serres, 2008; Jackson, 2008).

Phytohormone-mediated changes in the morphologies of etiolated rice seedlings suggest a complicated hormonal crosstalk in rice. ACC-treated etiolated rice seedlings showed unique ethylene-responsive phenotypes of an elongated coleoptile and inhibited root growth. Cytokinin and BR induce ethylene-responsive phenotypes in rice seedlings, which is consistent with their role in increasing ethylene biosynthesis. Interestingly, unlike what is observed in rice, BR does not trigger the triple response in dark-grown Arabidopsis seedlings, implying different actions of BR in rice and Arabidopsis, despite its common role in enhancing ethylene biosynthesis. The action of GA in rice seedlings is somewhat different from cytokinin and BR, although all of three hormones increase ethylene production. Unlike cytokinin and BR, GA promotes the growth of both coleoptiles and roots indicating that the ethylene-GA crosstalk may preferentially occur in the roots, or that ethylene differently works to promote root growth in the presence of GA. In fact, several studies showed that ethylene increases the growth of submerged roots of deep water rice by working together with GA in a synergistic manner (Jackson, 2008; Vriezen et al., 2003). Similarly, the synergistic interaction between GA and ethylene in coleoptiles has been shown to be an important adaptive feature of deep water rice to grow out of the water and survive flooding (Miro and Ismail, 2013; Watanabe et al., 2007). Interestingly, SA and ABA, which decreased ethylene biosynthesis in rice seedlings, promote enhanced ethylene-responses in roots, and have no effects on ethylene-responsive phenotypes in the coleoptiles of etiolated seedlings. In Arabidopsis, ABA inhibits the root growth of light-grown Arabidopsis seedlings by promoting ethylene biosynthesis via a calcium-dependent protein kinase-mediated phosphorylation of type-2 ACS6 (Luo et al., 2014). This result is somewhat different from the results from our previous study that ABA stabilizes type-1 and type-2 ACS, but the overall ethylene biosynthesis from ABA-treated seedlings is reduced due to a decrease in *ACO* mRNA levels. This discrepancy could be related to the growth condition of seedlings (light vs. dark), which might differently affect *ACO* gene expression. Similar to Arabidopsis etiolated seedlings, ABA decreased ethylene biosynthesis from rice etiolated seedlings likely through a reduction in a subset of *ACO* transcripts in rice (Fig. 3). However, surprisingly, ABA inhibits root growth without a concurrent increase in ethylene levels, indicating a different hormonal crosstalk between ethylene and ABA in rice plants. Likewise, SA appears to control the root elongation of rice through an ethylene-independent pathway, as the SA-induced reduction of ethylene did not influence root growth.

In summary, we show that the major phytohormones, with exception of IAA, influence ethylene biosynthesis in etiolated rice seedlings, through mechanisms that do not include the transcriptional changes of *ACS* genes, thus indicating the possibility of a post-transcriptional regulation of ACS proteins. In a similar manner to the *ACS*, the transcript levels of *ACO* were not altered by a subset of phytohormones including cytokinin, BR, and GA, implicating the potential involvement of the post-transcriptional control of ACO proteins. Moreover, we demonstrate that different phytohormones induced distinctive phenotypes in etiolated rice seedlings, which may or may not be directly related to the action of ethylene, despite their role in ethylene biosynthesis. Further understanding of the roles of phytohormones on the activity or stability of both ACS and ACO proteins will provide more insight into the regulatory mechanisms underlying ethylene biosynthesis in rice.

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

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