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ORIGINAL RESEARCH PAPER

Phytochrome-regulated *EBL1* contributes to *ACO1* upregulation in rice

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Abstract The 1-aminocyclopropane-1-carboxylate oxidase gene (*ACO1*) was upregulated in rice (*Oryza sativa* L.) *phyAphyBphyC* mutants lacking any phytochrome and containing the GCC box element, a binding site for rice ethylene-responsive element binding protein 1 (OsEREBP1), in its promoter region. Since the *OsEREBP1*-like gene *EBL1* (*OsEREBP1-LIKE 1*) was significantly downregulated in *phyAphyBphyC* mutants, *EBL1* was suspected to repress *ACO1* expression in wild-type plants. However, *ACO1* was downregulated in *EBL1* RNA interference plants, and the total length of these plants was slightly shorter than that of wild-type plants. This study shows that *EBL1* is positively regulated by phytochrome B and associated with *ACO1* upregulation.

Keywords *ACO1* · Ethylene-responsive element binding protein · Internode elongation · Phytochrome · Rice

Introduction

Red and far-red light-absorbing phytochromes are major photoreceptors that regulate the expression of light-responsive genes, and thus, influence many photomorphogenic events in higher plants. Rice (*Oryza sativa* L.) contains three phytochrome genes, *PHYA*, *PHYB*, and *PHYC*, and *phyAphyBphyC* triple mutants have been isolated and characterized (Takano et al. 2009). Although internodes start to elongate at the reproductive stage, *phyAphyBphyC* mutants have morphological changes and elongated internodes, even at the vegetative stage, indicating that phytochromes play an important role in inhibition of internode elongation at the vegetative stage in wild-type plants.

1-Aminocyclopropane-1-carboxylate (ACC) oxidase (EC 1.14.17.4) catalyzes the final step of ethylene biosynthesis in which the ACC precursor is converted to ethylene. A predominant ACC oxidase gene, ACC oxidase (*ACO1*), has been isolated from submerged internodes of deepwater rice, and high transcript levels are associated with internode elongation during submergence (Mekhedov and Kende 1996). We have recently shown that *ACO1* has an effect on internode elongation at the heading stage in rice (Iwamoto et al. 2010).

Expression of genes for ethylene-responsive element-binding proteins/factors (EREBPs/ERFs) inhibit growth (Hu et al. 2008; Wilson et al. 1996; Xu et al. 2006). We found that the upstream region of *ACO1* (−1,625 to +130, cDNA start site of *ACO1* being designated as +1), having promoter activity (Iwamoto et al. 2010), included a GCC box element (AGCCGCC), which is a binding site for EREBPs/ERFs. It has been reported that rice EREBP gene,

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OsEREBP1, binds to a GCC box element in rice (Cheong et al. 2003).

In this study, we examined an *OsEREBP1*-like gene, *EBL1* (*OsEREBP1-LIKE 1*), selected as a candidate gene associated with the regulation of *ACO1* expression. RNA interference (RNAi) transgenic plants for *EBL1* were generated to elucidate the relevance of *EBL1* to *ACO1* expression.

Materials and methods

Plant materials and growth conditions

Rice (*O. sativa* L. cv. Nipponbare) and its phytochrome-deficient mutants, *phyA-2*, *phyB-1*, *phyA-2phyC-1*, and *phyA-2phyB-1phyC-1*, were used in this study. Seedlings were maintained in a growth chamber at 28°C. White light (160 μmol m^{-2} s^{-1}) was provided by cool-white fluorescent lamps and monochromatic red light (55 μmol m^{-2} s^{-1}) was provided by a light-emitting diode panel (Model LED-R; Eyela). *EBL1* RNAi transgenic plants were grown into mature plants in an isolated glasshouse at 27°C.

Plant transformation

An *EBL1* fragment (441 bp) was amplified from the *EBL1* cDNA clone (002-163-C07), provided by the Rice Genome Resource Center, National Institute of Agrobiological Sciences, Japan, by polymerase chain reaction (PCR) using primers EBL1Ri-F (5′-CAC CAA GTC GAT GCC GAC GAC GAG-3′) and EBL1Ri-R (5′-TGC GAT GAA CTC ATG ACT GAA CAG-3′). PCR products were inserted in the pENTR/D-TOPO cloning vector (Invitrogen) and then in the pANDA vector (Miki and Shimamoto 2004) using the pENTR Directional TOPO Cloning Kit (Invitrogen) and the Gateway LR Clonase Enzyme Mix (Invitrogen), respectively, according to manufacturer's instructions. The resulting plasmids were introduced into *Agrobacterium tumefaciens* EHA105 (provided by Dr. E. Hood, ProdiGene), which was then used for transformation of rice plants according to methods of Hiei et al. (1994) and Toki (1997).

Reverse transcription-PCR (RT-PCR)

Total RNA was extracted from leaf blades using the RNeasy Mini Kit (Qiagen) according to manufacturer's instructions. RT-PCR was performed using one-step reactions (Superscript One-Step RT-PCR system; Invitrogen). Total RNA (50 ng) was used as a template and RT-PCR amplification reaction mixtures contained 0.2 mM of each dNTP, 1.2 mM $MgSO_4$, and 0.4 μM of each primer. The primer sets were ACO1-F (5′-CTG CGG CGA TGG AGC AGC TGG A-3′) and ACO1-R (5′-CAC GAA CTT GGG GTA CGC CAC GA-3′) for *ACO1*, OsEREBP1-F (5′-GAC GAT GAC GTC GTC GAG ATC AAG-3′) and OsEREBP1-R (5′-TGC GAG GAT CTC TGA TTT CAG CAG-3′) for *OsEREBP1* (Os02g0782700), AF190770-F (5′-AGT CCG ACG CCG ACG AGG CCA AG-3′) and AF190770-R (5′-CAC CTT TGC GAG GAT CTC TGA TTT CC-3′) for Os06g0194000, AF364176-F (5′-CTT TGA GGC CGA CTT CCG CGA G-3′) and AF364176-R (5′-CAT GTT GGA CAA GCT TTG TAG TCA AC-3′) for Os09g0434500, EBL1-F (5′-GAT CAG CGA CGA CGA GGA CTT CGA G-3′) and EBL1-R (5′-CTT GAT CGA TCG ATC GCC TCA CCA TG-3′) for *EBL1*, and nucleotides 4,158–4,177 and 4,374–4,355 having accession number AF184280 for the polyubiquitin gene (*RUBQ2*). PCR amplification was performed in a DNA thermal cycler (GeneAmp PCR system 9700, Applied Biosystems). Appropriate number of PCR cycles for each gene was determined prior to RT-PCR experiments. PCR products were electrophoresed on a 1.4% (w/v) agarose gel and visualized using ethidium bromide staining.

Results

Comparison of deduced amino acid sequences of OsEREBP1 and OsEREBP1-like proteins

A homology search was performed to detect genes having a high similarity to *OsEREBP1* using the cDNA sequence of *OsEREBP1* as a query. We detected three *OsEREBP1*-like genes (Os06g0194000, Os09g0434500, and Os10g0390800), and expectation values indicating similarity to *OsEREBP1* were 9e-62, 1e-17, and 1e-08 for Os06g0194000, Os09g0434500, and Os10g0390800, respectively. A multiple alignment of deduced amino acid sequences of OsEREBP1 and the three OsEREBP1-like proteins indicated that two domains in the *N*-terminal region (MCGGAI and EDFEADFEEFE) and an EREBP/AP2 DNA binding domain were highly conserved among the proteins (Fig. 1).

Expression of EREBP genes in phytochrome-deficient mutants

ACO1 is upregulated in *phyAphyBphyC* mutants (Takano et al. 2009). Expression analysis of *OsEREBP1* and the three *OsEREBP1*-like genes was performed to confirm whether any changes were present in their expression in *phyAphyBphyC* mutants. It was shown that transcript levels of Os10g0390800, named *EBL1*, were significantly decreased in *phyAphyBphyC* mutants, while those of *OsEREBP1*, Os06g0194000, and Os09g0434500 were nearly identical between *phyAphyBphyC* and *phyAphyC* mutants (Fig. 2a). phyB is the main photoreceptor responding to red light in rice (Takano et al. 2005). Under continuous red or white light, *phyB* mutants demonstrated significant down-regulation of *EBL1*expression and upregulation of *ACO1* expression (Fig. 2b). On the other hand, *EBL1* and *ACO1* transcript levels in *phyA* mutants were similar to those in wild-type plants under these conditions.

Alteration of *ACO1* expression and internode elongation in *EBL1* RNAi plants

From the results of Fig. 2, we deduced that *EBL1* acted as a repressor to regulate *ACO1* expression and that *EBL1* downregulation led to high *ACO1* transcript levels. To elucidate the role of *EBL1* in the regulation of *ACO1* expression, *EBL1* RNAi transgenic plants were produced and used. All five independent lines of *EBL1* RNAi plants had significantly decreased *EBL1* transcript levels (Fig. 3a). However, *ACO1* was unexpectedly downregulated in all of the *EBL1* RNAi plants. On the other hand, *OsEREBP1*, Os06g0194000, and Os09g0434500 transcript levels were nearly identical between wild-type and *EBL1* RNAi plants. *EBL1* RNAi plants had alterations in internode elongation, and the total length of *EBL1* RNAi plants was slightly shorter than that of wild-type plants (Fig. 3b). In addition, short elongation of the uppermost internode (first internode) caused panicle enclosure in the *EBL1* RNAi plants (Fig. 3c).

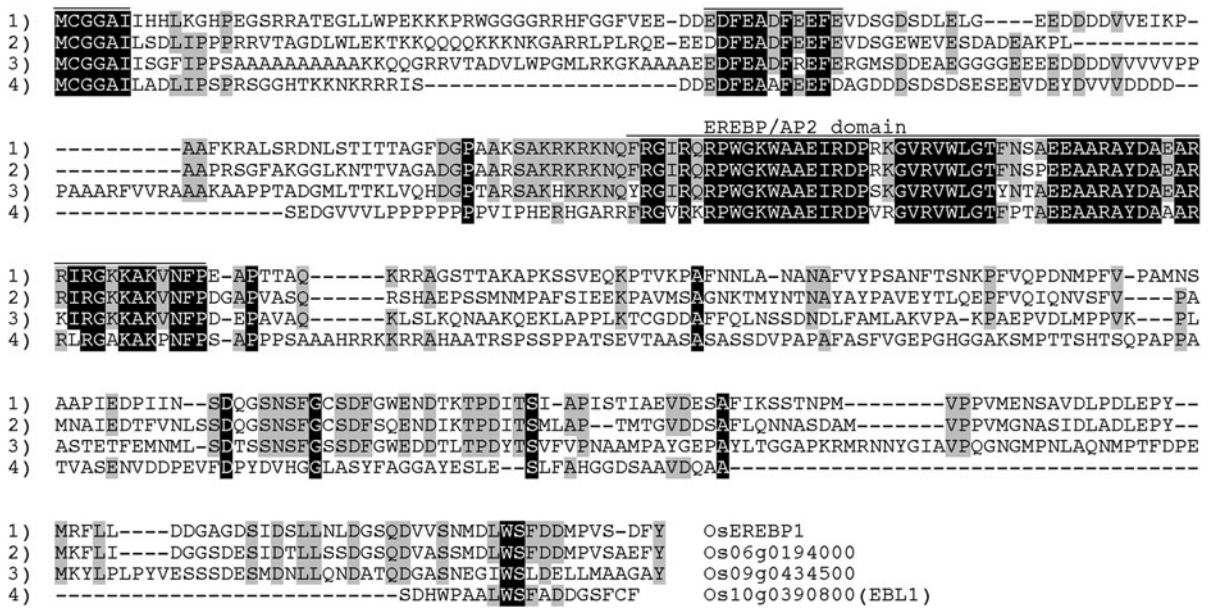

Fig. 1 Alignment of the deduced amino acid sequences of OsEREBP1 and OsEREBP1-like proteins. The amino acids conserved among the four proteins are indicated in *black* and those conserved among three of the four proteins are indicated in *gray*. *Lines* above the amino acid sequences indicate the conserved domains among the four proteins

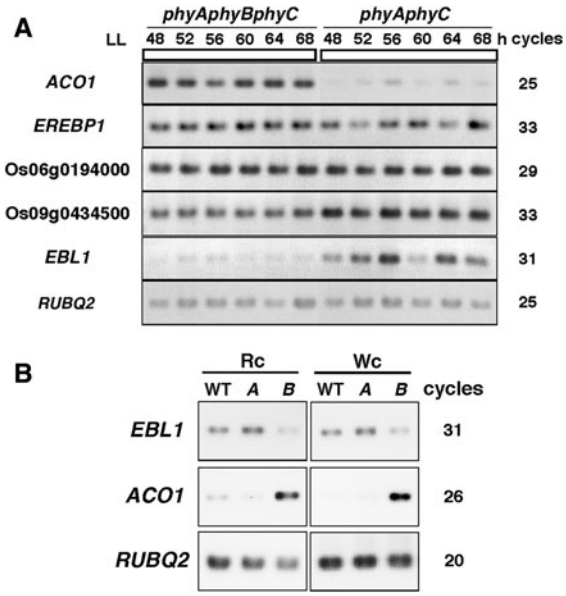

Fig. 2 Changes in *EBL1* expression in phytochrome-deficient mutants. **a** Expression of *OsEREBP1* and *OsEREBP1*-like genes in *phyAphyBphyC* or *phyAphyC* mutants. Seedlings grown under light/dark conditions were transferred to continuous light (LL) conditions at the end of the dark period, and leaf blades were sampled every 4 h. The total number of amplification cycles needed to detect RT-PCR products is indicated. RT-PCR amplification of *RUBQ2* was performed as a loading control. **b** Expression of *EBL1* and *ACO1* in wild-type (WT), *phyA* (*A*), or *phyB* (*B*) mutants under continuous red (Rc) or white light (Wc) conditions. The total number of amplification cycles needed to detect RT-PCR products is indicated. RT-PCR amplification of *RUBQ2* was performed as a loading control

Discussion

In this study, we showed that *EBL1* was a phytochrome-regulated gene that contributed to the regulation of *ACO1* expression. Since *EBL1* was downregulated in mutants lacking functional phyB (Fig. 2), *EBL1* expression was mainly regulated by phyB. It has been reported that EREBP/ERF genes have important roles in the regulation of internode elongation in deepwater rice plants. *SUBMERGENCE 1A-1* confers submergence tolerance and inhibits elongation growth during submergence (Xu et al. 2006) and *SNORKEL1* and *2* contribute to flooding-induced internode elongation and promote elongation during submergence (Hattori et al. 2009). To the best of our knowledge, this is the first report on an EREBP/ERF gene associated with internode

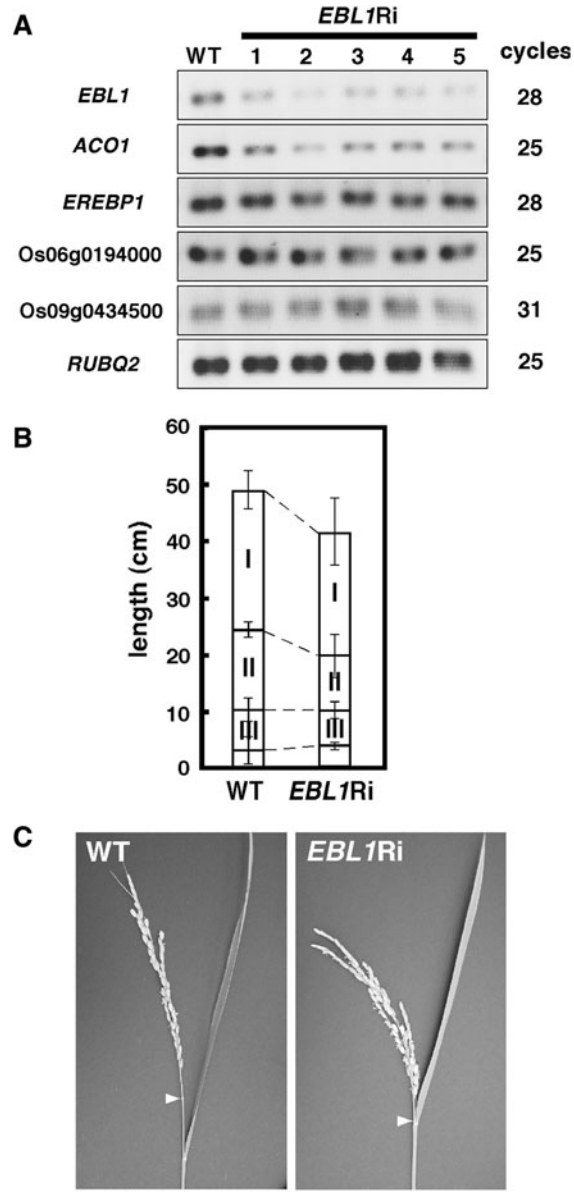

Fig. 3 Characterization of *EBL1* RNAi transgenic plants. **a** Expression of *EBL1*, *ACO1*, *OsEREBP1*, Os06g0194000, and Os09g043500 in leaf blades of wild-type (WT) and five independent lines of *EBL1* RNAi plants (*EBL1*Ri). Plants were grown under light/dark conditions (16 h of light and 8 h of dark). The total number of amplification cycles needed to detect RT-PCR products is indicated. RT-PCR amplification of *RUBQ2* was performed as a loading control. **b** Comparison between elongated internodes of wild-type (WT) and *EBL1* RNAi plants (*EBL1*Ri). I to III indicate the first to third internodes, respectively. Vertical bars indicate SE of the mean ($n = 4$). **c** Panicles of wild-type (WT) and *EBL1* RNAi plants (*EBL1*Ri). *Arrowheads* indicate panicle nodes

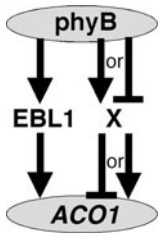

Fig. 4 Model for phyB-regulated *ACO1* expression in rice. EBL1 contributes to *ACO1* upregulation under the positive control of phyB. X represents an unknown transcription factor. The two possibilities of X-mediated *ACO1* regulation are as follows: X downregulates *ACO1* under positive control of phyB or upregulates *ACO1* under negative control of phyB

elongation at the reproductive stage in paddy field rice plants. *EBL1* RNAi transgenic plants incurred panicle enclosure because of short elongation of their first internodes (Fig. 3c). Since *ACO1* has effects on internode elongation (Iwamoto et al. 2010), *EBL1* is believed to be associated with internode elongation by regulating *ACO1* expression.

EREBPs/ERFs act as transcriptional activators or repressors of GCC box-mediated gene expression (Fujimoto et al. 2000). Prior to expression analysis using *EBL1* RNAi plants, we assumed that *EBL1* functioned as a repressor for *ACO1* downregulation in wild-type plants; however, *EBL1* contributed to *ACO1* upregulation, similar to an activator (Fig. 3a). These results indicate that two mechanisms regulate *ACO1* expression under the control of phyB: *EBL1*-dependent and -independent pathways (Fig. 4). The *EBL1*-dependent pathway plays a role in *ACO1* upregulation. The total length of *EBL1* RNAi plants was slightly shorter than that of wild-type plants (Fig. 3b), similar to *ACO1*-deficient plants (Iwamoto et al. 2010). On the other hand, the *EBL1*-independent pathway is necessary for *ACO1* downregulation, and unknown transcriptional factor(s) are believed to regulate *ACO1* expression. The *EBL1*-dependent pathway has a small effect on *ACO1* expression compared to the *EBL1*-independent pathway, since *ACO1* transcript levels in plants lacking phyB were higher than those in plants with phyB (Fig. 2). Further experiments are required to understand the transcriptional factor(s) associated with *ACO1* downregulation under the control of phyB.

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