Running title: Rice ERF OsEATB restricts GA biosynthesis

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Journal research area: Development and Hormone Action
Rice ethylene-response AP2/ERF factor OsEATB restricts internode elongation by down-regulating a gibberellin biosynthetic gene

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This work was supported by a grant from the Major State Basic Research Development Program of China (973 Program) (No. 2007CB109002), the Program of Conservation and a grant from the National High Technology Research and Development Program of China (863 program) (NO.2006AA10A102).

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ABSTRACT

Plant height is a decisive factor in plant architecture. Rice (Oryza sativa) plants have the potential for rapid internodal elongation, which determines plant height. A large body of physiological research has shown that ethylene and gibberellin (GA) are involved in this process. The AP2/ERF family of transcriptional factors (TFs) is only present in the plant kingdom. This family has various developmental and physiological functions. A rice AP2/ERF gene OsEATB (Oryza sativa ERF protein associated with tillering and panicle branching) was cloned from O. sativa L. subsp. Indica var. 9311. Bioinformatic analysis suggested this ERF has a potential new function. Ectopic expression of OsEATB showed that the crosstalk between ethylene and gibberellin, which is mediated by OsEATB, might underlie differences in rice internode elongation. Analyses of gene expression demonstrated that OsEATB restricts ethylene-induced enhancement of gibberellin responsiveness during the internode elongation process by down-regulating the gibberellin biosynthetic gene, ent-kaurene synthase A. Plant height is negatively correlated with tiller number, and higher yields are typically obtained from dwarf crops. OsEATB reduces rice plant height and panicle length at maturity, promoting the branching potential of both tillers and spikelets. These are useful traits for breeding high-yielding crops.
INTRODUCTION

The AP2/ERF family of transcription factors, which is only present in the plant kingdom, is characterized by the presence of a highly conserved DNA-binding domain (Riechmann et al., 1998). Members of the AP2/ERF family have various developmental and physiological functions. On the basis of the number and sequences of their AP2 domains this large gene family can be divided into five subfamilies (Sakuma et al., 2002). Among which, the ERF subfamily has a single AP2 domain and shows DNA-binding activity. Some ERF proteins have been shown to bind specifically to the GCC-box (AGCCG GCC), which is an ethylene-responsive element (ERE). The binding of ERF proteins to the GCC-box regulates ethylene-responsive transcription (Ohme-Takagi and Shinshi, 1995; Fujimoto et al., 2000). Recently, some ERF proteins were reported to have roles in biotic and abiotic stress responses of plants (Thara et al., 1999, Shinozaki et al., 2003). In rice, most ERF genes that have been identified and isolated are induced under abiotic stress conditions (Cao et al., 2006).

Rice (Oryza sativa) is a semiaquatic plant with a strong capacity for rapid internode elongation to facilitate aeration of submerged organs (Kende et al., 1998). The potential for rapid internodal elongation develops with the differentiation of each internode (Metraux and Kende, 1983). This growth response is mediated by at least two interacting hormones, namely ethylene and gibberellin (GA) (Fukao and Bailey-Serres, 2008). Ethylene triggers this rapid process, and increased responsiveness to GA is promoted by an increase in ethylene (Hattori et al., 2009). A number of genes encoding GA biosynthetic or signaling pathways have been identified as being associated with these processes (Yang and Hwa, 2008). ent-Kaurene oxidase (KO2), which encodes an enzyme involved in GA biosynthesis, is down-regulated in the gibberellin-deficient dwarf1 (gdd1) mutant (Li et al., 2011). The GIBBERELLIN INSENSITIVE DWARF1 (GID1) gene in rice encodes a soluble
receptor that mediates GA signaling (Ueguchi-Tanaka et al., 2005), and a mutation at this locus causes semi-dwarfism. Some rice ERFs were reported to have roles in regulating internode elongation. The deepwater rice ERF genes \textit{SNORKEL1} and \textit{SNORKEL2} trigger deepwater rapid internodal elongation via GA responses in response to ethylene signaling (Hattori et al., 2009). Another ERF gene, \textit{SUB1A}, restricts plant elongation at the seedling stage during flash floods (Xu et al., 2006). Further investigations of the molecular intricacies of the related signaling pathways are still required.

Rice plant height is an important agronomic trait linked to yield potential (Yang and Hwa, 2008; Huang et al., 1996). Greater production of rice is required to satisfy the growing food demands of the ever-increasing population (Sasaki et al., 2005). Yield is one of the most important and extensively investigated agronomical traits for rice productivity (Xue et al., 2008). The important components to determine rice yield are the number of panicles per plant, the number of spikelets per panicle, and grain weight. Although AP2/ERF proteins play an important role in plant development and physiology, little is known about the functions of AP2/ERFs in rice yield traits.

In this study, we isolated a rice AP2/ERF gene, \textit{OsEATB} (\textit{Oryza sativa} ERF protein associated with tillering and branching) from \textit{O. sativa} L. \textit{subsp. Indica var.} 9311. Transformation experiments demonstrated that \textit{OsEATB} influences rice plant height and branching, and therefore, affects plant architecture and yield. \textit{OsEATB} transgenic lines showed dwarf phenotypes, indicating that the internodal elongation process was suppressed by overexpression of \textit{OsEATB}. Our experimental results revealed that the rice ethylene-response AP2/ERF factor \textit{OsEATB} restricts internode elongation by down-regulating a gibberellin biosynthetic gene.

**RESULTS**

Features of rice \textit{OsEATB}
There are 161 known AP2/ERFs in *O. sativa* L. *subsp. Indica*, 103 of which are potential ERFs with one single complete AP2 domain (http://plntfdb.bio.uni-potsdam.de). Of these 103 potential ERFs, 38 cannot be classified into any of the four functional subgroups (Cao et al., 2006), for they lack typical group motifs. We selected *OsEATB* from these 38 ERFs for further investigation, because like the members of subgroup IV, it contains a nuclear localization signal (NLS) adjacent to the highly conserved AP2 domain (Fig. 1A, see Supplemental Figure S1 online). Multiple sequence alignment of *OsEATB* with other known ERF proteins showed that their similarity was restricted to the DNA-binding domain region, and *OsEATB* could not be classified into any of the ERF subgroups with previously defined functions (Fig. 1B). We inferred that *OsEATB* is a rice ERF that might have a potential new function.

Based on the data from NCBI GenBank database (http://www.ncbi.nlm.nih.gov/), *OsEATB* has no intron, and exists as a single copy gene. Using PCR, we isolated and cloned its full-length 825-bp open-reading frame (ORF) from genomic DNA of *O. sativa* L. *subsp. Indica var. 9311. Sequence data for this article have been deposited at GenBank under Accession no. EU622934. The 825-bp ORF encodes a protein consisting of 274 amino acids. We used the yeast one-hybrid system and electrophoretic mobility shift assays (EMSAs) to examine the binding activity of *OsEATB* to the GCC-box. As shown in Fig. 1C, the first section of each plate shows the yeast reporter strain harboring pHIS-GCC-box and pGAD-*OsEATB*, while the second section shows yeast cells harboring pHIS-muGCC-box and pGAD-*OsEATB*. The third section of each plate shows yeast cells transformed with pHIS-GCC-box and the negative control vector pGAD242, and the fourth section shows yeast cells transformed with pGAD-*OsEATB* and negative control vector pHIS2.1. The yeast reporter strain containing pHIS-GCC-box and pGAD-*OsEATB* grew well on both SD/Trp-Leu- medium and SD/Trp-Leu-His-/50mM 3-AT medium. The other three yeast reporter strains...
grew well on SD/Trp-Leu- medium, but not on SD/Trp-Leu-His-/50mM 3-AT medium. The band corresponding to the GCC-box-containing labeled probe and purified His fusion recombinant OsEATB protein complex showed a marked mobility shift compared with the free probe band (Fig. 1D). And mGCC-box-containing labeled fragment was served as competitor. These results demonstrated that OsEATB could specifically bind to the GCC-box.

If OsEATB acts as a transcription factor, then its NLS should localize it to the nucleus. To confirm the subcellular localization of OsEATB, we fused the coding sequence of OsEATB with that of green fluorescent protein (GFP) under the control of the 35S promoter (35S:OsEATB:GFP) and delivered the construct into onion epidermal cells by particle bombardment (Fig. 1E). The OsEATB protein localized the GFP signal to the nucleus (Fig. 1F). We investigated the tissue expression pattern of OsEATB by reverse transcriptase (RT)-PCR using rice total mRNA from roots, culms, leaves, and young panicles as the template. The results showed that OsEATB mRNA is expressed constitutively in these four tissues and is expressed at higher levels in roots and leaves than in culms and young panicles (Fig. 1G).

Expression of OsEATB in transgenic rice lines
To investigate the function of OsEATB, we introduced the plasmid 35S:OsEATB containing the OsEATB gene (Fig. 2A) into O. sativa L. subsp. Indica var. 9311. We produced transgenic plants over-expressing the sense strand of OsEATB. 35S:OsEATB transformants were screened on antibiotic selection medium containing hygromycin. The transgenic plants were checked by PCR using genomic DNA as the template and specific primers (as described in materials and methods). Eight independent transformants (T0), regenerated from hygromycin-resistant calli, were shown to contain OsEATB. Eight PCR-positive T1 lines (20 individual plants of each line) shared the same dwarf phenotype. Two transgenic lines (401003 and 401006) were selected for Southern hybridization analysis. A single, specific band of OsEATB transgene
was observed in 35S:OsEATB transgenic lines 401003 and 401006, respectively (Fig. 2B). These results showed that OsEATB was integrated into the rice genome.

The expression level of the OsEATB gene was examined in 3-leaf-stage transgenic plants and control plants by real-time PCR analyses (Fig. 2C). Expression of OsEATB was 9.77-fold greater in the 401006 transgenic plants, and 4.00-fold, 2.83-fold, 2.00-fold greater in the 401003, 401005, 401007 transgenic plants than in 9311 control plants, respectively. These results were further confirmed by RNA hybridization in situ of the 3-leaf-stage 401003 transgenic plants and 9311 control plants (Fig. 2D). All of these results demonstrated that the OsEATB gene is strongly expressed and genetically transmitted to the next generation.

**Effect of transgenic OsEATB on rice internode elongation**

Plant height is not only a decisive factor in plant architecture, but also an important agronomic trait that is directly linked to yield potential (Yang and Hwa, 2008; Huang et al., 1996). The OsEATB transgenic lines showed dwarf phenotypes, indicating that the internodal elongation process was suppressed by OsEATB over-expression. For experiments, we selected two transgenic lines: 401003 and 401006 (20 individual plants of each line). The transgenic plants and 9311 control plants were cultivated in the same conditions and plant height was determined at maturity. The average plant heights of the control, 401003, and 401006 lines were 121, 113, and 106 cm, respectively. Thus, 401003 and 401006 plants were 6.61 and 12.40% shorter than the control, respectively (Fig. 3A, see Supplemental Table S1 online). Furthermore, every elongated internode was shortened, especially the fourth internode, which was shortened by an average of 56.30% (Fig. 3A). OsEATB negatively regulated plant height at every growth stage of rice plants. The transgenic plants showed a dwarf phenotype from the 3-leaf-stage to the mature stage (Fig. 3B, Fig. 6A and 6B). The panicles also showed reduced elongation, demonstrating that
panicle internodes were shortened as well (Fig. 6C).

The plant hormone GA is involved in deepwater responsive internode elongation (Raskin and Kende, 1984). A number of genes encoding GA biosynthetic or signaling pathways have been identified as being associated with this rapid process (Yang and Hwa, 2008). To examine the regulation of GA sensitivity in OsEATB transgenic lines, we treated the wild-type and transgenic seedlings with 10 μM, 50 μM, and 100 μM GA₃ at the 4-leaf-stage. Under these conditions, both seedlings showed rapid elongation after the 24-h treatments, and OsEATB transgenic seedlings did not show negative sensitivity to GA treatments compared with the control seedlings during this process (Fig. 3B and 3C). Together, these results suggested that OsEATB negatively regulates the elongation process of every internode, in every organ, at every growth stage, and that the responsiveness of transgenic seedlings to GA is not impaired. Many GA-responsive dwarf plants that are deficient in the biosynthesis of active GAs have been characterized in various plant species (Ross et al., 1997; Hiroshi et al., 2004). These findings, together with our results, led us to conclude that there is negative regulation of GA biosynthesis in the transgenic plants.

To test this hypothesis, we used microarray analysis to compare expression of GA biosynthetic genes between OsEATB transgenic 401006 seedlings and control seedlings at the 4-leaf-stage. ent-Kaurene is an early intermediate in the GA biosynthesis pathway: ent-kaurene synthase A (CPS) catalyzes the cyclization of Geranylgeranyl diphosphate (GGDP) to ent-copalyl diphosphate (CDP), which is then converted to ent-kaurene by ent-kaurene synthase B (KS) (see Supplemental Figure S2 online) (Shinjiro et al., 1998). The microarray analysis results showed that the expression of rice ent-kaurene synthase A OsCPS2 in OsEATB transgenic seedlings was sharply down-regulated (10.32-fold less than the expression level in 9311 wild-type seedlings). In contrast, the expression of GA20ox2 was 2.77-fold
greater in *OsEATB* transgenic seedlings than in 9311 wild-type seedlings (Table I). Gibberellin 20-oxidase (*GA20ox*) is one of the major GA biosynthetic genes and the levels of GAs are homeostatically modulated through negative feedback regulation of *GA20ox* expression (Xu et al., 1995). In addition, no significant differences were found between the *OsEATB* transgenic seedlings and wild type in expression of *OsCPS1*, *OsKS1*, *OsKO2*, and *OsKAO*. To confirm these results, we used real-time PCR analysis to examine the expression of *OsCPS2* and *GA20ox2* genes in 4-leaf-stage transgenic line 401006 and 401003 plants and control plants (Fig. 3D). Expression of these two GA-biosynthesis-related genes was similarly affected by overexpression of *OsEATB* to the microarray results. We also analyzed the expression of *OsCPS1*, *OsKS1*, *OsKO2*, and *OsKAO*, and in agreement with the microarray data, no significant differences in expression of these genes were found (Fig. 3E).

**Effect of rice *OsEATB* on GA biosynthesis during internode elongation**

To examine the negative regulatory relationship between *OsEATB* and *OsCPS2* during internode elongation, we examined the expression of these two genes in elongating internodes of 64-day-old to 73-day-old 9311 plants (Fig. 4A). In elongating internodes of 64-day-old plants, *OsEATB* was expressed at a high level while *OsCPS2* was expressed at much lower level. The expression of *OsEATB* sharply decreased 3 days later, while that of *OsCPS2* increased approximately 10-fold. Expression of *OsEATB* was remarkably decreased from 67 to 73 days, while expression of *OsCPS2* was slightly increased and then remained almost stable.

We compared gene expression in elongating internodes of 67-day-old 401006 transgenic and control plants using real-time quantitative PCR analysis. The genes examined were involved in the GA biosynthesis and GA signaling pathways (Fig. 4B). Firstly, we reexamined expression of *OsCPS2*
and GA20ox2 at this growth stage. In elongating internodes of transgenic plants, OsCPS2 was down-regulated by 11.21-fold, while GA20ox2 was up-regulated by 2.03-fold, compared with their respective expression in the wild-type plants. In rice, the expression levels of XET (xyloglucan endotransglycosylase) and UROD (uroporphyrinogen decarboxylase) are up-regulated by GA treatment, while that of GOX (glycolate oxidase) is down-regulated (Yang et al., 2004). We investigated the effect of OsEATB on the expression of these three genes. Transgenic lines overexpressing OsEATB showed decreased expression of XET and UROD, but slightly increased expression of GOX, compared with their respective expression in the wild-type. These findings suggested that the GA response was suppressed in the transformants possibly due to the lack of bioactive GAs. SLENDER RICE1 [SLR1], the only DELLA protein in rice (Achard and Genschik, 2009), down-regulates the gibberellin signaling pathway (Fukao and Bailey-Serres, 2008). We evaluated the effect of OsEATB overexpression on this gene, and found that expression of SLR1 was down-regulated in transgenic plants. Together, all of these results confirmed that OsEATB negatively regulates the internode elongation process, not via down-regulating GA sensitivity, but through restricting GA biosynthesis.

**Over-expression of OsEATB decreases the endogenous GA level in rice**

*ent*-Kaurene synthase A catalyzes the cyclization of GGDP to CDP, which is then converted to *ent*-kaurene before going through a series of processes to produce GA and various GA derivatives. Since OsEATB represses expression of OsCPS2, we determined the endogenous GA level in 3-leaf-stage 401006 OsEATB transgenic plants and 9311 control plants, 6-leaf-stage 401006, 401003 OsEATB transgenic plants and 9311 control plants. As shown in Table II, the endogenous levels of all GAs tested (i.e. GA_{12}, GA_{53}, GA_{24}, GA_{19}, GA_{9}, GA_{20}, GA_{4}, and GA_{1}) were significantly lower in OsEATB transgenic plants.
than in the wild-type, while the levels of ABA and IAA were not significantly changed. Therefore, GA levels are decreased along with the suppression of OsCPS2 expression (see Supplemental Figure S2 online).

**OsEATB expression is negatively regulated by ethylene, ABA, and abiotic stress in rice**

OsEATB binds to the ethylene-responsive element, the GCC-box, resulting in decreases in the levels in GAs. While increased responsiveness to GA is often associated with an increase in ethylene (Hattori et al., 2009). Differential expression of OsEATB was observed in 18-day-old (3-leaf-stage) rice 9311 seedlings in response to 50 μM ethephon treatment (Fig. 5A). Ethephon treatment sharply decreased expression of OsEATB, and expression remained at low levels for 24 h after treatment. Because the expression level of OsEATB was down-regulated by ethephon treatment, and OsCPS2 is decreased by OsEATB, we examined the effect of ethylene treatment on OsCPS2. Real-time quantitative PCR analysis was used to investigate expression of OsEATB and OsCPS2 at 0, 3, 6, 12, and 24h h of ethephon treatment (Fig. 5B). Expression of OsCPS2 increased while that of OsEATB was sharply down-regulated by the treatment. Nevertheless, after 12 h treatment the expression of OsCPS2 was stabilized to normal level might via the self-balance regulation of other pathways for it is a key enzyme in GA biosynthesis. This result indicated that an increase in the amount of OsCPS2 in response to ethylene is correlated with the decrease in OsEATB expression responding to ethylene.

In rice, most ERF genes are induced by abiotic stress conditions (Cao et al., 2006). To investigate the effect of transgenic OsEATB on the response to abiotic stress in rice, 18-day-old seedlings of 9311 control and transgenic 401006 plants were treated with 200 mM NaCl or 100 μM ABA (Fig. 5C and 5D). The transgenic seedlings treated with 200 mM NaCl showed wilting and rolling of leaves, while 9311 control plants showed a significantly higher
survival rate (Fig. 5C). After 48 h of stress followed by 48 h of watering, almost all of the transgenic plants never recovered, whereas 42% of the control plants survived. The transgenic seedlings showed stronger chlorosis in response to the 100 \( \mu \text{M} \) ABA treatment, while the chlorosis of 9311 control line was slower (Fig. 5D). Thus, over-expression of \emph{OsEATB} made seedlings hypersensitive to NaCl and ABA. We also analyzed whether expression of \emph{OsEATB} was regulated by these two conditions. Seedlings (18-day-old) of 9311 subjected to a salt treatment (200 mM NaCl in Hoagland’s solution) showed rapid down-regulation of \emph{OsEATB} expression within 24 h after the treatment, compared with that in the water-treated control plants (Fig. 5A). Moreover, expression of \emph{OsEATB} was slightly decreased in 18-day-old 9311 seedlings in response to ABA treatment (100 \( \mu \text{M} \) ABA in Hoagland’s solution), compared with that in the water-treated control plants (Fig. 5A). \emph{OsERF3} is up-regulated by ethylene treatment (Jung et al., 2010), while ABA treatment induces expression of \emph{CPD} (Zhang et al., 2008). Therefore, we chose \emph{OsERF3} as a positive control for ethylene responsiveness and \emph{CPD} as a positive control for ABA responsiveness. These results strongly suggest that \emph{OsEATB} is responsive to ethylene and environmental stresses, and therefore, may be involved in regulation of ethylene-related responses.

**Effect of transgenic \emph{OsEATB} on rice tillering and panicle branching**

To investigate the function of rice \emph{OsEATB} on yield traits, we analyzed traits in two transgenic lines (401003 and 401006; 20 individual plants of each line). The transgenic lines and 9311 control plants were cultivated in the same conditions, and we evaluated four yield components; panicles per plant, panicle length, spikelets per panicle and grain number per panicle. The transgenic plants produced more tillers than the control 9311 lines at the 6-leaf stage (Fig. 6B). In rice, the tiller is a specialized grain-bearing branch that forms on the unelongated basal internode. The tiller grows independently of the mother stem (culm) by means of its own adventitious roots (Li, 1979). At
maturity, transgenic plants had produced 16.95% more panicles than wild-type plants (Fig. 6A and 6D; see Supplemental Table S1 online).

Spikelets are grass-specific, flower-bearing branches that form on each panicle branch. They consist of primary, secondary, and sometimes a higher order of panicle branches (Furutani et al., 2006). We counted the spikelets per panicle in transgenic plants and wild-type lines. The 35S:OsEATB transformants produced more spikelets than control plants (Fig. 6C and Fig. 6E). We also counted the numbers of the primary and secondary branches per panicle. Transgenic lines showed 8.31% more primary spikelets per panicle and 34.41% more secondary branches per panicle compared with the wild-type (Fig. 6D; see Supplemental Table S1 online). The finding that 35S:OsEATB transformants produced more secondary branches, more spikelets per panicle, and more panicles per plant suggested that OsEATB activity is responsible for branching in rice.

To investigate the effects of OsEATB on rice grain weight, we examined the 1000-grain weight at random and observed a slight change. The 1000-grain weight of the control and the transgenic lines is about 30.0g and 25.5g, respectively. Average numbers of grains per plant of the control, 401003 and 401006 lines were 776, 1096 and 1254. The calculated grain weights per plant were about 23.28g, 27.95g and 31.98g, respectively. The results indicated that 401003, 401006 possess an increase in grain yield per plant over the control of 20.06% and 37.37% (Fig. 6E; see Supplemental Table S1 online).

**DISCUSSION**

**OsEATB is a novel rice ethylene-response AP2/ERF factor**

ERFs are an important subfamily of AP2/ERF transcription factors, and they have a variety of functions. Several rice ERF genes have been isolated and identified; for example, OsEBP-89 regulates transcription of the rice Wx gene
(Zhu et al., 2003), and OsEREBP1 is related to regulation of defense responses (Cheong et al., 2003). In the present study, we investigated and confirmed the function of a rice AP2/ERF gene, OsEATB. ERFs can be classified into different subgroups based on their function and group motif. Subgroup I, which includes Arabidopsis AtERF1 and AtERF2 and tomato Pti4, functions as transcriptional activators. Subgroup II, which includes tobacco NtERF3 and Arabidopsis AtERF3 and AtERF4, functions as transcriptional repressors. However, the functions of ERF subgroups III and IV remain unclear (Cao et al., 2006). OsEATB cannot be classified into any of the function-known ERF subgroups (Fig. 1). Our data show that OsEATB encodes a transcriptional factor that localizes to the nucleus and is constitutively expressed in various tissues (Fig. 1). Experimental data from transgenic plants showed that OsEATB plays a crucial role in regulating the rice internode elongation process (Fig. 2, 3 and 4).

Ethylene is perceived by a family of histidine kinase-like receptors, and downstream, by EIN2-a novel protein containing an integral membrane domain. In the nucleus, the EIN3 family of DNA-binding proteins regulates ethylene-responsive transcription, and an immediate target of EIN3 is the AP2/ERE family (Chang and Shockey, 1999). As a result, most ERFs are regulated by ethylene. Many ERF proteins have been shown to bind to the specific ethylene-responsive element, the GCC-box. In our study, we examined the binding activity of OsEATB to the GCC-box (Fig. 1). At the same time, we found that the expression level of OsEATB was sharply decreased by ethylene (Fig. 5). Consequently, our results show that this gene is involved in regulation of ethylene-related responses. OsEATB over-expression dramatically suppressed the internode elongation process (Fig. 3) and made seedlings hypersensitive to NaCl and ABA. In addition, the expression of this gene was down-regulated by both NaCl and ABA (Fig. 5). Ethylene is mostly reported to trigger the internodal elongation process via GA (Fukao and Bailey-Serres, 2008; Hattori et al., 2009) and many ERF genes are induced by
abiotic stress conditions in rice (Cao et al., 2006; Thara et al., 1999). Thus, rice OsEATB might be a negative balance regulator of the ethylene-responsive pathway.

**OsEATB negatively regulates ethylene-induced enhancement of GA responsiveness by reducing GA biosynthesis**

Plant height is a decisive factor in plant architecture. In rice, there is the potential for rapid internodal elongation, and the degree of elongation determines plant height. This rapid growth response is best demonstrated in deepwater rice, especially in the process of submergence tolerance (Kende et al., 1998). Sub1A is an ERF that confers submergence tolerance to rice. This tolerance is mediated by SLR1, which restricts the response to gibberellin. Sub1A augments SLR1 and SLRL1 gene expression, which counteract the elevated responsiveness to GA promoted by the increase in ethylene (Xu et al., 2006; Fukao and Bailey-Serres, 2008). The ERF genes SNORKEL1 (SK1) and SNORKEL2 (SK2) allow rice to adapt to deep water. Under deepwater conditions, ethylene accumulates in the plant and induces expression of these two genes. These SK genes encoding ERFs trigger internode elongation in deepwater rice via GA. In contrast to Sub1A, SK1 and SK2 may stimulate GA responses (Hattori et al., 2009). Both SK genes and SUB1A encode ERFs and are related to GA, but they have opposing functions in regulating plant height in response to flooding. It is interesting that gene family members in the same subgroup confer different functions related to internode elongation. In conclusion, there is a self-balance of ethylene-induced enhancement of the GA response during the internodal elongation process.

It is possible that crosstalk mediated by OsEATB between ethylene and GA underlies differences in rice internode elongation. The OsEATB transgenic seedlings did not show negative sensitivity to GA treatments (Figure 3), and the expression level analysis of the genes involved in GA biosynthesis and GA signaling pathways showed that the GA response is suppressed via
down-regulating a key enzyme involved in GA biosynthesis, not through up-regulating the important GA sensitivity repressor (Figure 4). The Arabidopsis GA-deficient mutant dwarf and delayed-flowering (ddf1) phenotypes are caused by increased or ectopic expression of a putative AP2 transcription factor DDF1. DDF1 is involved in the down-regulation of GA biosynthesis (Hiroshi et al., 2004). As a potential negative balance regulator of the ethylene-responsive pathway, OsEATB suppresses the internode elongation process through restriction of GA biosynthesis, specifically down-regulating the expression of OsCPS2 (Table I, Fig. 3). The mutation line of ent-kaurene synthase A OsCPS1 showed a dwarf phenotype without flower or seed development, which is a typical phenotype of GA-deficient rice dwarf mutants (Tomoaki et al., 2004). Evidence is presented indicating that OsCPS2 is involved in related secondary metabolism producing defensive phytochemicals (Slajdha et al., 2004). The expression of OsCPS2 is sharply negative-related with OsEATB expression, and the endogenous GA level is decreased in OsEATB transgenic plants alongside suppressed expression of OsCPS2 (Table II). No significant differences between the OsEATB transgenic seedlings and wild type in expression of OsCPS1, OsKS1, OsKO2, and OsKAO were found, and the expression of GA20ox2 was slightly up-regulated for the feedback of GA deficiency. All these experimental results strongly demonstrated that OsCPS2 is also associated with GA biosynthesis. Promoter regions (1,300 bp upstream of the translation site) of OsCPS2 do not contain the GCC-box motif, suggesting that this gene may not be a direct target of OsEATB. We propose a model for OsEATB-dependent hormonal regulation of internode elongation in rice (Fig. 7). It was suggested that the important function of OsEATB is to negatively regulate ethylene-induced enhancement of GA responsiveness during the internode elongation process by decreasing GA biosynthesis.
*OsEATB* regulates rice yield components through promotion of tillering and panicle branching

Food security for the ever-increasing world population largely relies on the grain yield of crop plants (Xue et al., 2008). The critical components to determine rice yield include the grain number and grain weight. Grain number is contingent on the number of spikelets per panicle and the number of panicles per plant. The number of panicles is mainly determined by the plant architecture and the spikelets per panicle by panicle morphology (i.e. the number of primary/secondary/tertiary branches on each panicle) (Zha et al., 2009). Over-expression of *OsEATB* decreased plant height (Fig. 3), and increased the numbers of panicles per plant and spikelets per panicle (Fig. 6). More panicles and spikelets resulted in a 37.37% increase in grain yield notwithstanding the 1000-grain weight was 15.00% lower in transgenic lines than the control (Fig. 6). The results of this study show that *OsEATB*, as a member of AP2/ERF family, positively regulates rice yield components through promotion of rice tillering and panicle branching.

The basic structure of a rice panicle is determined by the pattern of branch formation. In rice, the *MONO CULM 1 (MOC1)* and *LAX PANICLE (LAX)* genes are necessary for branch meristem formation (Furutani et al., 2006). *MOC1* positively regulates tillering by promoting axillary meristem outgrowth (Li et al., 2003; Leyser, 2005). Although the molecular mechanisms that underlie the crosstalk between plant height and branching are poorly understood, it is well known that rice plant height is strongly negatively correlated with tiller number (Hong et al., 2003; Booker et al., 2004). Higher yields are typically obtained from dwarf crops (Spielmeyer et al., 2002). The finding that transgenic rice plants harboring the *MOC1* gene are dwarf but produce more tillers than wild-type plants (Li et al., 2003) provides a good opportunity to investigate the genetic control network. Our results indicate that over-expression of *OsEATB* gene (Fig. 2) reduces rice plant height and panicle length at maturity, promoting rice branching potential in both tillers and
spikelets, possibly via regulation of both shoot elongation and axillary outgrowth. The short stature reflects the decreased growth of the mother stem, which allows growth of more tillers, leaves, and panicles, and enhances the energy utilization ratio and biomass production. The functions of OsEATB in regulating rice plant architecture include its effects on plant height (decreased internode elongation) and panicle morphology (increased tiller formation).

Studies on the rice AP2/ERF gene OsEATB provide an opportunity to identify agriculturally important functions that can be used to improve rice yield components. In addition, this gene provides a model for investigating crosstalk between ethylene and GA in the internodal elongation process.

**MATERIALS AND METHODS**

**Plant material and transformation**

We used the binary plasmid vector pCAMBIA1304 (Center for the Application of Molecular Biology of International Agriculture, Canberra, ACT, Australia), which carries the kanamycin resistance gene (Kan') for bacterial selection and the hygromycin phosphotransferase gene (hyp) for selection of transformed plants. The binary plasmid was constructed by inserting the cDNA of the rice OsEATB gene between the Bgl II and Pml I sites in the sense orientation driven by the CaMV 35S promoter and Nos-3' terminator (Fig. 2). Mature seeds of rice (*O. sativa* L. subsp. *Indica* var. 9311) were dehusked and surface-sterilized by immersion in 70% (v/v) ethanol for 10 min, followed by washing with sterile distilled water. Seeds were then soaked in 0.1% (w/v) HgCl₂ for 20 min with regular shaking, rinsed with several changes of sterile distilled water, dried on sterilized filter paper, and then sown on medium for induction of embryogenic callus (Attia et al., 2005). Approx. 30 pieces per dish were selected for two rounds of bombardment. Successfully transformed calli were selected by culturing on selection medium containing 50 mg L⁻¹ hygromycin for 2 weeks. Drug-resistant calli were transferred into regeneration medium to grow into plants in the greenhouse.
Phenotype analysis of transgenic rice

Primary transformants (T0 generation) were grown in the greenhouse at 28°C under a 14-h light/10-h dark photoperiod. Seeds from eight T0 lines and equivalent control plants were collected and germinated by soaking in water for 2 d at 37°C. Germinating seeds were sown in pots and grown in the greenhouse as described above. At the 5-leaf stage, 20 seedlings of these T1 (first transgenic progeny) lines and equivalent control plants were transplanted into larger pots under the same conditions.

PCR and Southern blot analyses to identify transgenic plants

Genomic DNAs were extracted from leaf tissues of wild-type and transgenic rice and used as templates for PCR as described elsewhere (Sheu et al., 1996). In the transgenic lines, the primers 5’-GGGTAGTCATCAGGCTCCG-3’ (forward) and 5’-GCCGTTTCTTTGGCTGGG-3’ (reverse) were used for amplification. For Southern hybridization, 20 μg total genomic DNA from leaf tissue of 401003, 401006 transgenic and control plants was digested with appropriate restriction endonucleases EcoR I. DNA fragments were separated by electrophoresis on 1% (w/v) agarose gels, and then transferred to nylon membranes (Amersham Bioscience) according to standard protocols.

RT-PCR and real-time quantitative analysis

Total RNAs were extracted from rice using the RNAprep Plant kit (Tiangen, China). First-strand cDNA was generated using Primerscript RT reagent (perfect real time) (TaKaRa, Japan). The cDNAs of interest were specifically amplified with the following primers: OsEATB-S (5’-GAACGACCTCAAGCACACTAC-3’) (forward) and OsEATB-A (5’-AAGTCGCACCCGAGACAG-3’) (reverse). The PCR conditions were as follows: amplification at 95°C for 5 min, followed by 26 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 1 min, and then final extension at 72°C for 10 min. As a control for RT-PCR, a 500-bp cDNA fragment encoding rice Actin was amplified with the primers OsActin-S (5’–TCCATCTTGGCATCTCTCAG-3’) (forward) and OsActin-A (5’-CATGGGAGTAGTCCGCTAGAC-3’) (reverse). PCR products were verified by sequencing, fractionated on 1% agarose gels, and stained with ethidium bromide.
In this experiment, real-time quantitative PCR analysis was carried out using a BioRad iCycler IQ Real-Time PCR Detection System (Bio-Rad, USA) and a Primerscript RT reagent (perfect real time) kit (TaKaRa, Japan). Each 25 µl reaction mixture contained 1 × SYBR Premix ExTaq, 0.5 µl cDNA template, and 200 nM gene-specific primers. Reactions were run in duplicate under the following conditions: 40 cycles of 95°C for 5 s, 61°C for 20 s, after an initial activation step at 95°C for 10 s, according the manufacturer's instructions. Melting curves and standard curves were calculated and analyzed for OsEATB and Actin. The relative mRNA expression levels were normalized against Actin gene expression levels.

Yeast one hybrid assay

The full open reading frame (ORF) of OsEATB was generated by PCR using the primers OsEATB-AD-S (5’-CATATGAACGACCTCAAGCACAATAG-3’) (forward) and OsEATB-AD-A (5’-GGATCCGCCTCCAGTAATCTAATCCAAAC-3’) (reverse). The ORF was then fused in-frame into pGAD424 to construct pGAD-OsEATB. To determine whether OsEATB interacted with the GCC-box, the construct was transformed into the yeast strain YM4271 (Clontech), which contained the reporter vector pHis-4 and the GCC-box (5’-TAAGAGCCGCC-3’). The same yeast strain containing pHis-4 and muGCC-box (5’-TAAGATCCTCC-3’), which had a 2bp substitution mutation at the center of the GCC-box, was used as the negative control.

Electrophoretic mobility shift assays (EMSA)

Nucleotide sequence of the double-stranded oligonucleotides of GCC-box element (2 × 5’-TAAGAGCCGCC-3’) was treated as the probe, and the double-stranded oligonucleotides of mGCC-box element (2 × 5’-TAAGATCCTCC-3’) was served as the competitor. Then, we used a DIG Gel Shift kit (Roche, Switzerland) to examine whether recombinant OsEATB protein could interact with the GCC-box element. The probe in a final volume of 20 µl DNA-protein complex was allowed to bind for 20 min at 20°C, and then proteins were separated on an 8% (w/v) polyacrylamide gel in 0.5 TBE. The gel was sandwiched and transferred to N+ nylon membrane (Millipore) in 0.5 TBE buffer at 380
mA at 4°C for 60 min. Bands were visualized by autoradiography.

**Subcellular localization assay**

The coding sequence of *OsEATB* was amplified with the cDNA clone as the template using the primers *OsEATB*-GFP-S (5’-GGATCCCGACCTCAAGCACACTACTAGC-3’) (forward) and *OsEATB*-GFP-A (5’-ACTAGTAAAACCTAGACGCTGCACCGG -3’) (reverse). The PCR product was inserted into the pCAMBIA 1304 vector fused with GFP in-frame, under the control of the CaMV 35S promoter. The fused plasmid coprecipitated with tungsten particles and was introduced into onion epidermal cells by particle bombardment using the PDS-1000 system (Xinzhi, China) at 1,100 psi helium pressure. We observed GFP fluorescence under a fluorescence conversion microscope (Olympus, Japan) after incubation of transformed cells in the dark for 36 h.

**RNA Hybridization in situ**

Freshly collected plant tissues were fixed in 4% (w/v) formaldehyde at 4°C for 2–4 h and 10 µm microtome sections were mounted on RNase-free glass slides. The primers used to prepare the probe were 5’-GGGTAGTCATCAGGCTCCG-3’ (forward) and 5’-GCCGTTTTCTTTGGCTGGG -3’ (reverse). The fragment was linked to pGEM-T (Promega, Madison, WI, USA) and the RNA probes were then produced by T7 and SP6 transcriptase labeled with digoxigenin (Roche, Switzerland). RNA hybridization in situ and immunological detection were carried out according to the instructions for the Enhanced Sensitive ISH Detection kit (POD, Boster Biotech, Wuhan, China).

**Stress and hormone treatments**

For stress and hormone treatments, 3-leaf-stage seedlings of wild-type and transgenic rice were incubated in Hoagland’s solution containing 200 mM NaCl, 50 μM ethephon, and 100 μM ABA at 28°C under a 14-h light/10-h dark photoperiod. For 200 mM NaCl treatment, the rate of seedlings without wilting and rolling was calculated every 6 h for 3 trials with 50 individuals for each trial. For 100 μM ABA treatment, the rate of seedlings without chlorosis was calculated every 6 h for 3 trials with 50 individuals for each trial. The
seedlings calculated are the ones with no visible change of phenotype. These experiments were repeated twice and similar results were obtained each time.

**Microarray analysis**

Control and transgenic seedlings were grown to the 4-leaf-stage in half-strength Murashige and Skoog medium under a 14/10 h light/dark cycle at 28°C. RNA samples were extracted using an RNeasy mini kit (Cat#74106, Qiagen, GmBH, Germany) following the manufacturer’s instructions. To determine integration of RNA, the RNA integrity number (RIN) was determined using an Agilent Bioanalyzer 2100 (Agilent technologies, Santa Clara, CA, USA). Microarray analyses were carried out using a competitive hybridization method using the Affymetrix microarray system. All procedures were carried out according to the manufacturer’s protocols. To obtain biotin-labeled cRNA, 1 μg total RNA was amplified, labeled, and purified using the GeneChip 3’IVT Express kit (Cat#901229, Affymetrix, Santa Clara, CA, USA) according to the manufacturer’s instructions. Array hybridization and washing was performed using the GeneChip® Hybridization, Wash and Stain kit (Cat#900720, Affymetrix, Santa Clara, CA, US) in the 645 Hybridization Oven (Cat#00-0331-220V, Affymetrix, Santa Clara, CA, US) and the Fluidics Station 450 (Cat#00-0079, Affymetrix, Santa Clara, CA, US) according to the manufacturer’s instructions. Slides were scanned by a GeneChip® Scanner 3000 (Cat#00-00212, Affymetrix, Santa Clara, CA, US) and analyzed with Command Console Software 3.1 (Affymetrix, Santa Clara, CA, US) with default settings. Raw data were normalized by the MAS 5.0 algorithm with Gene Spring Software 11.0 (Agilent technologies, Santa Clara, CA, US). The normalized log2 signal intensity data was generated by the MAS 5.0 algorithm. All microarray data have been deposited in a public database. The dataset was normalized by the LOWESS dye normalization method. Spots with low fluorescence intensity (i.e. <60) were excluded from analyses. Genes with a Log2Ratio>=1 or <=-1 were filtered for further analyses.

**Quantification of endogenous GAs**

Rice leaves (3 g) were frozen in liquid nitrogen, ground to a fine powder, and extracted
with 15 ml 80% (v/v) methanol at 4°C for 12 h. Before grinding of plant tissues, the following labeled GAs were added as internal standards: \([\text{d}^2\text{H}_2\text{]}\) GA\(_1\) (1.00 ng/g), \([\text{d}^2\text{H}_2\text{]}\) GA\(_4\) (2.00 ng/g), \([\text{d}^2\text{H}_2\text{]}\) GA\(_{12}\) (2.00 ng/g), \([\text{d}^2\text{H}_2\text{]}\) GA\(_{24}\) (6.00 ng/g), and \([\text{d}^2\text{H}_2\text{]}\) GA\(_{53}\) (4.00 ng/g). After centrifugation (10,000 g, 4°C, 20 min), the supernatant was collected and passed through a C-18 SPE-cartridge (12 ml, 1.5 g) preconditioned with 8 ml water, 8 ml methanol, and 8 ml 80% (v/v) methanol. The eluate was pooled and evaporated under a nitrogen gas stream and redissolved in 3 ml water. The solution was acidified with 360 \(\mu\)l 0.1 mol/L hydrochloric acid and repeatedly extracted with ethyl ether (10 \(\times\) 0.5 ml). The ether phases were combined, dried under nitrogen gas, and redissolved in 112 \(\mu\)l acetonitrile. Then, 180 \(\mu\)l Et\(_3\)N (20 \(\mu\)mol/mL) and 108 \(\mu\)l 3-bromoacetyltrimethylammonium bromide (20 \(\mu\)mol/mL) were added and the reaction solution was vortexed for 10 min. The mixture was evaporated to dryness under a stream of nitrogen gas, and then the residue was dissolved in 30 \(\mu\)l water. The resulting sample solution was injected by 25 kV \(\times\) 1 min and separated by 100-cm amino groups, coated capillary electrophoresis coupled with electrospray ionization quadrupole-time of flight mass spectrometry for analysis (Chen et al., 2011).

**Data Availability**

All microarray data from this work are available from the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) (www.ncbi.nlm.nih.gov/geo) under the series entry GSE28229.

**Accession Numbers**

Sequence data from this article can be found in the GenBank/EMBL database under the following accession numbers: OsEATB, EU622934; OsCPS2, AK072928; GA20ox2, AY114310; OsCPS1, AK100333; OsKS1, AK119442; OsKO2, AK071743; OsKAO, AK120757; XET, AF443603; UROD, AF119232; GOX, AF022740; SLR1, AB262980; OsERF3, AB036883; CPD, AK111418; OsERF15, ABH04236; OsBIERF1, AAV98700; OsBIERF2, AAV98701; OsBIERF3, CAC39058;
OsBIERF4,AAK92632; AtERF1,BAA32418; AtERF2,BAA32419; AtERF3,BAA32420; AtERF4,BAA32421; AtERF5,BAA32422; AtERF6,BAA97157; AtERF7,ABB51576; AtERF8,NP175725; AtERF9,NP199234; AtERF10,NP171876; AtERF11,NP174159; AtERF12, NP174158; NtERF1,BAA07321; NtERF2,BAA07324; NtERF3,BAA07322; NtERF4,BAA07323; NtERF5,AAU81956; NsERF2,BAA97122; NsERF3,BAA97123; NsERF4,BAA97124; PtI4,AAC50047.

Supplemental Data

The following materials are available in the online version of this article.

**Supplemental Figure S1.** Multiple sequence alignment of OsEATB and other known ERF proteins.

**Supplemental Figure S2.** Pathway of GA biosynthesis.

**Supplemental Table S1.** Phenotypic analysis of control and transgenic plant lines overexpressing OsEATB.

**Supplemental Table S2.** Primers used in this work.

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FIGURE LEGENDS

Figure 1. Rice AP2/ERF gene OsEATB with a potential new function.

A ,  Genome structure of OsEATB. NLS, nuclear localization signal.

B ,  Phylogenetic tree analysis of OsEATB protein sequence with those of other known ERF proteins from various plant species. Sequences used for calculating the relationships were from Oryza sativa OsERF3; OsBIERF1-4 (Cao et al. 2006); OsERF15 (GenBank: ABH04236); Arabidopsis AtERF1-12 (Riechmann and Meyerowitz, 1998; Fujimoto et al., 2000); Nicotiana tabacum NtERF1-5 (Ohme-Takagi and Shinshi, 1995; Suzuki et al., 1998; Ohta et al., 2000; Nishiuchi et al., 2002); N. sylvestris NsERF2-4 (Kitajima et al., 2000); and Lycopersicon esculentum Pti4 (Chakravarthy et al., 2003). The amino acid sequences were aligned using ClustalX, and the phylogenetic tree was constructed using the neighbor-joining method.

C ,  GCC-box binding activity analysis of OsEATB using the yeast one-hybrid system. Sketch maps show construction of vectors used in this experiment. Photographs show growth behavior of transformants on SD/Trp-Leu- medium and SD/Trp-Leu-His/-50mM 3-AT medium. 1. pHIS-GCC-box + pGAD-OsEATB; 2. pHIS-muGCC-box + pGAD-OsEATB; 3. pHIS-GCC-box + pGAD242; 4. pGAD-OsEATB + pHIS2.1.

D ,  Purification of His fusion recombinant OsEATB protein (arrowhead), and competition assay showing formation of OsEATB and the GCC-box element complex.

E ,  Linear map of pCAMBIA1304 binary plasmid constructed to determine subcellular location of OsEATB.

F ,  Subcellular localization of GFP and GFP-OsEATB fusion protein. Constructs were transiently expressed in onion epidermal cells. 1, Bright field image; 2, fluorescence
Figure 2. Expression of transgenic OsEATB in rice.

A, Linear map of pCAMBIA1304 binary plasmid containing OsEATB gene. 35S promoter, cauliflower mosaic virus 35S promoter; Nos T, nopaline synthase terminator; Kanr, kanamycin resistance gene; hpt, hygromycin phosphotransferase gene as a plant selection marker gene; Bgl II and Pml I, restriction enzyme cleavage sites; LB: left border; RB: right border.

B, Southern hybridization analysis of 35S:OsEATB transformants. Transgenic lines 401003 and 401006 show a single specific copy of OsEATB transgene, respectively. Lane 1, 9311 control; Lane 2, 401003; Lane 3, 401006. Arrows show the DNA fragments containing OsEATB transformants.

C, Real-time PCR analysis of OsEATB expression in 401003, 401005, 401006, and 401007 transgenic lines, Values are mean ± SD, n = 3 individuals.

D, In situ hybridization of OsEATB in 18-day-old 9311 control and 401003 transgenic seedlings. Left panels show cytohistological structures of materials stained with 0.25% toluidine blue O; Right panels show In situ hybridization results. OsEATB was strongly expressed in 401003 transgenic seedlings, but constitutively expressed at a basal level in 9311 control seedlings. Bar=100 μm.

Figure 3. Effect of transgenic OsEATB on rice internode elongation.

A, OsEATB transgenic plant (right) showing dwarf phenotype. Every internode is shortened: first internode by an average of 2.6%, second internode by 18.5%, third internode by 26.9%, fourth internode by 56.3%, and fifth internode by 5.0%.

B and C, OsEATB transgenic seedlings did not show negative sensitivity to GA treatments compared with the control seedlings. The 4-leaf-old transgenic and control seedlings are
treated with 10 μM, 50 μM, and 100 μM GA3. 1, 9311 Control; 2, 9311 Control with 24-h 100 μM GA3 treatment; 3, OsEATB transgenic plant with 24-h 100 μM GA3 treatment; 4, OsEATB transgenic plant. The seedling height was recorded every 6 h. Values are mean ± SD, n = 20 individuals.

**D** and **E**, Expressions of OsCPS2 and GA20ox2 were examined in transgenic line 401006, 401003 and control plants by real-time PCR analysis. Values are mean ± SD, n = 3 individuals. Expressions of OsCPS1, OsKS1, OsKO2, and OsKAO in transgenic and wild type seedlings were also examined. Values are mean ± SD, n = 3 individuals. One asterisk indicates a significant difference (P<0.05); Two asterisks indicate a significant difference (P<0.01).

**Figure 4.** Effect of rice OsEATB on GA biosynthesis during the internode elongation process.

**A**, Expression-level traits of rice OsEATB and OsCPS2 in elongating internode of 9311 control plants from 64-days-old to 73-days-old. Values are mean ± SD, n = 3 individuals.

**B**, Real-time quantitative PCR analysis of expressions of genes involved in GA biosynthesis and GA signal response pathway. Values are mean ± SD, n = 3 individuals.

**Figure 5.** OsEATB expression is negatively regulated by ethylene, ABA, and abiotic stress in rice.

**A**, Expression of OsEATB was decreased sharply by NaCl and ethephon treatments and slightly decreased by ABA treatment. OsERF3 was chosen as the positive control for ethylene responsiveness **CPD** as the positive control for ABA responsiveness. Values are mean ± SD, n = 3 individuals.

**B**, Expression levels of OsEATB and OsCPS2 at 0, 3, 6, 12, and 24 h of ethephon treatment. Values are mean ± SD, n = 3 individuals.

**C**, Seedlings (18-day-old) of 9311 control and transgenic plants were treated with 200 mM NaCl for 48 h. 0 day, no treatment; 2 day, 48 h treatment; +2 day, 48 h treatment
followed by 48 h watering. The rate of seedlings without wilting and rolling for both seedlings was recorded every 6 h. Values are mean ± SD, \( n = 3 \) trials, 50 individuals for each trial.

**D**, Seedlings (18-day-old) of 9311 control and transgenic lines were treated with 100 μM ABA for 96 h. 0 day, no treatment; 2 day, 48 h treatment; 4 day, 96 h treatment. The rate of seedlings without chlorosis for both seedlings was recorded every 6 h. Values are mean ± SD, \( n = 3 \) trials, 50 individuals for each trial.

**Figure 6.** Effect of transgenic *OsEATB* on rice yield traits.

**A**, Gross morphology of 9311 control (left) and transgenic lines 401006 (right) at maturity. Transgenic lines produced more panicles than wild-type plants.

**B**, Comparison of tillering between wild-type (left) and transgenic 401006 T2 lines (right) at the 6-leaf-stage. Transgenic plants produced more tillers than control 9311 lines.

**C**, Effect of *OsEATB* on primary and secondary branching. Main panicle of 9311 control (left) and transgenic 401006 lines (right) after seed production.

**D**, Yield components of transgenic and 9311 control plants. Left to right: panicles per plant, panicle length, spikelets per panicle, and grain number per panicle. Values are mean ± SD (least significant difference test: \( P < 0.05 \), \( n = 20 \) individuals).

**E**, Comparison of panicle structure and seed size between 9311 control (left) and transgenic line 401003 (right).

**Figure 7.** Model of *OsEATB*-dependent hormonal regulation of internode elongation. *OsEATB* acts as a negative balance regulator of ethylene-induced enhancement of GA responsiveness during the internode elongation process.

**TABLES**
Table I. Expressions of GA biosynthesis-related genes in OsEATB transgenic plants

| Probe ID\(^a\)               | Annotation | Accession number | Fold change\(^b\) |
|------------------------------|------------|------------------|-------------------|
| Os.27751.1.S1_at             | OsCPS2     | AK072928         | -10.32            |
| Os.19823.1.S1_at             | GA20ox2    | AY114310         | 2.77              |
| Os.16326.1.S1_at             | OsCPS1     | AK100333         | 1.72              |
| OsAffx.27508.22.S1_s_at      | OsKS1      | AK119442         | -1.32             |
| Os.53276.1.S1_at             | OsKO2      | AK071743         | 1.70              |
| Os.50329.1.S1_s_at           | OsKAO      | AK120757         | 1.16              |

\(^a\)Describes name of probe set on Affymetrix Rice GeneChip.

\(^b\)Fold change measures the relative expression level for each probe ID between wild-type and 35S: OsEATB 401006 transgenic plants. Two replicates were performed.

Table II. Decreased GAs abundance in OsEATB transgenic plants

|                   | 3-leaf-stage | 6-leaf-stage |
|-------------------|--------------|--------------|
|                   | 9311 control | 35s: OsEATB 401006 | 9311 control | 35s: OsEATB 401006 | 35s: OsEATB 401003 |
| GA\(_{12}\)       | n.q.(1.07-3.55) | n.d.(<1.07) | 0.54 ± 0.09 | n.d.(<0.08) | n.d.(<0.08) |
| GA\(_{33}\)       | n.q.(0.45-1.49) | n.d.(<0.45) | n.d.(<0.52) | n.d.(<0.52) | n.d.(<0.52) |
| GA\(_{19}\)       | n.q.(0.08-0.25) | n.d.(<0.08) | n.d.(<0.03) | n.d.(<0.03) | n.d.(<0.03) |
| GA\(_{24}\)       | 9.55±0.37     | 0.73±0.02     | 6.75 ± 0.18  | 0.28 ± 0.02  | 0.29 ± 0.03  |
| GA\(_9\)          | 0.20±0.01     | n.d.(<0.04)   | 3.00 ± 0.21  | 0.13 ± 0.01  | 0.12 ± 0.01  |
| GA\(_{20}\)       | 1.18±0.05     | n.d.(<0.07)   | 1.16 ± 0.11  | n.d.(<0.44)  | n.d.(<0.44)  |
| GA\(_4\)          | 1.18±0.05     | n.d.(<0.07)   | n.d.(<0.34)  | n.d.(<0.34)  | n.d.(<0.34)  |
| GA\(_1\)          | 0.93±0.06     | n.d.(<0.07)   | 0.87 ± 0.05  | n.d.(<0.20)  | n.d.(<0.20)  |
| ABA               | 81.78±3.17    | 86.39±2.15    | 46.08 ± 1.71 | 42.16 ± 4.34 | 45.08 ± 7.65 |
| IAA               | 11.21±0.75    | 13.15±0.37    | 11.51 ± 0.71 | 9.22 ± 0.57  | 8.54 ± 0.37  |

n.d., not detected due to low abundance; n.q. detected but not quantified; Data are means ± SD from three trials (ng/g fresh weight).
Figure 1. Rice AP2/ERF gene OsEATB with a potential new function.

A. Genome structure of OsEATB. NLS, nuclear localization signal

B. Phylogenetic tree analysis of OsEATB protein sequence against those of other known ERF proteins from various plant species. Sequences used for calculating the relationships were from Oryza sativa OsERF3; OsERF14 (Cao et al. 2006); OsERF15 (GenBank: ABH04236); Arabidopsis AtERF1-12 (Riechmann and Meyerowitz, 1998; Fujimoto et al., 2000); Nootiana tabacum NiERF1-5 (Ohme-Takagi and Shinshi, 1995; Suzuki et al., 1995; Ohita et al., 2000; Nishiochii et al., 2002); N. sylvestris NsERF2-4 (Kitajima et al., 2000); and Lycopersicon esculentum Pi4 (Chakrarvary et al., 2003). The amino acid sequences were aligned using ClustalX, and the phylogenetic tree was constructed using the neighbor-joining method.

C. GCC-box binding activity analysis of OsEATB using the yeast one-hybrid system. Sketch maps show construction of vectors used in this experiment. Photographs show growth behavior of transformants on SD/Trp-Leu medium and SD/Trp-Leu-His/+50mM 3-AT medium. 1. pHis-GCC-box + pGAD-OsEATB, 2. pHIS-muGCC-box + pGAD-OsEATB, 3. pHIS-GCC-box + pGAD242, 4. pGAD-OsEATB + pHIS21.

D. Purification of His fusion recombinant OsEATB protein (arrowhead), and competition assay showing formation of OsEATB and the GCC-box element complex.

E. Linear map of pCambia1304 binary plasmid constructed to determine subcellular location of OsEATB

F. Subcellular localization of GFP and GFP-OsEATB fusion protein. Constructed right in exhibited expression of OsEATB under the 35S promoter.

G. RT-PCR analysis of expression of rice OsEATB in various organs.

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Figure 2

Expression of transgenic OsEATB in rice.

A. Linear map of pCAMBIA1304 binary plasmid containing OsEATB gene. 35S promoter, cauliflower mosaic virus 35S promoter; Nos T, nopaline synthase terminator; Kanr, kanamycin resistance gene; hpt, hygromycin phosphotransferase gene as a plant selection marker gene; Bgl II and Pml I restriction enzyme cleavage sites; LB, left border; RB, right border.

B. Southern hybridization analysis of 35sOsEATB transformants. Transgenic lines 401003 and 401006 show a single specific copy of OsEATB transgene, respectively. Lane 1, 9311 control; Lane 2, 401003; Lane 3, 401006. Arrows show the DNA fragments containing OsEATB transformants.

C. Real-time PCR analysis of OsEATB expression in 401003, 401005, 401006, and 401007 transgenic line. Values are mean ± SD, n = 3 individuals.

D. In situ hybridization of OsEATB in 18-day-old 9311 control and 401003 transgenic seedlings. Left panels show the cytohistological structures of materials stained with 0.25% toluidine blue O; Right panels show the in situ hybridization results. OsEATB is strongly expressed in 401003 transgenic seedlings, but constitutively expressed at a basal level in 9311 control seedlings. Bar=100 μm.
Figure 3

(A) OsEATB transgenic plant (right) showing dwarf phenotype. Every internode is shortened first internode by an average of 26%, second internode by 18.5%, third internode by 26.9%, fourth internode by 55.1%, and fifth internode by 50.6%.

(B) OsEATB transgenic seedlings did not show negative sensitivity to GA treatments compared with the control seedlings. The 4-week-old transgenic and control seedlings are treated with 10 μM, 500 μM, and 100 μM GA3. 1, 931 Control; 2, 931 Control; 3, OsEATB transgenic plant with 24 h 100 μM GA treatment; 4, OsEATB transgenic plant. The seedling height was recorded every 2 h.

(C) Expression level of OsCPS2 and GA20ox in transgenic line 400003, 400006 plants and control plants by real-time PCR analysis. Values are mean ± SD, n = 3 individuals. Expression of OsCPS2, OsKSI, OsKO2, and OsKAO in transgenic and wild type seedlings. Values are mean ± SD, n = 3 individuals. One asterisk indicates a significant difference (P<0.05).

Two asterisks indicate a significant difference (P<0.01).
Figure 4. Effect of rice OsEATB on GA biosynthesis during the internode elongation process.

A. Expression-level traits of rice OsEATB and OsCPS2 in elongating internode of 9311 control plants from 64-days-old to 73-days-old.

Values are mean ± SD, n = 3 individuals.

B. Real-time quantitative PCR analysis of expressions of genes involved in GA biosynthesis and GA signal response pathway.

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Figure 5. OsEATB expression is negatively regulated by ethylene, ABA, and abiotic stress in rice.

A. Expression of OsEATB was decreased sharply by NaCl and ethylene treatments and slightly decreased by ABA treatment. OsERF3 was chosen as the positive control for ethylene responsiveness. CPD as the positive control for ABA responsiveness. Values are mean ± SD, n = 3 individuals.

B. Expression levels of OsEATB and OsCPS2 at 0, 3, 6, 12, and 24 h of ethylene treatment. Values are mean ± SD, n = 3 individuals.

C. Seedlings (18-day-old) of 9311 control and transgenic plants were treated with 200 mM NaCl for 48 h. 0 day, no treatment; 2 day, 48 h treatment; +2 day, 48 h treatment followed by 48 h water. The rate of seedlings without wilting and rolling for both seedlings was recorded every 6 h. Values are mean ± SD, n = 3 trials. 50 individuals for each trial.

D. Seedings (18-day-old) of 9311 control and transgenic lines were treated with 100 μM ABA for 96 h. 0 day, no treatment; 2 day, 48 h treatment; 4 day, 96 h treatment. The rate of seedlings without chlorosis for both seedlings was recorded every 6 h. Values are mean ± SD, n = 3 trials. 50 individuals for each trial.
Figure 6. Effect of transgenic OsEATB on rice yield traits.

A. Gross morphology of 9311 control (left) and transgenic line 401008 (right) at maturity. Transgenic lines produced more panicles than wild-type plants.

B. Comparison of tillering between wild-type (left) and transgenic 401008 T2 lines (right) at the three-leaf stage. Transgenic plants produced more than their control 9311 lines.

C. Effect of OsEATB on primary and secondary branching. Main panicle of 9311 control (left) and transgenic 401008 (right) after seed production.

D. Yield components of transgenic and 9311 control plants. Left to right: panicles per plant, panicle length, spikelets per panicle, and grain number per panicle. Values are mean ± SD (least significant difference test; P < 0.05, n = 20 individuals).

E. Comparison of panicle structure and seed size between 9311 control (left) and transgenic line 401003 (right).
Figure 7. Model of OsEATB-dependent hormonal regulation of internode elongation. OsEATB acts as a negative balance regulator of ethylene-induced enhancement of GA responsiveness during the internode elongation process.