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Ethylene Promotes Submergence-Induced Expression of OsABA8ox1, a Gene that Encodes ABA 8'-Hydroxylase in Rice†

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A rapid decrease of the plant hormone ABA under submergence is thought to be a prerequisite for the enhanced elongation of submerged shoots of rice (Oryza sativa L.). Here, we report that the level of phaseic acid (PA), an oxidized form of ABA, increased with decreasing ABA level during submergence. The oxidation of ABA to PA is catalyzed by ABA 8'-hydroxylase, which is possibly encoded by three genes (OsABA8ox1, -2 and -3) in rice. The ABA 8'-hydroxylase activity was confirmed in microsomes from yeast expressing OsABA8ox1. OsABA8ox1–green fluorescent protein (GFP) fusion protein in onion cells was localized to the endoplasmic reticulum. The mRNA level of OsABA8ox1, but not the mRNA levels of other OsABA8ox genes, increased dramatically within 1 h after submergence. On the other hand, the mRNA levels of genes involved in ABA biosynthesis (OsZEP and OsNCEDs) decreased after 1–2 h of submergence. Treatment of aerobic seedlings with ethylene and its precursor, 1-aminocyclopropane-1-carboxylate (ACC), rapidly induced the expression of OsABA8ox1, but the ethylene treatment did not strongly affect the expression of ABA biosynthetic genes. Moreover, pre-treatment with 1-methylcyclopropene (1-MCP), a potent inhibitor of ethylene action, partially suppressed induction of OsABA8ox1 expression under submergence. The ABA level was found to be negatively correlated with OsABA8ox1 expression under ACC or 1-MCP treatment. Together, these results indicate that the rapid decrease in ABA levels in submerged rice shoots is controlled partly by ethylene-induced expression of OsABA8ox1 and partly by ethylene-independent suppression of genes involved in the biosynthesis of ABA.

Keywords: ABA 8'-hydroxylase — Ethylene — Rice — Submergence.

Introduction

Many plants suffer from submergence stress caused by flooding and heavy rain. During complete submergence, the energy supply becomes insufficient because aerobic respiration and photosynthesis are inhibited. Some terrestrial plants [e.g. semi-aquatic plants such as rice (Oryza sativa L.) and Rumex palustris] have evolved adaptation and avoidance mechanisms to survive under such temporal submerged conditions.

Rice is one of the few crops that can temporarily survive under complete submergence. Two common ecotypes of rice, deepwater rice (or floating rice) and lowland rice, use different strategies to overcome temporal flooding (Kende et al. 1998, Sauter 2000). Deepwater rice grows in areas where water depth increases slowly and often remains at >1 m for several months. When submerged, deepwater rice varieties enhance the ability of internode elongation to allow the leaf tips to reach the water surface (Kende et al. 1998, Sauter 2000). Lowland rice varieties, cultivated in rain-fed areas, also elongate their shoots under submergence. However, shoot elongation in lowland varieties provides little advantage during flooding, which is usually a transient event. Instead, shoot elongation uses up the energy supply, and results in lodging and less resistance to pests after

Abbreviations: ABA-GE, ABA glucose ester; ACC, 1-aminocyclopropane-1-carboxylate; DPA, dihydrophaseic acid; ER, endoplasmic reticulum; GFP, green fluorescent protein; LC-MS/MS, liquid chromatography–tandem mass spectrometry; 1-MCP, 1-methylcyclopropene; NCED, nine-cis-epoxycarotenoid dioxygenase; PA, phaseic acid; qRT–PCR quantitative reverse transcription–PCR; ZEP, zeaxanthin epoxidase.

We dedicate this paper to the memory of Dr. Hans Kende.

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recovery from submerged conditions (Jackson and Ram 2003; Das et al. 2005). Thus, in submergence-tolerant cultivars of lowland rice, such as FR13A, shoot elongation under submergence is reduced (Setter and Laureles 1996). Submergence-1 (Sub1), which is derived from the cultivar FR13A, is a major quantitative trait locus contributing to submergence tolerance and is located on chromosome 9 (Xu and Mackill 1996). Sub1 has been identified as a member of a gene family encoding ethylene response factors (ERFs) (Fukao et al. 2006, Xu et al. 2006).

In submerged plants, at least three plant hormones, ethylene, gibberellin and ABA, are involved in the regulation of shoot elongation (Kende et al. 1998, Sauter 2000, Voesenek et al. 2003). The initial event of submergence responses in plants is thought to be triggered by the physical entrapment of gaseous compounds such as ethylene that cannot easily diffuse into water (Jackson 1985). Ethylene plays a key role in the submergence responses partly through regulating gibberellin and ABA, which are positive and negative regulators of shoot elongation, respectively. After the ethylene concentration rises, there is first a decrease in the level of ABA (Hoffmann-Benning and Kende 1992, Azuma et al. 1995, van der Straeten et al. 2001), and then an increase in the level of endogenous gibberellin (Hoffmann-Benning and Kende 1992, Rijnders et al. 1997, van der Straeten et al. 2001). The rapid decrease in ABA levels is thought to be a prerequisite for submergence-induced gibberellin biosynthesis or increased responsiveness to gibberellin, thereby promoting internode or petiole elongation (Kende et al. 1998, Benschop et al. 2006).

In higher plants, the level of ABA is finely controlled by the balance between the rates of ABA biosynthesis and catabolism (Nambara and Marion-Poll 2005). The key enzyme in ABA synthesis is nine-cis-epoxy-carotenoid dioxygenase (NCED), which converts 9'-cis-neoxanthin and 9-cis-violaxanthin to xanthoxin. Zeaxanthin epoxidase (ZEP), which converts zeaxanthin to violaxanthin via antheraxanthin, is also postulated to have a regulatory role in ABA biosynthesis in non-photosynthetic organs (Schwartz and Zeccaart 2004). The rice genome has a single ZEP gene (OsZEP) (Agrawal et al. 2001) and three putative NCED genes (OsNCED1, -2 and -3) (Tan et al. 2003). In the case of ABA catabolism, the major pathway is thought to be hydroxylation to 8'-hydroxy ABA by cytochrome P450, followed by spontaneous isomerization into phaseic acid (PA) and reduction to dihydropyaseic acid (DPA) (Krochko et al. 1998, Cutler and Krochko 1999, Nambara and Marion-Poll 2005). On the other hand, ABA is inactivated by conjugation with sugars. ABA glucose ester (ABA-GE) is one of the predominant ABA conjugates (Cutler and Krochko 1999).

The mechanism responsible for the submergence-induced ABA decrease in semi-aquatic plants has not been clearly described, although in R. palustris it has been speculated to be due to both activation of ABA catabolism and down-regulation of ABA biosynthesis (Benschop et al. 2005). Recently, Yang and Choi (2006) reported that a gene encoding ABA 8'-hydroxylase (CYP707A5) in deepwater rice was up-regulated by ethylene treatment, suggesting that CYP707A5 decreases the ABA level during ethylene-induced stem elongation. In this study, to obtain direct evidence about the mechanism responsible for the submergence-induced ABA decrease in lowland rice, we examined (i) the function of ABA in the shoot elongation of lowland rice under submergence; (ii) the ethylene inducibility of ABA biosynthetic and catabolic genes; and (iii) the expression of genes involved in the biosynthesis and catabolism of ABA under submergence. Our results indicate that the induction of OsABA8ox1 (i.e. CYP707A5) is the primary means by which ethylene regulates ABA levels in submerged lowland rice.

**Results**

**Effects of ABA on shoot elongation in submerged rice**

To determine the effects of ABA on shoot elongation in submerged rice, we measured the lengths of shoots (i.e. plant height) of Nipponbare, a lowland rice cultivar, at 6 d after submergence in various concentrations of ABA solutions (10^{-9}–10^{-5} M). The rate of shoot elongation during submergence was negatively correlated with the ABA concentration (Fig. 1A), suggesting that exogenous ABA dose-dependently suppresses shoot elongation under submergence, in agreement with previous reports on deepwater rice varieties (Hoffmann-Benning and Kende 1992, Azuma et al. 1995). Furthermore, we examined whether shoot elongation in an ABA-deficient mutant is enhanced under submergence. We used a Nipponbare mutant, oszep, in which the retrotransposon Tos17 is inserted into one of the introns of an ABA biosynthetic gene OsZEP (Agrawal et al. 2001). In the oszep mutant, the level of OsZEP mRNA is dramatically decreased, and the endogenous ABA level is about 20% of that in the wild type (Agrawal et al. 2001). Shoot elongation in the oszep homozygous mutant (−/− in Fig. 1B) was higher than that in wild homozygous plants (+/+ in Fig. 1B) under both submerged and aerobic conditions. The elongation rates were higher under complete submergence than under aerobic conditions in each line (Fig. 1B). These results indicate that ABA functions as a negative regulator of enhanced shoot elongation of lowland rice (Nipponbare) under submergence.
Change of ABA and PA levels in submerged rice shoots

Endogenous ABA levels decreased by 30% at 1 h after submergence and then by 50% at 2–4 h after submergence (Fig. 2A). On the other hand, PA levels increased by 50% at 1 h after submergence and then gradually decreased (Fig. 2B). The levels of DPA or ABA-GE changed little during the first 4 h of submergence (Fig. 2C and D). These data suggest that, in response to submergence, endogenous ABA is catabolized to PA, and little if any of it is converted to ABA-GE. The levels of ABA, PA, DPA and ABA-GE showed no remarkable changes under aerobic conditions (data not shown).

Expression analysis of ABA 8'-hydroxylase genes under submergence

The data shown in Fig. 2 suggested that the reduction in the ABA levels was caused by the activation of the ABA 8'-hydroxylation pathway. To address this point, we checked the mRNA levels of three CYP707A genes from Nipponbare, designated as OsABA8ox1, -2 and -3, by quantitative reverse transcription–PCR (qRT–PCR; see Materials and Methods). The mRNA level of OsABA8ox1 increased remarkably 1 h after the start of submergence.
decreased temporarily, and then increased transiently at 24 h after submergence (Fig. 3A). On the other hand, the mRNA levels of OsABA8ox2 and OsABA8ox3 did not increase, but instead decreased gradually (Fig. 3A).

Expression of ABA biosynthetic genes under submergence

We examined the expression of four genes involved in the biosynthesis of ABA. The mRNA levels of OsZEP and OsNCED3 began to decrease 1 h after submergence, and those of OsNCED1 and OsNCED2 began to decrease 2 h after submergence (Fig. 3B and C). Expression of all four genes tended to decrease gradually, although the expression of OsZEP and OsNCED2 showed slight increases 8–12 h after submergence.

Characterization and functional analyses of the OsABA8ox1 gene

The data shown in Fig. 3A suggested that OsABA8ox1 protein is responsible for the rapid decline of endogenous ABA levels under submergence. Thus, we focused on further characterization of the OsABA8ox1 gene. For functional analyses, the OsABA8ox1 cDNA was amplified from 1-aminocyclopropane-1-carboxylate (ACC)-treated Nipponbare shoots by RT–PCR and its sequence was determined (accession No. AB277270).

Recombinant OsABA8ox1 expressed in yeast, when incubated with NADPH and (+)-S-ABA, produced a product that was identified as PA by HPLC (Fig. 4A and B) and liquid chromatography–tandem mass spectrometry (LC-MS/MS) (data not shown). In contrast, yeast expressing a recombinant protein that is not related to ABA
catabolism (CYP88A3, a cytochrome P450) did not produce PA (Fig. 4C). These results indicate that the PA production is OsABA8ox1 dependent.

In onion epidermal cells transformed with OsABA8ox1–green fluorescent protein (GFP) fusion protein, GFP fluorescence had a mesh-like appearance (Fig. 5A), which is a typical morphology of plant endoplasmic reticulum (ER) (Hayashi et al. 2001). The pattern of GFP fluorescence was the same as the pattern observed in cells transformed with a gene encoding GFP fused with an ER-targeting signal, which was used as a positive control (Fig. 5B). In contrast, GFP fluorescence in cells transformed with a gene encoding GFP without any targeting signals was dispersed in the cytosol (Fig. 5C). These results confirm that OsABA8ox1 protein is localized in the ER.

Regulation of OsABA8ox1 expression by ethylene and its precursor ACC

Treatment of rice seedlings with ethylene, which has been shown to trigger the decrease of endogenous ABA during submergence (Hoffmann-Benning and Kende 1992), or treatment with its precursor ACC caused OsABA8ox1 mRNA levels to increase within 1 h in rice shoots under aerobic conditions (Fig. 6A and B, respectively). In addition, treatment of seedlings with ACC for 1 h caused ABA levels to decrease by 50% and caused PA levels to increase by 100% (Fig. 6C).

Under aerobic conditions, ethylene did not seem to affect the amounts of mRNA of the ABA biosynthetic genes (OsZEP and OsNCED1, -2 and -3) within 4 h after the treatment (Fig. 7A–D).

Pre-treatment of rice seedlings with 1-methylcyclopropene (1-MCP), a potent inhibitor of ethylene action, reduced the submergence-induced increase in the mRNA levels of OsABA8ox1 by 40–50% (Fig. 8A). Prior to the submergence, the ABA levels in the

![Fig. 5](image)

**Fig. 5** Intercellular localization of OsABA8ox1–GFP fusion protein. The cells of onion epidermal cells were bombarded with the plasmids (A) 35Spro::OsABA8ox1-GFP, (B) 35Spro::GFP-HDEL (GFP fused with the ER targeting signal and retention signal peptide) or (C) 35Spro::GFP. Each panel shows higher magnification images of a part of an epidermal cell. Scale bars indicate 10 μm.

![Fig. 6](image)

**Fig. 6** Effect of ethylene on OsABA8ox1 expression and decrease in ABA content. Eight-day-old seedlings were treated with 50 p.p.m. ethylene or 50 μM ACC for the indicated time under aerobic conditions. (A, B) A qRT–PCR analysis of the OsABA8ox1 gene in a shoot treated with ethylene (A) and ACC (B). The qRT–PCR analysis was done as in Fig. 3. (C) The endogenous levels of ABA and PA in rice shoots after ACC treatment. Rice seedlings were treated with 50 μM ACC for 1 h under aerobic conditions. The endogenous ABA (filled bars) and PA (open bars) levels compared before and after ACC treatment. Three independent samples were analyzed and the averages are shown with the SE.
1-MCP-pre-treated shoots were twice as high as those in the control shoots (Fig. 8B). This result suggests that 1-MCP inhibited ABA catabolism aerobically in an opposite manner to the ethylene action. During submergence, the ABA amounts were always higher in the 1-MCP-treated shoots than in the control shoots (Fig. 8B). The submergence-induced increase in the PA levels at 1 h was reduced in the 1-MCP-pre-treated shoots (Fig. 8C). Thus, it seems likely that the treatment with 1-MCP inhibited the catabolism of ABA to PA in the submerged shoots.

**Discussion**

In this study, we found that expression of *OsABA8ox1*, which encodes an ABA 8'-hydroxylase, dramatically increased in shoots of lowland rice within 1 h after the onset of submergence, and this induction was controlled partly by ethylene. Recently, Yang and Choi (2006) showed that ethylene strongly induced expression of *OsABA8ox1* (i.e. CYP707A5) in internodes of deepwater rice under aerobic conditions. Based on this result, they proposed that
increased concentration of ethylene in the internodes of deepwater rice under submergence triggers induction of OsABA8ox1 gene expression, thereby inducing internode elongation. Here, we showed that expression of OsABA8ox1 was indeed induced by the onset of submergence, and that the induction was reduced by the pre-treatment with 1-MCP, indicating that it is ethylene dependent. Furthermore, we found that mRNA levels of genes involved in ABA biosynthesis, OsZEP and three variants of OsNCED, began to decrease 1–2 h after the start of submergence. Interestingly, the decreases in the mRNA levels of the OsZEP gene and the OsNCED genes do not appear to be controlled by ethylene, because the mRNA levels of the ABA biosynthetic genes were not affected by ethylene treatment under aerobic conditions (Fig. 7). Thus, we propose that the rapid reduction of ABA that occurs upon submergence in rice is due to ABA 8'-hydroxylation, which is activated through ethylene-induced up-regulation of OsABA8ox1 expression, as well as the ethylene-independent suppression of ABA biosynthesis.

Catabolism of ABA to PA in rice shoots under complete submergence

A rapid decrease in ABA level in response to complete submergence is a common phenomenon in semi-aquatic plants such as deepwater rice (where the decrease is observed in internodes), lowland rice (where the decrease is observed in shoots) and submergence-tolerant Rumex species, such as R. palustris (where the decrease is observed in petioles) (Hoffmann-Benning and Kende 1992, van der Straaten et al. 2001, Benschop et al. 2005). In R. palustris, the rapid decrease of ABA was recently found to derepress expression of the gene for the gibberellin biosynthetic enzyme gibberellin 3-oxidase, which allowed GA1 levels to rise (Benschop et al. 2006). This rapid decrease is thought to be a prerequisite for submergence-induced gibberellin biosynthesis or increased responsiveness to gibberellin, which promotes internode or petiole elongation (Kende et al. 1998, Benschop et al. 2006). The role of ABA in the regulation of internode or petiole elongation under submergence has been analyzed by examining the effects of exogenously applied ABA or fluridone, an ABA biosynthesis inhibitor (Hoffmann-Benning and Kende 1992, Benschop et al. 2005). In this study, we confirmed that ABA was a negative regulator of enhanced shoot elongation of submerged lowland rice (cv. Nipponbare) based on two findings. First, externally applied ABA suppressed shoot elongation of rice seedlings under submergence (Fig. 1A). Secondly, shoot elongation of an ABA-deficient rice mutant was faster than that in the wild homozygote under complete submergence or aerobic conditions. However, the elongation rates of each line were higher under submergence than under aerobic conditions (Fig. 1B). The low ABA level in the oszep mutant is further decreased during submergence due to the normal breakdown of ABA that occurs during submergence. This may explain why shoot elongation in this mutant was faster under submergence than under aerobic conditions. However, we cannot rule out the possibility that an ABA-independent mechanism contributes to submergence-induced shoot elongation in the oszep mutant. In agreement with previous findings that ABA acts as a negative regulator of elongation of internodes in deepwater rice (Hoffmann-Benning and Kende 1992) and petioles in R. palustris (Benschop et al. 2005), our results indicate that ABA acts as a negative regulator of submerged shoot elongation of a lowland rice cultivar (Nipponbare). Therefore, the rapid decrease in ABA levels of this cultivar (Fig. 2A) might be required for shoot elongation under submergence. Elongated lowland rice shoots that consume limited energy stocks are thought to have reduced productivity and survival when rescued from submergence stresses (Setter and Laureles 1996, Das et al. 2005). In most cases, submergence stress on lowland rice is a temporal event. Therefore, genetically engineering a lowland rice cultivar that maintains high ABA levels under submergence might be a useful strategy for conferring tolerance to temporal flooding.

Prior to this study, little was known about the molecular mechanism for the rapid decrease in the ABA levels in rice under submerged conditions. In this study, we found that, concomitant with the decrease in the ABA levels, the PA levels increased at 1 h after the onset of submergence and then gradually decreased (Fig. 2A and B). However, the levels of ABA-GE were relatively unchanged during the first several hours after submergence (Fig. 2D). These results suggest that the rapid decrease of ABA contents in rice shoots under submergence occurs mainly by activation of the ABA 8'-hydroxylation pathway rather than by the conjugation pathway to produce ABA-GE. In R. palustris, a 70% decrease in ABA level that occurred 1 h after submergence was accompanied by only a small (10–25%) increase in the PA levels (Benschop et al. 2005). Moreover, an ABA analog, 8'-acetylene ABA, which potentially acts as an inhibitor of ABA 8'-hydroxylase, was 10 times more active in inhibiting petiole elongation in R. palustris under submergence than ABA (Benschop et al. 2005). Hence, these data indicate that the ABA catabolic pathway via ABA 8'-hydroxylation is activated under submergence and this pathway mainly contributes to the submergence-induced ABA breakdown in R. palustris as well as in rice.

Characterization of the OsABA8ox1 gene

Our expression analysis of the three OsABA8ox genes depicted in Fig. 3A suggests that OsABA8ox1 protein is responsible for the decrease of ABA levels in submerged
rice shoots. OsABA8ox1 protein has ABA 8'-hydroxylase activity (Fig. 4), as was recently shown by Yang and Choi (2006), and is localized to the ER (Fig. 5). In maize, ABA 8'-hydroxylase was detected in the microsomal pellet extracted from suspension cells (Krochko et al. 1998), suggesting that this enzyme functions in the ER. In Arabidopsis, β-glucosidase (AtBG1), which produces active ABA by hydrolyzing ABA-GE, was also localized in the ER (Lee et al. 2006). Our finding that OsABA8ox1 is localized in the ER indicates that the ER plays an important role in both biosynthesis and catabolism of ABA.

Control of ABA synthesis in rice shoots under submergence

The mRNA level of OsABA8ox1 dramatically increased (Fig. 3A), but the mRNA levels of OsZEP and OsNCED3 genes began to decrease (Fig. 3B and C) within 1 h after submergence treatment, suggesting that the rapid decrease in the ABA levels was controlled by both the ABA catabolic pathway and the ABA biosynthetic pathway in rice shoots. Furthermore, at later time points (i.e. 2–4 h) after submergence, the mRNA levels of both the ABA biosynthetic genes (OsZEP or OsNCEDs) and the ABA catabolic gene (OsABA8ox1) decreased (Fig. 3). In agreement with the decrease of these mRNAs, the amounts of both ABA and PA decreased at 2–4 h after submergence (Fig. 2). In R. palustris, the expression of several RpNCED genes decreased within 2 h after the start of submergence treatment and remained at a low level, suggesting that the submergence-induced ABA reduction is accompanied by both activation of ABA catabolism and down-regulation of the ABA biosynthetic genes (Benschop et al. 2005). Taken together, these findings lead us to propose, that, at the beginning of submergence, activation of ABA catabolism as well as the suppression of ABA biosynthesis are important for a rapid decrease in ABA, and that the suppression of ABA biosynthesis becomes more important for maintaining low ABA than the ABA catabolism in rice during longer submergence times.

Curiously, the mRNA levels of OsABA8ox1, OsZEP and OsNCEDs, after decreasing at 2–4 h after onset of submergence, temporarily increased (Fig. 3). However, the expression of OsZEP and OsNCEDs peaked at 8–12 h after submergence and OsABA8ox1 expression peaked at 24 h. Although the mechanisms underlying the fluctuation of mRNA levels of OsABA8ox1, OsZEP and OsNCEDs at later time points of submergence are unclear, it is possible that these expression patterns reflect an unknown homeostatic regulation of endogenous ABA levels in water.

Involvement of ethylene in the induction of OsABA8ox1 gene expression under submergence

Ethylene often reduces the ABA levels in vegetative plants such as in Xanthium (Zeevaart 1983), deepwater rice (Hoffmann-Benning and Kende 1992), R. palustris (Benschop et al. 2005) and Arabidopsis (Ghassemian et al. 2000). This mechanism is thought to be involved in the submergence responses of aquatic and semi-aquatic plants. In the aquatic plant Ludwigia arcuata, the shapes of leaves are changed by submergence or re-aeration, and this reaction is regulated by a decrease in the ABA content promoted by ethylene (Kuwabara et al. 2003). In deepwater rice (Hoffmann-Benning and Kende 1992) and R. palustris (Benschop et al. 2005), the ABA contents decreased under submergence conditions or by ethylene treatments, thereby enhancing internode and petiole elongation, respectively. Benschop et al. (2005) showed that the ABA 8'-hydroxylation pathway plays a pivotal role in the growth habit of the submerged R. palustris. Yang and Choi (2006) showed that ethylene-induced internode elongation of deepwater rice was caused by activation of the ABA 8'-hydroxylation pathway under aerobic conditions. In addition, the results presented in this study show how induction of OsABA8ox1 gene expression by ethylene gas entrapped in lowland rice shoots contributes to shoot elongation under submergence.

The increase in ethylene concentration that occurs in submerged plants is known to be caused by at least two mechanisms. One is entrapment, which causes the ethylene concentration to increase transiently, and another is enhancement of de novo ethylene biosynthesis (Sauter 2000, Vriezen et al. 2003, Voeseke et al. 2006). Ethylene biosynthesis upon submergence is promoted by the induction of genes encoding ACC synthase (ACS) such as OsACS1 and OsACS5, followed by the enzymatic activation of ACS (Cohen and Kende 1987, Zarembinski and Theologis 1997, van der Straeten et al. 2001). OsACS5 mRNA was accumulated within 1 h after submergence, indicating that ethylene biosynthesis is enhanced upon short-term submergence (van der Straeten et al. 2001). As shown in Figs. 6 and 8, the induction of OsABA8ox1 gene expression under submergence was controlled partly by ethylene. Because OsABA8ox1 mRNA began to accumulate as early as 10 min after submergence (data not shown), it is most likely that the induction of OsABA8ox1 gene expression observed just after submergence is caused by the entrapped ethylene rather than newly synthesized ethylene. High ethylene levels, which are produced by submergence-induced de novo ethylene biosynthesis, may contribute to the maintenance of the high mRNA levels of OsABA8ox1 under a longer period of submergence.

The gene expression of rice Universal stress protein 1 (OsUSP1) (Sauter et al. 2002) and AcIreductone dioxygenase 1 (OsARD1) (Sauter et al. 2005) was induced within 1 h after submergence, as was observed with OsABA8ox1. The gene expression of OsUSP1 or OsARD1 may be induced by entrapped ethylene upon
short-term submergence. The upstream sequences of these genes contain sequences similar to the binding site of ETHYLENE-INSENSITIVE3 (EIN3) (Sauter et al. 2005 and data not shown), which is one of the ethylene-responsive transcription factors in Arabidopsis (Solano et al. 1998). EIN3 protein is quickly degraded through a ubiquitin–proteasome pathway in the absence of ethylene, and the EIN3 protein levels rapidly increase in response to ethylene (Guo and Ecker 2003, Potuschak et al. 2003, Gagne et al. 2004). These results suggest that expression of the three genes, OsABA8ox1, OsUSP1 and OsARD1, is rapidly induced by rice EIN3-like proteins, which might be stabilized in response to the entrapped ethylene under submergence.

Materials and Methods

Plant materials and treatments

Rice (O. sativa L., a lowland rice cultivar Nipponbare) was used in this study. Rice seedlings were grown aerobically in constant light at 28°C for 8 d. For submergence treatments, 8-day-old seedlings were completely submerged in water in the dark at 28°C. For the treatment with ACC (Wako Pure Chemical Industries, Tokyo, Japan), 8-day-old seedlings were grown aerobi-
cally under 50 μM ACC solutions in the light at 28°C. For the ethylene treatment, the vessel in which 8-day-old seedlings were grown was filled with 50 p.p.m. ethylene gas (Suzuki Shokan, Tokyo, Japan) in the light at 28°C. For the 1-MCP treatment, 8-day-old seedlings were transferred into a vessel filled with 300 p.p.m. 1-MCP (Rohm and Haas Japan K.K., Kawasaki, Japan) in the light at 28°C for 6 h and the seedlings were completely submerged. For extraction of RNA or extractions of ABA and its catabolites, rice seedlings were harvested, immediately frozen in liquid N2 and stored at –80°C.

Measurement of lengths of submerged shoots treated with ABA

Eight-day-old seedlings were completely submerged in various concentrations (10−9–10−3 M) of ABA solutions in the dark at 28°C. After 6 d of submergence, the lengths of shoots (i.e. plant height) were measured.

Measurement of lengths of submerged shoots of the OsZEP-deficient line ND7980

Seeds obtained from heterologous lines of the OsZEP-deficient mutant ND7980 (Agrawal et al. 2001) were germinated and seedlings were grown aerobi-
cally at 30°C/25°C [14 h (day time)/10 h (night time)] in a greenhouse for 8 d. The aerobi-
cally grown seedlings were completely submerged in the dark at 26°C and the lengths of shoots were measured every 1 h (to 8 h after the onset of submergence). As a control, aerobi-
cally grown seedlings were transferred to darkness under aerobic conditions at 26°C and lengths of shoots were measured every 1 h. The submerged seedlings were transferred to aerobic conditions and were grown for 7 d in a greenhouse. To check genotypes (i.e. the presence or absence of Tos17 insertion in the OsZEP) of the individual lines, total DNA was extracted from shoots with TPS buffer [100 mM Tris–HCl (pH 8.0), 10 mM EDTA (pH 8.0) and 1 M KCl]. PCR was performed with a primer set of OsZEP-F and OsZEP-R1 (Table 1) for detection of original OsZEP or a primer set of OsZEP-F1 and OsZEP-R1-F for detection of Tos17 insertion (Table 1) for detection of Tos17 insertion into OsZEP. Based on the PCR experiments, homozygous plants for Tos17 insertion (−/−) and wild homozygous plants without Tos17 insertion (+/+ ) were classified. Just before submergence,

### Table 1 Oligonucleotides used in this study

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<td>OsNCED2-F</td>
<td>TCATTCCAAAACACC TTCCA</td>
</tr>
<tr>
<td>OsNCED2-R</td>
<td>TCCGGGGAACCTCT ATGTAT</td>
</tr>
<tr>
<td>OsNCED3-F</td>
<td>CGCAACAGTAAAAAG AATACACG</td>
</tr>
<tr>
<td>OsNCED3-R</td>
<td>TATACACACACCGG TCGTT</td>
</tr>
<tr>
<td>17S rRNA-F</td>
<td>TCCTACCGATTGAAT GGTCC</td>
</tr>
<tr>
<td>17S rRNA-R</td>
<td>CTTGTTACGACTTCT CTTCTC</td>
</tr>
</tbody>
</table>

For cloning

OsABA8ox1-F1 | CAAACCACCCGCCC ATTTC |
OsABA8ox1-R   | CCGTGCTAATACCGA ATCCA |

For genotyping

OsZEP-F1     | GGTGCGATAACGTC GTTGATC |
OsZEP-R1     | GTATGGTCTATAAG TGTTAGC |
Tos17-F      | CATCCCGATGTCCA GTCCAT |

Tos17-F and OsZEP-R1 (Table 1) for detection of Tos17 insertion into OsZEP. Based on the PCR experiments, homozygous plants for Tos17 insertion (−/−) and wild homozygous plants without Tos17 insertion (+/+) were classified. Just before submergence,
OsABA8ox1 expression in submerged rice

shoot lengths of plants homozygous for Toss17 insertion were 3.9 ± 2.5 cm (mean ± SD) and those of wild homozygous plants were 9.3 ± 2.6 cm.

Identification of rice ABA8'-hydroxylase genes

A BLAST search and a search of the Rice Annotation Project Database (RAP-DB, http://rapdb.lab.nig.ac.jp/index.html) for rice genes homologous to the Arabidopsis CYP707A gene family (Kushiro et al. 2004, Saito et al. 2004) found three putative OsABA8ox genes, Os02g0703600, Os08g0472800 and Os09g0457100. The Os09g0457100 gene has not been registered as a gene for cytochrome P450 in this database. In this study, Os02g0703600, Os08g0472800 genes correspond to CYP707A5 and CYP707A6, respectively, in the Cytochrome P450 Homepage (http://drnelson.utmem.edu/cytochromeP450.html). The Os09g0457100 gene has not been registered as a gene for cytochrome P450 in this database. In this study, Os02g0703600, Os08g0472800 and Os09g0457100 were designated OsABA8ox1, OsABA8ox2 and OsABA8ox3, respectively.

Cloning of OsABA8ox1 cDNA

Total RNA was extracted from shoots of the young seedlings treated with 50 μM ACC for 0.5 h, and was used for reverse transcription using the primer OsABA8ox1-R (Table 1) with Superscript II (Invitrogen, Carlsbad, CA, USA). The resultant cDNA was amplified using a primer set of OsABA8ox1-F1 and OsABA8ox1-R (Table 1) with a high-fidelity DNA polymerase KOD-Plus (Toyobo, Osaka, Japan), and then was cloned into pDONR207 (Invitrogen). The sequence of the cDNA clone was read with an ABI3100 sequencer (Perkin Elmer ABD, Foster City, CA, USA) and was assembled with SEQMAN Software (DNASTAR Inc., Madison, WI, USA).

Extraction of total RNA and qRT–PCR

Total RNA was extracted from frozen shoots with an RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA). Transcript levels of each gene were measured by real-time qRT–PCR using a LightCycler (Roche Diagnostics, Mannheim, Germany) and the QuantiTect SYBR Green RT-PCR kit (Qiagen) according to the manufacturer's protocols. Primers used for RT–PCR are listed in Table 1. A PCR fragment of each gene was used to make standard curves for quantification. Experiments were repeated twice and similar results were obtained.

Extraction and mass spectrometric analysis of ABA and its catabolites

ABA and its catabolites were extracted for analysis as described (Priest et al. 2006) with some modifications. Tissue (approximately 300 mg) was frozen by liquid N2 and ground to powder in 14 ml polypropylene round-bottomed tubes using 10 mm alumina beads with vortexing. Samples were homogenized with 2 ml of 80% acetone:20% water (v/v) containing 0.1 mg ml⁻¹ 2,4-di-tert-butyl-4-methylphenol. Deuterium-labeled d₆-ABA was purchased from ICON SERVICES (Summit, NJ, USA) and deuterium-labeled d₅-PA, d₅-DPA and d₅-ABA-GE were gifted from Dr Suzanne Abrams (Plant Biotechnology Institute, Saskatoon, Canada), were used as internal standards. After adding 1 ng of internal standards, the homogenate was incubated in darkness for 12 h at 4 °C and centrifuged at 2,580 × g for 10 min at 4 °C. The precipitate was re-extracted for 1 h, and the combined supernatant was dried under vacuum. Following resuspension in 1 ml of 99% isopropanol:1% acetic acid (v/v) by vortexing and sonication, samples were centrifuged (16,000 × g for 5 min, 4 °C), and the supernatant was transferred to a fresh tube and then dried again. Samples were dissolved in 50 μl of methanol and 450 μl of 1% acetic acid solution (v/v) was added. Oils in the samples were removed by partitioning using 1 ml of hexane, and following centrifugation (16,000 × g for 5 min, 4 °C), the remaining aqueous extracts were again removed to a fresh tube and dried by centrifugation under vacuum. Extracts were dissolved in 100 μl of methanol, and 900 μl of 1% acetic acid solution (v/v) was added. Oasis HLB 1 ml solid-phase extraction cartridges (Waters, Milford, MA, USA) were conditioned with 1 ml of acetonitrile followed by 1 ml of methanol and equilibrated with 1 ml of 1% acetic acid solution (v/v). Samples were loaded, followed by a wash with 1 ml of 1% acetic acid solution (v/v). ABA and its metabolites were eluted using 1 ml of 50% acetonitrile:49% water:1% acetic acid (v/v) before samples were dried under vacuum. The resulting sample mixture was dissolved in 20 μl of water and 10 μl was injected. ABA and its catabolites were quantified by an LC-MS/MS/Q-Tof Premier system. The LC equipped with a 2.1 mm × 50 mm × 1.7 mm ACCQUITY UPLC BEH C₁₈ column (Waters) was used with a binary solvent system comprising water (A) and acetonitrile containing 0.05% acetic acid (B). Separations were performed using a gradient of increasing solvent B content with a flow rate of 0.2 ml min⁻¹. The gradient was increased linearly from 3% B to 20% B over 25 min and then 98% B at 30 min. After 10 min of 98% B, the initial condition was restored and allowed to equilibrate for 5 min. The retention times of the compounds were 9.1 min (DPA and d₅-DPA), 15.5 min (PA and d₅-PA), 16.5 min (ABA-GE and d₅-ABA-GE), 23.3 min (d₆-ABA) and 23.6 min (ABA-GE). MS/MS conditions were as follows: capillary (kV)=2.6, source temperature (°C)=80, desolvation temperature (°C)=400, cone gas flow (liter h⁻¹)=0, desolvation gas flow (liter h⁻¹)=500, collision energy=8.0 (ABA and d₅-ABA), 12.0 (PA and d₅-PA, ABA-GE and d₅-ABA-GE), 18.0 (DPA and d₅-DPA), MS/MS transition (m/z): 263/153 (ABA), 269/159 (d₅-ABA), 279/139 (PA), 282/142 (d₅-PA), 281/171 (DPA), 284/174 (d₅-DPA), 425/263 (ABA-GE) and 430/268 (d₅-ABA-GE). The amount of each compound was determined by spectrometer software (MassLynx™ v. 4.1, Micromass).

Functional expression of OsABA8ox1 in yeast

An OsABA8ox1 cDNA was inserted into a yeast expression plasmid, pYeDP60, and the plasmids were transformed into Saccharomyces cerevisiae strain WAT11 (Pompon et al. 1996). OsABA8ox1 function was analyzed as described by Kushiro et al. (2004). An Arabidopsis CYP88A3, which is responsible for gibberellin biosynthesis (Helliwell et al. 2001), was used as a negative control.

Intercellular localization of OsABA8ox1

To link GFP to the C-termini of OsABA8ox1, the open reading frame of OsABA8ox1 (deleted stop codon, TAG) was cloned into the Gateway™ destination binary vector pH7YWG2 (Karimi et al. 2002). This gene was expressed under the control of the cauliflower mosaic virus (CaMV) 35S promoter. This constructed plasmid 35Spro:OsABA8ox1-GFP, the plasmid containing ER-localized GFP (35Spro:GFP-HDEL; GFP fused with the ER targeting signal and retention signal peptide) or the plasmid containing GFP without any targeting signal (35Spro:GFP) was introduced into onion epidermal cell on agar plates by a helium-driven particle accelerator (PDS/1000; Bio-Rad, Hercules, CA, USA) with all basic adjustments set according to the manufacturer’s recommendations. Bombardment parameters were the
following: 1,100 p.s.i. bombardment pressure, 1.6 μm gold particles, a distance of 12 cm from macrocarrier to samples, and a decomposition vacuum of 95,000 Pa. The bombarded onion cells were incubated for 1 d under dark conditions at 22 °C and observed with a fluorescence microscope (Nikon TE2000-U and a 100 x 1.3 numerical aperture objective) and a confocal laser scanning microscope system (MicroRadiance MR/AG-2,Bio-Rad). The samples were illuminated with an argon ion laser (488 nm wavelength) for GFP. All the images were obtained by superimposing several images of the same position, except that the z-axis position of the focal plane was varied by steps of 1.0 μm. All the images were prepared with Photoshop 7.0 (Adobe Systems, Mountain View, CA, USA).

Acknowledgments

We are deeply saddened by the news of the recent death of Dr. Hans Kende, whose initial work on the effect of ethylene on the ABA levels in rice provided the basis for many of our studies. We thank Mr. B. Mitsui (Rohm and Haas Japan K.K., AgroFresh Inc.) for kindly providing 1-MCP, Dr. S. Abrams (Plant Biotechnology Institute, National Research Council of Canada, Saskatoon, Canada) for kindly providing the deuterium-labeled ABA cabalotes, and Dr. T. Ueda (The University of Tokyo) for kindly providing the expression vector of ER targeting GFP (GFP-HDEL). We also thank Dr. K. Ishizawa (Miyagi University of Education) for his stimulating discussions, and Ms. Y. Ozaki (The University of Tokyo) for her assistance. This work was partly supported by grants-in-aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and by a grant of the Ministry of Agriculture, Forestry and Fisheries of Japan to M.N.

References


(Received October 16, 2006; Accepted December 24, 2006)