Journal of Plant Biology, October 2007, 50(5) : 595-599

Ethylene-induced Stem Growth of Deepwater Rice Is Correlated with Expression of Gibberellin- and Abscisic Acid-biosynthetic Genes

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Ethylene decreases the content of endogenous abscisic acid (ABA) and increases the level of bioactive gibberellin A_1 (GA_1) in the submerged internodes of deepwater rice. During partial submergence, internodes of deepwater rice undergo rapid elongation as a result of ethylene accumulation in the internodal lacunae. In an *in vitro* experiment using stem sections from deepwater rice, treatment with 5 $\mu L\ L^{-1}$ ethylene promoted stem growth by up to 3.2-fold times over air treatment. Expression patterns were analyzed for genes that encode GA- and ABA-biosynthesis enzymes to determine any possible molecular basis for the changes observed in GA_1 and ABA contents as a result of ethylene action. Expression of the *OsGA20ox2* and *OsGA20ox4* genes, which encode GA 20-oxidase, and of the *OsGA3ox2* gene, which encodes the enzyme that converts GA_{20} to GA_1, was up-regulated, whereas that of three ABA-biosynthetic genes -- *OsNCED1*, *OsNCED2*, and *OsNCED5* -- was down-regulated in the presence of ethylene. These results indicate that GA and ABA contribute equally to the submergence- or ethylene-induced stem elongation of deepwater rice via the coordinated and opposite regulation of biosynthesis.

Keywords: abscisic acid, deepwater rice, ethylene, gibberellin, stem elongation

Deepwater rice is used as a model system for studying plant growth because of its remarkable ability to elongate stems under partial submergence (Kende et al., 1998). This rapid stem growth is induced by an environmental signal (submergence) and is mediated by the interactions of at least three hormones: ethylene, abscisic acid (ABA), and gibberellins (GA) (Kende et al., 1998). Upon submergence, the gas atmosphere and the hormone ratio in the internodes change dramatically, i.e., the ethylene level increases, which leads to a rapid reduction (by 75%) in ABA content while the amount of GA_1, which is the bioactive form in rice, increases four-fold (Hoffmann-Benning and Kende, 1992). Because ABA is a strong antagonist of GA action in those internodes, its reduced level renders them more responsive to GA (Raskin and Kende, 1984; Hoffmann-Benning and Kende, 1992). Therefore, gibberellin is considered the ultimate plant hormone that promotes the elongation of deepwater rice internodes. GA also induces ethylene production in those tissues (Azuma et al., 1994), indicating a synergistic interaction between these two growth regulators.

Even though physiological explanations have been presented for submergence-induced stem elongation, little is known about the regulation of hormone metabolism at the molecular level. Rice GA metabolism genes have been well characterized (Grennan, 2006). Among the enzymes involved in GA metabolism, GA 20-oxidase, GA 3-oxidase, and GA 2-oxidase are critical to the modulation of endogenous GA levels, and the genes that encode them comprise a multigene family (Sakamoto et al., 2004). Benschop et al. (2006) have shown that submergence enhances expression of the *RpGA3ox1* gene encoding GA 3-oxidase in *Rumex palustris*, and have suggested that this signal induces petiole elongation by regulating GA-biosynthetic genes, thus implying that ethylene can control GA metabolism at the gene expression level.

Although ethylene is generally thought to suppress plant growth, there is recent evidence for a positive role as well (Rose-John and Kende, 1985; Azuma et al., 1990; Furukawa et al., 1997; Voesenek et al., 1997; Kim, 2006; Steffens et al., 2006; Benschop et al., 2007). Pierik et al. (2006) have proposed a biphasic response model in which ethylene either inhibits or promotes development in a dose-dependent manner. Ethylene is known to interact or cross-talk with ABA, especially in biosynthesis and signaling pathways (Hansen and Grossmann, 2000; Sharp, 2002; LeNoble et al., 2004; Tanaka et al., 2005).

Genes encoding ABA 8′-hydroxylases, the key enzymes in ABA catabolism, have now been identified and characterized in rice (Yang and Choi, 2006; Saika et al., 2007). At least one of them is tightly up-regulated by ethylene treatment, indicating that those genes are responsible for the decreased amount of endogenous ABA in the presence of ethylene. However, that level is regulated not only by ABA catabolism but also by its biosynthesis. In the latter process, zeaxanthin epoxidase (ZEP) and 9-*cis*-epoxycarotenoid dioxygenase (NCED) are ABA-specific, and they mediate critical reaction steps toward ABA biosynthesis (Xiong and Zhu, 2003). Benschop et al. (2005) have observed that ethylene down-regulates the expression of an ABA-biosynthetic gene, *RpNCED*, which partly explains why endogenous ABA decreases under submergence or in the presence of ethylene. In the growth of lowland rice seedlings, ethylene does not strongly change the expression level of ABA-biosynthetic genes, whereas submergence reduces their expression (Saika et al., 2007).

The objective of this study was to analyze the expression patterns of genes that encode enzymes for GA metabolism and ABA biosynthesis during ethylene-induced stem elongation in deepwater rice.

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MATERIALS AND METHODS

Plant Material

Rice (*Oryza sativa* L., cv Pin Gaew 56) seeds obtained from the International Rice Research Institute (Los Baňos, Philippines) were germinated on wet filter paper in the dark at 30°C for 4 d. The plants were grown in a growth chamber according to the method of Stünzi and Kende (1989).

Ethylene Treatment, Growth Measurement, and RNA Preparation

To assess the effect of ethylene on growth, 15-cm-long stem sections were prepared as described previously (Yang and Choi, 2006), but were incubated in closed glass jars rather than within a continuous gas flow system. Stem sections were pre-incubated for 2 h and then further incubated in the presence of ethylene (5 μL L^{-1}) or air. During incuba-

Table 1. Primer sequences used in this study

Gene name	Primer	Primer sequence (5′ – 3′)	Reference
1. Primers for semi-quantitative RT-PCR of genes for GA metabolism enzymes			
OsGA20ox1	HK1289	AATGAGCATGGTGGTGCAGCAGGAGCAG	Sakamoto et al., 2004
	HK1290	GTTAACCACCAGGAAGAAGCCGTGCCTC	
OsGA20ox2	HK1291	TACTACAGGGAGTTCTTCGCGGACAGCA	Sakamoto et al., 2004
	HK1292	TGTGCAGGCAGCTCTTATACCTCCCGTT	
OsGA20ox3	HK1293	GTGGAAGGAGACCATGTCGTTCAACTGC	Sakamoto et al., 2004
	HK1294	TCATCACGTCGCAGTACTCCTGGTACAC	
OsGA20ox4	HK1295	AACAACTCCTCCACCGTCGCCGATTACT	Sakamoto et al., 2004
	HK1296	TGATCGAGCTGCTGTCCTCGAAGAACTC	
OsGA3ox1	HK1283	ATGGAGGAGTACGACTCGTCGATGAGAG	Sakamoto et al., 2004
	HK1284	CTCTGCAGGATGAAGGTGAAGAAGCCTG	
OsGA3ox2	HK1285	TCTCCAAGCTCATGTGGTCCGAGGGCTA	Sakamoto et al., 2004
	HK1286	TGGAGCACGAAGGTGAAGAAGCCCGAGT	
OsGA2ox1	HK1297	CGAGCAAACGATGTGGAAGGGCTACAGG	Sakamoto et al., 2004
	HK1298	TGGCTCAGGCGGAGTGAGTACATTGTCG	
OsGA2ox3	HK1299	TTCTTCGTCAACGTCGGCGACTCGTTGC	Sakamoto et al., 2004
	HK1300	TCTCAAACTGGGCCAGCCTGTTGTCTCC	
OsGA2ox4	HK1301	GCGTGCGAGAGGTTTGGGTTCTTCAAGG	Sakamoto et al., 2004
	HK1302	CTCCGCCACCATCTCCAGCACCGTCC	
OsAct1	HK1273	GGCTATGAAAGCCTCGACAA	
	HK1274	GTGTGATATTCCGTAGATTGA	
2. Primers for semi-quantitative RT-PCR of genes for ABA-biosynthesis enzymes			
OsZEP	ZEP-F1	GGTGCGATAACGTCGTTGATC	Saika et al., 2007
	ZEP-R1	GTATGGTCTATAAGTGGTAGC	
OsNCED1	NCED1-F2	CGAGAACCGGTTCGTGGTGA	
	NCED1-R2	TCTCGAAGTATGTGTGCACTT	
OsNCED2	NCED2-F2	AGAGAGTTGGTTTGTGGTGA	
	NCED2-R2	TCAAATGCTGGAAAGTACAT	
OsNCED3	HK1324	CAAACCATCCAAACCGACGA	
	HK1325	GCATATCCTGGCGTCGTGAT	
OsNCED4	HK1326	GAATCCATCTCCTTCTCCCT	
	HK1327	CTCGCACCCTGCTTGATCTT	
OsNCED5	NCED5-F3	AACTTCGCGCCGGTGGGGAGCAG	
	NCED5-R3	CGGTGAAGCGGCAGGCGTAGGACT	
17s rRNA	17s F	TCCTACCGATTGAATGGTCC	Saika et al., 2007
	17s R	CTTGTTACGACTTCTCCTTCCTC	

tion, the relative humidity was kept near 100% by means of wet filter paper inside the jars. At various intervals, stem sections were removed, and their growth measured. The basal 1-cm portions containing the intercalary meristems and elongation zones in the stem sections were then harvested and immediately frozen in liquid nitrogen.

Semi-quantitative RT-PCR

First-strand cDNA was prepared from total RNA isolated from frozen stem sections, as described previously (Choi et al., 2004), and was used as template for semi-quantitative RT-PCR. The optimum number of PCR cycles for each gene was determined empirically, and was adopted in semi-quantitative PCR reactions. PCR was performed with TaKaRa Ex Taq™ DNA polymerase (Takara Bio Inc., Otsu, Shiga, Japan) and various primer sets (Table 1) over cycles of 98°C for 10 s, 52°C for 30 s, and 72°C for 30 s. The *OsGA20ox* and *OsGA3ox* gene families were chosen for GA biosynthesis and the *OsGA2ox* gene family was used for GA catabolism. To check ABA biosynthesis, *OsZEP*, *OsNCED1*, *OsNCED2*, *OsNCED3*, *OsNCED4*, and *OsNCED5* genes were analyzed. Nucleotide sequences for the *OsNCED* genes were retrieved from the GenBank database (Accession Numbers: *OsNCED1*, AY838897; *OsNCED2*, AY838898; *OsNCED3*, AY838899; *OsNCED4*, AY838900; and *OsNCED5*, AY838901) (http://www.ncbi.nlm.nih.gov/), and corresponding primer sets were designed for PCR reactions.

Primer sequences for the GA metabolism genes and the *OsZEP* gene were those reported by Sakamoto et al. (2004) and Saika et al. (2007). Semi-quantitative RT-PCR was repeated at least three times for each experiment using RNAs from three independent biological samples.

RESULTS

Effect of Ethylene on Stem Growth

To investigate the effect of ethylene on the elongation of stem tissues from deepwater rice, excised sections were pre-incubated for 2 h to eliminate the effects of wounding and endogenous ethylene, then further incubated in the presence of air or ethylene. Treatment with ethylene for 4 h and 12 h promoted stem elongation by 3.2-fold and 2.5-fold, respectively, compared with the air-only treatment (Fig. 1).

Expression Patterns of GA Metabolism Genes in the Presence of Ethylene

The expression of two groups of genes for GA metabolism enzymes was analyzed by semi-quantitative RT-PCR (Fig. 2). *OsGA20ox* and*OsGA3ox* encode GA synthesis enzymes while the *OsGA2ox* genes encode enzymes that inactivate bioactive GAs. Among the four *OsGA20ox* genes, *OsGA20ox2* and *OsGA20ox4* were tightly up-regulated by ethylene treatment whereas transcripts could not be detected for *OsGA20ox1* or *OsGA20ox3*, with or without ethylene. *OsGA3ox2* also was expressed at 30 min in response to ethylene while no expression was found for *OsGA3ox1*. In contrast, expression of the *OsGA2ox* genes for GA_1-inactivating GA2-oxidases was not influenced or detected following ethylene treatment.

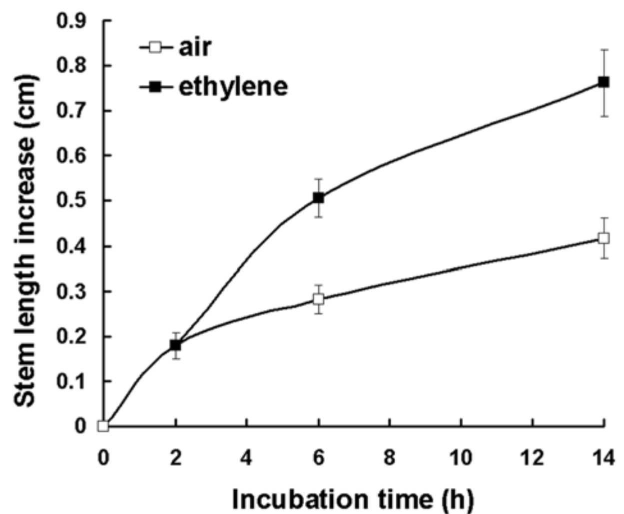

Figure 1. Growth of excised stems in presence or absence of ethylene. Sections (15 cm long) were first incubated for 2 h to eliminate wound-induced and endogenous ethylene effects, then transferred to closed glass jars that were filled with ethylene-free air or air containing 5 mL L^{-1} ethylene, and further incubated for 4 h and 12 h. At each time point, 10 to 15 stem sections were measured. The values represent the means standard error.

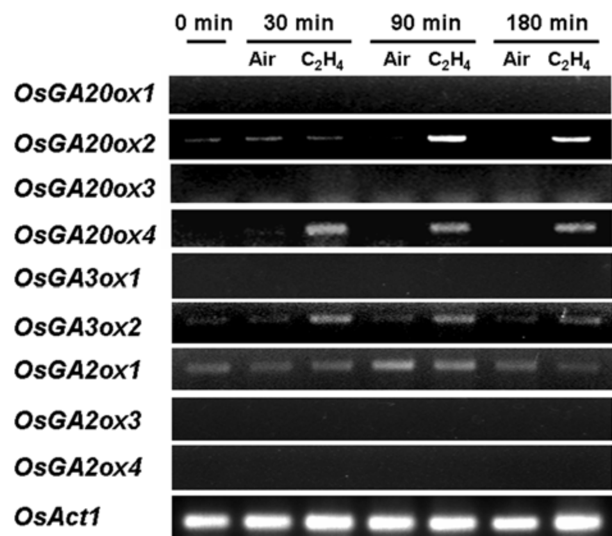

Figure 2. Expression of GA metabolism genes in response to ethylene treatment. Stem sections, 15 cm long, were first incubated for 2 h to remove wound-induced and endogenous ethylene effects, then further incubated for 30, 90, and 180 min in closed glass jars filled with ethylene-free air or air containing 5 mL L^{-1} ethylene. Semi-quantitative RT-PCR was conducted, and *OsAct1* served as internal control. Results are representative of those from repeated experiments.

Expression Patterns of ABA-biosynthetic Genes in the Presence of Ethylene

Expression levels of the ABA-biosynthetic genes were analyzed in the presence of ethylene. Among the five family members, *OsNCED1*, *OsNCED2*, and *OsNCED5* were remarkably down-regulated by ethylene at 180 min. However, expression of *OsZEP*, *OsNCED2*, and *OsNCED5* was low in the beginning of incubation and then increased at 30 min whether or not ethylene was present.

DISCUSSION

It has previously been demonstrated that the accumulation of ethylene is a prerequisite for the submergence-induced stem growth of deepwater rice (Rose-John and Kende, 1985; Hoffmann-Benning and Kende, 1992). Raskin and Kende (1983) have shown that rice seedlings in closed containers did not show any considerable difference in growth over 24 h compared to those in flow-through containers. That is why a closed system was selected for the ethylene research described here. Ethylene-treated stem sections in the glass jars exhibited more elongation than did the control tissues in the glass jars, thus confirming that a closed system provides an excellent alternative system for studying ethylene-induced stem growth.

In deepwater rice, GA_{20} and GA_1 levels increase in submerged internodes (Hoffmann-Benning and Kende, 1992), the former rising by three-fold within 24 h compared with a four-fold elevation in the latter within 3 h of submergence. In this study, expression of *OsGA20ox2* and *OsGA20ox4* appeared to be tightly up-regulated by ethylene (Fig. 2), suggesting that this hormone improves GA_1 biosynthesis partly by enhancing the expression level of those two genes. That effect then leads to greater production of GA_{20} molecules, the immediate precursors to GA_1. In addition, judging by the intensity and promptness of its expression, *OsGA20ox4* may participate early, before *OsGA20ox2* takes control at a later stage of ethylene-induced GA biosynthesis. It is noteworthy that *OsGA20ox2*, also known as *SD1*, is referred to as the 'Green Revolution gene' (Sasaki et al., 2002).

OsGA3ox2 expression was also tightly regulated by ethylene treatment (Fig. 2). That is consistent with a report by Itoh et al. (2001) that *OsGA3ox2* is preferentially expressed in fast-growing organs such as elongating leaves in rice. In the experiment using recombinant enzymes, OsGA3ox2 showed strong specificity over OsGA3ox1 for the reaction products (Itoh et al., 2001). That is, the recombinant protein of *OsGA3ox2* produced only GA_1 from GA_{20}, whereas that of *OsGA3ox1* produced GA_1, GA_3, and GA_8 from GA_{20} (cf., Itoh et al., 2001). Considering that GA_1 is an active gibberellin in rice, it is conceivable that strong expression of *OsGA3ox2* results in a sufficient supply of bioactive GA_1 to induce the growth of deepwater rice stems. Therefore, one aspect of this ethylene action can be attributed, in part, to the enhancement of *OsGA3ox2* expression. Taken together with the expression study of *OsGA20ox* genes described above, these results support the notion that ethylene enhances GA biosynthesis by up-regulating the genes that encode GA biosynthetic enzymes, which ultimately increases the level of GA_1, an active gibberellin in rice.

Rumex palustris has been well studied for its submergence-induced petiole elongation. Hormonal analysis has suggested that ethylene decreases endogenous ABA levels by promoting ABA catabolism and repressing ABA biosynthesis (Benschop et al., 2005). Previously, Yang and Choi (2006) demonstrated that ethylene promotes ABA catabolism by enhancing the expression of *CYP707A5,* which encodes ABA 8′-hydroxylase. In the current study, the expression levels of ABA-biosynthetic genes *OsNCED1*, *OsNCED2,* and *OsNCED5* were decreased significantly within 180 min in response to ethylene (Fig. 3). Therefore, both the up-regulation of ABA catabolism genes and the down-regulation of ABA-biosynthetic genes by ethylene provide the molecular mechanism by which ethylene contributes to the reduction in ABA content during ethylene-induced stem elongation in deepwater rice (Fig. 3). Unexpected behavior was seen from *OsZEP*, *OsNCED2*, and *OsNCED5* during the 30-min incubation period. In fact, these genes were probably induced by an unknown environmental stress when the stem sections were being prepared from whole plants at 2.5 h before incubation. Nevertheless, one can conclude that *OsNCED1*, *OsNCED2,* and *OsNCED5* are down-regulated by ethylene treatment. This result differs from that of Saika et al. (2007), who reported that ethylene does not strongly affect the expression of ABA-biosynthetic genes. Such a discrepancy may be due to physiological differences in growth between lowland rice seedlings and deepwater rice stems.

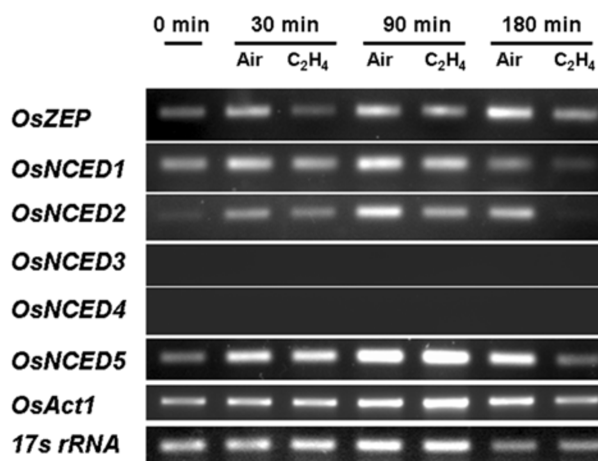

Figure 3. Expression of ABA-biosynthetic genes in response to ethylene treatment. Stem sections, 15 cm long, were first incubated for 2 h to remove wound-induced and endogenous ethylene effects, then further incubated for 30, 90, and 180 min in closed glass jars filled with ethylene-free air or air containing 5 mL L^{-1} ethylene. Semi-quantitative RT-PCR was conducted, and *OsAct1* and *rice 17s rRNA* genes were used as internal controls. Results are representative of those from repeated experiments.

In summary, molecular evidence now exists for the ethylene-mediated stem elongation found in deepwater rice. It is proposed here that ethylene may regulate the endogenous levels of GA and ABA by promoting GA-biosynthetic gene expression while simultaneously suppressing the expression of ABA-biosynthetic genes. This dual effect of ethylene seems to maximize the responsiveness of stem tissues to GA by increasing GA content and sensitivity.

ACKNOWLEDGEMENT

This work was supported by a research grant from the Crop Functional Genomics Center of the 21st Century Frontier Research Program (Code No. CG2151).

Received August 28, 2007; accepted September 7, 2007.

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