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【Short Report】

Cloning of a Cytochrome P450 Gene Induced by Ethylene Treatment in Deepwater Rice (*Oryza sativa* L.)

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Deepwater rice (*Oryza sativa* L.) is a subsistence crop in some areas of Southeast Asia which is flooded during the rainy season. This rice is of great agricultural importance, since it is the only crop that can be grown in this area. Therefore, an understanding of the growth physiology of deepwater rice is significant. Survival of this rice depends on elongating rate when it becomes submerged. In the field, growth rates of up to 25 cm d^{-1} have been recorded (Vergara et al., 1976). It has been reported that submerged plants respond to an altered internal gas atmosphere (Kende et al., 1998). Ethylene production is required for the stimulation of growth in submerged deepwater rice plant, and in fact, exogenously applied ethylene enhances internodal elongation of deepwater rice (Metraux and Kende, 1983).

In plants, cytochrome P450 constituted the largest group of enzymes associated with syntheses and metabolism of including second metabolites and plant hormone, such as gibberellin (Donaldson and Luster, 1991; Rojas et al., 2001) and brassinolide (Sakamoto and Matsuoka, 2006). However, knowledge regarding the role of cytochrome P450 in internode elongation of deepwater rice in terms of ethylene physiology has not yet been known.

We have already isolated an *Os-ERL1* gene from deepwater rice encoding an ethylene receptor gene, similar to those of *Arabidopsis thariana* ETR2 and EIN4 (Watanabe et al., 2004). In addition to the previous report (Watanabe et al., 2004), to understand the regulation of growth in deepwater rice in detail, we here isolated a cDNA clone for the first time encoding for the cytochrome P450 gene whose expression was increased by ethylene treatment in deepwater rice.

Materials and Methods

Seeds of deepwater rice (*Oryza sativa* L. cv. Pin Gaew 56) were obtained from the International Rice Research Institute (Los Baños, Philippines). Rice was germinated and grown as described by Stunzi and Kende (1989). Twenty-centimeter-long stem sections containing the growing internodes were excised from 12-week-old plants. Stem sections were placed in an upright position in a 300 ml glass beaker containing 40 ml distilled water. Each beaker containing the sections was placed in a 5.5 L desiccator with a glass inlet tube fitted with a rubber cap through which ethylene was introduced using a gas-tight syringe when necessary. Stem sections were incubated for 3 hr in 10 μl l^{-1} ethylene or under ethylene-free conditions as control (Metraux and Kende, 1983; Suge, 1985). For maintaining ethylene-free conditions, three 50 ml glass beakers filled with Purafil (Purafil Inc., Atlanta, GA) were enclosed into a desiccator to deplete any endogenously evolved ethylene. Total RNA was isolated from ethylene-treated and control rice internodes (Verwoerd et al., 1989). Differential display analysis (Liang and Pardee, 1992) was performed using the RNAimage™ Kit (GenHunter Corp., Nashville, TN).

Results and Discussion

Firstly, a partial-length cDNA was cloned by differential display with H-T_{11}A (AAGCT_{11}A) as a 3' primer and CGCCATTCGG as a 5' primer. This cDNA gene contained the PFG motif (PFGXGRRCXG), which is a highly conserved domain in the heme-binding region of cytochrome P450 gene (Holton

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Abbreviations: Acc. No., accession number; EIN4, ETHYLENE INSENSITIVE 4; EST, expressed sequence tag; EtBr, ethidium bromide; ETR2, ETHYLENE RESPONSE 2; *Os-ERC1*, *Oryza sativa* ETHYLENE RESPONSE CYTOCHROME 1; *Os-ERL1*, *Oryza sativa* ETHYLENE RESPONSE 2 LIKE 1; PCR, polymerase chain reaction.

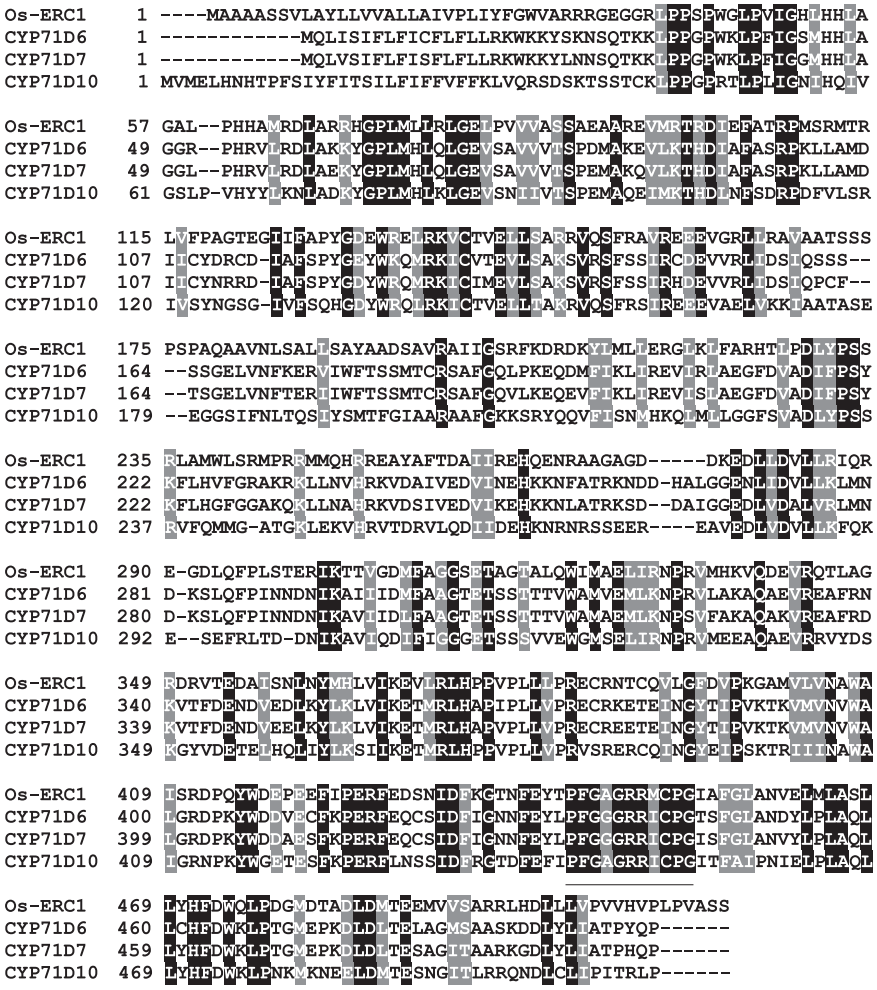

Fig. 1. Alignment of Os-ERC1 with CYP71D sequences. The Os-ERC1 amino acid sequence is aligned with CYP 71D6 (Acc. No. P93530) and CYP 71D7 (Acc. No. P93531) from *Solanum chacoense* (Hutvagner et al., 1997), CYP71D10 (Acc. No. O48923) from *Glycine max* (Siminszky et al., 1999), by ClustalW 1.8 program available at http://mbcr.bcm.tmc.edu/searchlauncher. The heme-binding domain is underlined. -, gaps to align the amino acid sequence.

and Lester, 1996; Schopfer and Ebel, 1998). The rice expressed sequence tag (EST: S0564), whose deduced amino acid sequence shows a similarity to the partial-length cDNA, was obtained from the National Institute of Agro Biological Resources, Tsukuba, Japan. Sequence analysis of S0564 indicated a full-length cDNA. Subsequently, we designed PCR primers derived fromS0564,CAGAATTCGAGCTCCTTCAGTTCA ATCC(fwd)andTCATCGATGTTCGCCGTTGGACT TTA (rev), and PCR amplifications were carried out using total RNA from ethylene-treated stem segments. PCR products were purified by gel electrophoresis and cloned into pBluescript Ⅱ SK(−) for sequencing. We isolated a full-length cDNA and designated the gene as *Os-ERC1* (*Oryza sativa* ETHYLENE RESPONSE CYTOCHROME 1. Acc. No. AB290211). *Os-ERC1* contained a PFG motif, which is a highly conserved domain in the heme-binding region of cytochrome P450 gene. The isolated *Os-ERC1* has an open reading frame for a protein of 516 amino acids, and a predicted molecular weight of 58 kDa. The rice genomic databases according to the procedure proposed by Yuan et al. (2000) revealed that the *Os-ERC1* gene probably resided 99.2 cM from the top of chromosome IV. Comparing the alignment of the derived amino acid sequences between *Os-ERC1* and known full-length cytochrome P450, revealed that *Os-ERC1* had a relatively low similarity with cytochrome P450, such as CYP71D7 (38% identity; Hutvagner et al., 1997), CYP71D10 (38% identity; Siminszky et al., 1999), CYP71D6 (36% identity; Hutvagner et al., 1997) (Fig. 1). The function of these proteins is unknown. Given its low degree of similarity with these (<40% identity) and other plant P450s, this *Os-ERC1* of cytochrome P450 has been placed in a new cytochrome P450 family (Nelson et al., 1996). Consequently, this cytochrome

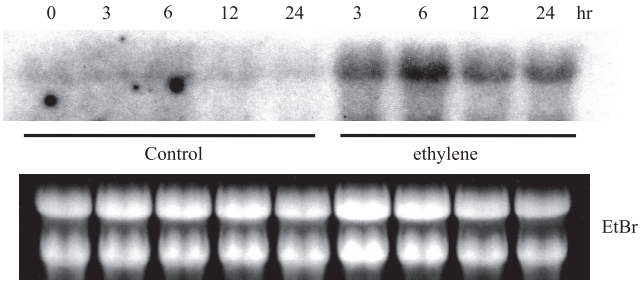

Fig. 2. Northern blot analysis of the *Os-ERC1* gene in rice. Rice stem sections were incubated in with or without 10μl/l ethylene for the times indicated above the lanes. Thirty micrograms of total RNA isolated from control and ethylene treated deepwater rice stem sections including intercalary meristem were electrophoresesed on a 1.2% agarose-formaldehyde gel, blotted onto a Hybond-N membrane, and hybridized to ^{32}P-labelled cDNA probes for *Os-ERC1* gene. Also shown is a photograph of an ethidium bromide-stained gel of the RNA used for the experiment (EtBr).

P450 gene was assigned CYP71K9 based on the nomenclature of Nelson et al. (1996). RNA gel blot analysis showed that *Os-ERC1* mRNA levels increased by ethylene treatment compared to that of the control, and the maximum increment of its transcript was observed 6 hrs after ethylene treatment (Fig. 2).

Cytochrome P450 is involved in biosyntheses and metabolism of plant hormone, such as gibberellin (Donaldson and Luster, 1991; Rojas et al., 2001) and brassinolide (Sakamoto and Matsuoka, 2006). Expression of this gene was also induced by abscisic acid and jasmonate in *Solanum chacoense* leaves. (Hutvagner et al., 1997).

From the above results, it appears that *Os-ERC1* belongs to a novel class of cytochrome P450, and may play a role, directly or indirectly, in ethylene-induced internode elongation of deepwater rice. However, further expression analysis of *Os-ERC1* in several systems (ex. the yeast two-hybrid system) needs to define the function clearly of the protein product of this gene.

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