Molecular Plant • Volume 5 • Number 6 • Pages 1359–1374 • November 2012 RESEARCH ARTICLE

OsDMI3 Is a Novel Component of Abscisic Acid Signaling in the Induction of Antioxidant Defense in Leaves of Rice

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ABSTRACT **Ca^{2+} and calmodulin (CaM) have been shown to play an important role in abscisic acid (ABA)-induced antioxidant defense. However, it is unknown whether Ca^{2+}/CaM-dependent protein kinase (CCaMK) is involved in the process. In the present study, the role of rice CCaMK, OsDMI3, in ABA-induced antioxidant defense was investigated in leaves of rice (*Oryza sativa*) plants. Treatments with ABA, H_2O_2, and polyethylene glycol (PEG) induced the expression of *OsDMI3* and the activity of OsDMI3, and H_2O_2 is required for the ABA-induced increases in the expression and the activity of OsDMI3 under water stress. Subcellular localization analysis showed that OsDMI3 is located in the nucleus, the cytoplasm, and the plasma membrane. The analysis of the transient expression of *OsDMI3* in rice protoplasts and the RNA interference (RNAi) silencing of *OsDMI3* in rice protoplasts showed that OsDMI3 is required for ABA-induced increases in the expression and the activities of superoxide dismutase (SOD) and catalase (CAT). Further, the oxidative damage induced by higher concentrations of PEG and H_2O_2 was aggravated in the mutant of *OsDMI3*. Moreover, the analysis of the RNAi silencing of *OsDMI3* in protoplasts and the mutant of *OsDMI3* showed that higher levels of H_2O_2 accumulation require OsDMI3 activation in ABA signaling, but the initial H_2O_2 production induced by ABA is not dependent on the activation of OsDMI3 in leaves of rice plants. Our data reveal that OsDMI3 is an important component in ABA-induced antioxidant defense in rice.**

Key words: OsDMI3; abscisic acid; antioxidant defense; H_2O_2; oxidative stress; signal transduction.

INTRODUCTION

Water stress, including drought and salinity, is one of the most important environmental factors that affect plant growth and development, and limit crop production. Plants can respond and adapt to water stress by perceiving the stimulus, generating and transmitting the signals, and initiating various defense mechanisms. The plant hormone abscisic acid (ABA) is the central regulator of water stress resistance in plants, and coordinates a complex regulatory network enabling plants to cope with decreased water availability (Cutler et al., 2010; Hubbard et al., 2010; Joshi-Saha et al., 2011). Under drought or salinity stress conditions, plants accumulate increased amounts of ABA, which stimulate stomatal closure, changes in gene expression, the accumulation of osmo-compatible solutes, and the up-regulation of antioxidant defense systems, thus increasing the plant's capacity to cope with stress conditions (Seki et al., 2007; Cutler et al., 2010; Hubbard et al., 2010; Joshi-Saha et al., 2011).

It has been documented that calcium (Ca^{2+}), reactive oxygen species (ROS), and nitric oxide (NO) are important signal molecules of ABA signaling in plant cells (Cho et al., 2009). Cytosolic Ca^{2+} is a universal second messenger and acts as a mediator of stimulus–response coupling in the regulation of plant growth, development, and responses to environmental stresses. Various stimuli, such as cold, heat shock, salinity, drought, mechanical disturbances, ABA, hydrogen peroxide (H_2O_2), and pathogen elicitors, trigger changes in the cytosolic Ca^{2+} concentration (Yang and Poovaiah, 2003; Zhang

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doi:10.1093/mp/sss068, Advance Access publication 5 August 2012
Received 4 February 2012; accepted 21 May 2012

and Lu, 2003; Lecourieux et al., 2006; DeFalco et al., 2010). Transient Ca^{2+} elevations are sensed by several Ca^{2+} sensors such as calmodulin (CaM) and CaM-like protein (CML), calcium-dependent protein kinase (CDPK), calcineurin B-like protein (CBL), and Ca^{2+}/CaM-dependent protein kinase (CCaMK) (Yang and Poovaiah, 2003; Zhang and Lu, 2003; Harper et al., 2004; Kim et al., 2009; DeFalco et al., 2010). These Ca^{2+} sensors function to regulate diverse downstream targets, which result in various physiological responses (Kim et al., 2009; DeFalco et al., 2010).

CCaMK is thought to be a decoder of Ca^{2+} spiking, on the basis of its domain structure, which is composed of a serine/threonine kinase domain, a calmodulin (CaM) binding domain, and three EF-hand motifs that potentially trap Ca^{2+} ions (Levy et al., 2004; Yang et al., 2007; DeFalco et al., 2010; Hayashi et al., 2010). CCaMKs have been isolated from lily, tobacco, maize, rice, wheat, *Lotus japonicus*, *Medicago trunculata*, and *Sesbania rostrata* (Yang and Poovaiah, 2003; Zhang and Lu, 2003; Harper et al., 2004; Levy et al., 2004; Mitra et al., 2004; Godfroy et al., 2006; Tirichine et al., 2006; Chen et al., 2007; Capoen et al., 2009; DeFalco et al., 2010; Hayashi et al., 2010; Yang et al., 2011). However, no CCaMK has been identified in the *Arabidopsis thaliana* genome (Harper et al., 2004; DeFalco et al., 2010). In *Medicago trunculata* and *Lotus japonicus*, *CCaMK* (termed *MtDMI3* and *LjCCaMK*, respectively) is a component of the common symbiosis genes that are required for both root nodule (RN) and arbuscular mycorrhiza (AM) symbioses (Levy et al., 2004; Mitra et al., 2004; Tirichine et al., 2006; Messinese et al., 2007; Yano et al., 2008; Hayashi et al., 2010). In rice, *CCaMK* (*OsDMI3*) is not only required for AM symbiosis, but also is able to rescue *MtDMI3* knockouts, indicating an equivalent role of *MtDMI3* orthologs in non-legumes (Godfroy et al., 2006; Chen et al., 2007). In addition, the preferential expression of CCaMK in developing anthers and root tips (Patil et al., 1995; Poovaiah et al., 1999) suggested that CCaMK may play a role in mitosis and meiosis (Yang and Poovaiah, 2003). A recent study showed that the wheat CCaMK gene, *TaCCaMK*, was down-regulated by ABA, NaCl, and PEG treatments in wheat seedlings roots (Yang et al., 2011). The overexpression of *TaCCaMK* in *Arabidopsis* reduced ABA sensitivity of *Arabidopsis* during seed germination and seedling growth. These results suggest that *TaCCaMK* is a negative regulator for ABA signaling involved in abiotic stress responses in wheat.

Previous studies showed that Ca^{2+}/CaM is required for ABA-induced antioxidant defense, including the up-regulation in the expression and the activities of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and glutathione reductase (GR), and the crosstalk between Ca^{2+}/CaM and H_2O_2 and NO plays a pivotal role in the ABA signaling in leaves of maize seedlings (Jiang and Zhang, 2003; Hu et al., 2007; Sang et al., 2008). In animal cells, Ca^{2+}/CaM-dependent protein kinases (CaMKs) have been shown to be involved in H_2O_2 signal transduction that results in the regulation of various cellular processes (Nguyen et al., 2004; Bouallegue et al., 2009; Palomeque et al., 2009). These findings let us investigate whether CCaMK is involved in the ABA signaling leading to the up-regulation of antioxidant defense systems in plants. Here, we show that rice CCaMK, OsDMI3, is required for ABA-induced antioxidant defense and oxidative stress tolerance in leaves of rice plants exposed to water stress. These results identify a novel function of CCaMK in plants.

RESULTS

ABA, H_2O_2, and PEG Induce the Expression of *OsDMI3* and the Activity of OsDMI3 in Rice Leaves

To investigate the effects of ABA, H_2O_2, and polyethylene glycol (PEG) on the induction of *OsDMI3* gene in leaves of rice seedlings, relative quantitative real-time PCR analysis was performed on total RNA isolated from rice plants treated with 100 μM ABA, 10 mM H_2O_2, and 10% PEG. All these treatments induced a rapid increase in the expression of *OsDMI3* (Figure 1A). Treatments with ABA and H_2O_2 induced a biphasic response, in which the first peak occurred after 15 min (H_2O_2 treatment) or 30 min (ABA treatment) of treatments, and the second peak appeared within 240 min of treatments, in the expression of *OsDMI3* (Figure 1A). However, PEG treatment only induced a peak appeared after 60 min of PEG treatment.

To investigate the effects of ABA, H_2O_2, and PEG on the activation of OsDMI3 in leaves of rice plants, immunoprecipitation, and in-gel kinase assay were carried out on protein extracts from the leaves of rice seedlings treated by ABA, H_2O_2, and PEG, using OsDMI3 antibody raised against the C-terminal peptide, histone S-III as a substrate. Treatments with ABA, H_2O_2, and PEG also caused a rapid increase in the activity of OsDMI3 in rice leaves (Figure 1B), and a similar change pattern between the activity of OsDMI3 and the expression of *OsDMI3* (Figure 1B) was observed in leaves of rice plants exposed to ABA, H_2O_2, and PEG.

To prove the specificity of the antibody, immunoprecipitations with or without peptide competitors were carried out and immune complexes were assayed for kinase activity. Proteins that could phosphorylate histone S-III were precipitated from extracts of leaves of rice plants. The immune complexes were competed out by the peptide used to raise the antibody against the C-terminal region of OsDMI3 but not by the peptide that corresponds to the N-terminal segment of OsDMI3 (Figure 1C).

H_2O_2 Is Required for the ABA-Induced Increases in the Expression and the Activity of OsDMI3 under Water Stress

To determine whether PEG-induced increases in the expression of *OsDMI3* and the activity of OsDMI3 are related to the action of endogenous ABA, rice seeds were pretreated by an inhibitor of ABA biosynthesis, fluridone, and then the pretreated plants exposed to PEG treatment. Pretreatment with fluridone blocked the PEG-induced increases in the expression