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Functional characterization of xanthoxin dehydrogenase in rice

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ABSTRACT

Abscisic acid (ABA) is a phytohormone that plays a key role in biotic and abiotic stress responses. ABA metabolic genes are promising targets for molecular breeding work to improve stress tolerance in crops. The accumulation of ABA does not always improve stress tolerance since stress-induced accumulation of ABA in pollen inhibits the normal course of gametogenesis, affecting grain yields in cereals. This effect highlights the importance of manipulating the ABA levels according to the type of tissues. The aim of this study was to assign an ABA biosynthetic enzyme, xanthoxin dehydrogenase (XanDH), as a functional marker to modulate ABA levels in rice. XanDH is a member of the short-chain dehydrogenase/reductase family that catalyzes the conversion of xanthoxin to abscisyl aldehyde (ABAld). Previously, this enzyme had only been identified in *Arabidopsis*, as *AtABA2*. In this study, a XanDH named OsABA2 was identified in rice. Phylogenetic analysis indicated that a single gene encodes for *OsABA2* in the rice genome. Its amino acid sequence contains two motifs that are essential for cofactor binding and catalytic activity. Expression analysis of *OsABA2* mRNA showed that the transcript level did not change in response to treatment with ABA or dehydration. Recombinant OsABA2 protein expressed in *Escherichia coli* converted xanthoxin to ABAld in an NAD-dependent manner. Moreover, expression of OsABA2 in an *Arabidopsis aba2* mutant rescued the *aba2* mutant phenotypes, characterized by reduced growth, increased water loss, and germination in the presence of paclobutrazol, a gibberellin biosynthesis inhibitor or high concentration of glucose. These results indicate that OsABA2 is a rice XanDH that functions in ABA biosynthesis.

Introduction

Abscisic acid (ABA) is a phytohormone that regulates various physiological processes, including stress responses, seed maturation, seed dormancy, and stomatal closure (Nambara and Marion-Poll, 2003; Kim et al., 2010; Fujita et al., 2011). ABA is also involved in plant developmental processes, such as lateral root growth (Brady et al., 2003; Deak and Malamy, 2005; Duan et al., 2013) and guard cell formation (Arend et al., 2009; Tanaka et al., 2013). To adapt to their environments, plants actively change their ABA levels in response to environmental or developmental stimuli by controlling the balance between ABA biosynthesis and catabolism.

Rice is an important cereal worldwide and is a staple food for a significant part of the world's population. Reductions in rice yield caused by abiotic stresses are a continuous problem threatening the stability of food production and the food supply. To resolve these problems, we need to generate a rice variety with improved stress tolerance. Genetic manipulation of ABA biosynthetic genes has been used to develop stress tolerance by increasing the level of ABA in plants (Iuchi et al., 2001; Qin and Zeevaart, 2002; Hwang et al., 2010).

Grain yields of cereals are severely decreased when cereals are subjected to stress at the flowering stage. Ji et al. (2011)

Abbreviations: ABA, abscisic acid; ABAld, abscisyl aldehyde; AO, aldehyde oxidase; CBB, Coomassie brilliant blue; HA, human influenza hemagglutinin; HPLC, high-performance liquid chromatography; LC–MS, liquid chromatography–mass spectrometry; MOSU, molybdenum cofactor sulfurase; NCED, 9-*cis*-epoxycarotenoid dioxygenase; QRT-PCR, quantitative reverse transcription polymerase chain reaction; SDR, short-chain dehydrogenase/reductase; Xan, xanthoxin; XanDH, xanthoxin dehydrogenase; ZEP, zeaxanthin epoxidase.

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demonstrated that a drought-tolerant wheat variety produced a higher grain yield than a drought-susceptible variety under drought stress at the flowering stage, and that the tolerance was strongly associated with a reduction in ABA in pollen. This finding revealed that reducing the amount of ABA in pollen is necessary in order to improve plant stress tolerance at the flowering stage. Therefore, manipulation of ABA biosynthesis may improve stress tolerance not only by increasing ABA levels in some tissues but also by decreasing ABA in other tissues.

The ABA biosynthetic pathway in higher plants has been largely established (Nambara and Marion-Poll, 2005). Isolation and characterization of ABA-deficient mutants have revealed that ABA is synthesized from epoxycarotenoid in plastids. The epoxycarotenoid violaxanthin is synthesized by zeaxanthin epoxydase (Marin et al., 1996; Xiong et al., 2002). Violaxanthin is converted to neoxanthin by neoxanthin synthase, a reaction that requires *AtABA4* (Dall'Osto et al., 2007; North et al., 2007). Neoxanthin and violaxanthin are converted to 9-*cis* isomer by an unknown isomerase. A rate-limiting step in ABA biosynthesis is the oxidative cleavage of 9-*cis*-epoxycarotenoid to produce xanthoxin (Xan), catalyzed by 9-*cis*-epoxycarotenoid dioxygenase (NCED) in plastids (Schwartz et al., 1997b; Tan et al., 1997). In the cytosol, xanthoxin dehydrogenase (XanDH) catalyzes the conversion of Xan to abscisyl aldehyde (ABAld), which is converted to ABA by aldehyde oxidase (AO) (Seo et al., 2000; Cheng et al., 2002; González-Guzmán et al., 2002; Seo et al., 2004). *Arabidopsis ABA3* encodes a molybdenum cofactor sulfurase, that modulates AO activity (Bittner et al., 2001; Xiong et al., 2001b). The genes that code for NCED have been extensively characterized in various plant species (Schwartz et al., 1997b; Burbidge et al., 1999; Qin and Zeevaart, 1999; Chernys and Zeevaart, 2000; Iuchi et al., 2000, 2001; Zhu et al., 2007; Qin et al., 2008; Chen et al., 2011; Xian et al., 2014). The genes associated with XanDH, an enzyme involved in ABA biosynthesis, have been identified only in *Arabidopsis* as *AtABA2/GIN1/AtSDR1* (Cheng et al., 2002; González-Guzmán et al., 2002).

Arabidopsis aba2 mutants have been used extensively for physiological analysis, due to the clear and ABA-specific phenotypes in *Arabidopsis*. The ABA-deficient mutant phenotype is obvious because a single gene in the *Arabidopsis* genome codes for *Arabidopsis* XanDH (Cheng et al., 2002). In contrast, the *nced3* and *aao3* mutants display mild ABA-deficient phenotypes because other, redundant genes exist (Seo et al., 2004; Frey et al., 2012). The *Arabidopsis aba1* and *aba3* mutants show ABA-independent phenotypes, such as abnormal carotenoid compositions and a loss of activity in AOs and xanthine dehydrogenases, respectively (Rock and Zeevaart, 1991; Schwartz et al., 1997a). Due to stringent and ABA-specific phenotypes, *Ataba2* mutants have been isolated using various genetic screens, including gibberellin biosynthesis inhibition and salt- or sugar-resistant germination or growth (Léon-Kloosterziel et al., 1996; Nambara et al., 1998; Laby et al., 2000; Cheng et al., 2002; González-Guzmán et al., 2002). Therefore, the XanDH gene appears to be an excellent target for molecular breeding work that seeks to fine-tune ABA levels in plants.

XanDH (EC 1.1.1.288) is a cytosolic enzyme that belongs to a short-chain dehydrogenase/reductase (SDR) superfamily, NAD(P)(H)-dependent oxidoreductase, which is present in most organisms (Jörnvall et al., 1995). The members of the SDR superfamily comprise approximately 250–300 amino acid residues that have two conserved motifs, namely, the cofactor binding and active sites (Jörnvall et al., 1995). The enzymes categorized in the SDR family function in essential metabolic pathways, such as those involved in retinoic acid, prostaglandin, and hydroxysteroid metabolism in animals (Cho et al., 2005; Wu et al., 2007; Lee et al., 2011). In flowering plants, the SDR superfamily has approximately 200 members. The diversification of plant SDR families is believed to be correlated with the development of secondary metabolism in higher plants (Moummou et al., 2012a). Many recent studies characterizing plant SDRs have determined that they participate in hormone biosynthesis or secondary metabolism (Shimura et al., 2007; Acosta et al., 2009; Scherbak et al., 2011; Jirschitzka et al., 2012; Moummou et al., 2012b). However, the majority of predicted SDRs in plant genomes still await functional annotation.

In the present study, we investigated XanDH in rice. To date, XanDH has been isolated and characterized only in *Arabidopsis* (Cheng et al., 2002; González-Guzmán et al., 2002), and no additional studies have reported the isolation of *AtABA2* orthologs. Recent advances in sequencing technology enable us to utilize information from the complete genome, not only from model plants but also from varieties of plant species. This advance motivated us to characterize XanDH orthologs. To uncover regulatory mechanisms of ABA biosynthesis in rice, we first sought to isolate XanDH from rice and then performed biochemical and complementation analyses using an *Arabidopsis aba2* mutant in order to achieve a functional characterization of XanDH in rice.

Materials and methods

Plant materials

The rice cultivar *Oryza sativa* L. cv. Hayayuki was used in this study. *Arabidopsis thaliana*, wild-type (Col-0 accession), and *aba2-2* (Col-0 background) were used for complementation experiments.

Phylogenetic analysis and sequence alignment

Amino acid sequences of AtABA2 orthologs were obtained from plant genome databases for *Arabidopsis*, rice, maize, sorghum, and *Brachypodium*, respectively. Ten sequences with high homology against AtABA2 were collected from each database. A maximum-likelihood tree was firstly constructed by aligning 54 full-length amino acid sequences using the MEGA5 package with default settings (Fig. S1) (Tamura et al., 2011). Tree nodes were evaluated using bootstrap analysis with 500 replicates. 17 short-chain dehydrogenase/reductases (SDRs) belonging to groups of xanthoxin dehydrogenase (XanDH), OsMAS and TS2 were used to reconstruct the smaller tree in the same way (Fig. 1A). Amino acid sequences of putative AtABA2 orthologs were aligned by using the CLC sequence viewer 6. Gene IDs of all sequences are listed in Table S1.

RNA extraction and quantitative reverse transcription PCR

Imbibed rice seeds were incubated for 2 days (d) at 28 °C in the dark, after which germinating seeds were hydroponically grown in a plant growth chamber for 14 d under a long-day condition (light for 16 h at 25 °C and dark for 8 h at 20 °C). Abscisic acid (ABA) treatment was performed by replacing hydroponic media with water containing 100 μM ABA and then plants were incubated for 6 or 12 h under the growth condition as described above. To subject the plants to dehydration stress, the plants were taken out from the hydroponic media and excess water was removed using paper towels. The plants were transferred to a plastic tray covered with plastic wrap and the tray was incubated for either 6 or 12 h under dim light at room temperature. Plants in each treatment group were frozen using liquid nitrogen and stored in a −80 °C freezer until use. Total RNA was extracted according to the instructions of an RNeasy Mini kit (QIAGEN). RNA was purified by chloroform extraction and LiCl precipitation to obtain the total RNA with a high level of purity. First strand cDNA was synthesized from 500 ng of total RNA by using PrimeScript RT Master Mix (TaKaRa).

The LightCycler carousel-based system (Roche) was used for quantitative reverse transcription PCR with LightCycler TaqMan Master (Roche) and TaqMan probe as a fluorescent reporter. To