Australian Journal of Crop Science AJCS
AJCS 9(8):744-753 (2015) ISSN:1835-2707

Molecular and biochemical characterization of superoxide dismutase (SOD) in upland rice under drought

Karinne Evaristo de Deus[1], Anna Cristina Lanna[2], Fernanda Raquel Martins Abreu[3], Ricardo Diógenes Dias Silveira[4], Wendell Jacinto Pereira[1], Claudio Brondani[4], Rosana Pereira Vianello[4*]

[1]Institute of Biological Science, Universidade Federal de Goiás (Federal University of Goias), Goiânia, GO, 74001-970, Brazil
[2]Laboratory of Physiology Laboratory, Embrapa Arroz e Feijão (Embrapa Rice and Beans), Santo Antônio de Goiás, GO, 75375-000, Brazil
[3]School of Agronomy and Food Engineering, Universidade Federal de Goiás, Goiânia, GO, 74001-970, Brazil
[4]Laboratory of Biotechnology, Embrapa Arroz e Feijão, Santo Antonio de Goiás, GO, 75375-000, Brazil

***Corresponding author: rosana.vianello@embrapa.br, +55 62 3533 2154 (office); +55 62 3533 2100 (fax)**

Abstract

Drought is a major cause of reduced yield in upland rice farming in many regions of the world and cause an increased production of reactive oxygen species (ROS), followed by activation of a complex antioxidant system. In this study, the superoxide dismutase (SOD) enzymatic activity and the gene expression levels of eight isoforms of SOD were evaluated in drought-tolerant and drought-sensitive varieties of upland rice, *Oryza sativa* japonica (the Primavera and Douradão cultivars), including shoot and root tissues in two stages of plant development, grown under normal and restricted irrigation. The SOD activity was determined by *spectrophotometric method* and the gene expression by qPCR experiments. In the tolerant cultivar, SOD enzymatic activity increased significantly ($p \leq 0.05$) only in the root tissue in the reproductive stage (268.00 SOD UN mg-1), whereas in the sensitive cultivar, SOD increased in the leaf (112.17 SOD UN mg-1) and root (172.56 SOD UN mg-1) tissues in the reproductive stage. The genes *CuZnSOD4* and *MnSOD* showed significant increases in expression ($p \leq 0.05$) in both tissues and developmental stages in the tolerant cultivar under drought, whereas *FeSOD1* showed increased expression ($p \leq 0.05$) only in the tolerant genotype (vegetative stage/leaf and root) under water restriction. The different patterns of SOD activity and/or gene expression in upland rice plants should be strongly considered to elucidate the cellular mechanisms of drought tolerance; these findings may ultimately inform breeding programs aimed at more efficiently developing cultivars that are better adapted to areas prone to water deficits.

Keywords: *Oryza sativa*; water stress; oxidative stress; gene expression; enzymatic activity.
Abbreviations: ROS_reactive oxygen species; SOD_Superoxide dismutase; p_p-value; FAO_Food and Agricultural Organization of the United Nations; AsA_ascorbate; GSH_glutathione; KCN_potassium cyanide; FC_field capacity; DAE_days after emergence; NBT_Nitroblue Tetrazolium; PVP_polyvinylpyrrolidone; EDTA_Ethylenediamine tetraacetic acid; TAE_Tris-acetate-EDTA; EEF-1α_Elongation factor 1-alpha 1; bp_base pairs; RIN_RNA integrity; Ct_threshold cycle; NCBI_National Center for Biotechnology Information; CDS_coding sequence of DNA; EMBOSS_European Molecular Biology Open Software Suite, ACT_actin; GAPDH_glyceraldehyde-3-phosphate dehydrogenase; UDG_Uracil DNA Glycosylase; ANOVA_Analysis of variance; RGAP_Rice Genome Annotation Project.

Introduction

Among the many climatic factors affecting agriculture worldwide, drought is one of the most significant problems. Drought conditions have a tendency to increase due to population growth, especially in developing countries (FAO - Water, 2013). Rice (*Oryza sativa* L.) is a cereal crop of high socioeconomic value and a staple food for over half of the world's population (Hadiarto and Tran, 2011). It is estimated that approximately 20% of the area planted with cereal crops in Brazil is affected by drought, resulting in a production loss of over 23.7 million tons per year. In specific locations and years, there is a near total loss of production (Guimarães et al., 2006). Worldwide, approximately 164 million hectares of rice are grown in different soil and climate conditions, producing approximately 720 million tons, of which Brazil ranks among the top ten rice producers, with more than two million hectares of planted area (FAOSTAT, 2013). In Brazil, the Central-west, North, and Northeast regions grow upland rice, representing 60% of the planted area. This cropping system depends on rainfall or irrigation, and there is growing demand from an economic and environmental perspective for an increase in rain-fed rice production due to reduced water availability (Quan et al., 2010). When facing periods without rain during their life cycle, plants use a complex redirection of metabolism related to different and sometimes complementary physiological and biochemical mechanisms, such as stomatal closure, leaf rolling, the abscisic acid signaling pathway, reductions in the photosynthetic and transpiration rates, solute accumulation, the production of free radicals and characteristic metabolites, and changes in antioxidant enzymes and their gene expression levels (Serraj et al., 2011; Tian et al., 2011; Ji et al., 2012; Aydin et al., 2013). Furthermore, the severity, duration, and frequency

with which drought stress is imposed, as well as the developmental stage of the plant, will be decisive in the different levels of response (Fritsche-Neto et al., 2011). One of the primary biochemical processes observed in plants due to low soil water availability is excess production of reactive oxygen species (ROS); this effect has been widely described in the literature (Levine et al., 1994; Scandalios, 2005; Gill and Tuteja 2010; Sharma et al., 2012). Excess ROSs trigger the activity of the antioxidant defense system, which tends to modulate the redox state of the cell in an attempt to maintain cellular homeostasis (Morita et al., 2011; Ma et al., 2013). Metabolic processes in aerobic organisms, such as respiration, photosynthesis, and photorespiration, lead to the continuous production of ROSs in mitochondria, chloroplasts, and peroxisomes, as described by Sharma et al. (2012). Under optimum physiological conditions, ROS production and degradation are in equilibrium due to several mechanisms of cellular detoxification (Alscher et al., 1997). Disruption of this balance, which may occur due to a number of biotic and abiotic factors, results in an increase in the intracellular concentration of ROSs (production rate higher than the degradation rate). Increased cellular ROSs may in turn lead to a series of oxidative injuries to biomolecules (such as lipids, proteins, and DNA), and these injuries can even result in cell death, depending on the severity of the stress (Mittler, 2002; Sharma et al., 2012). Oxidative stress at the cellular level is characterized by excessive ROSs, which may be formed by free radicals (such as superoxide ($O_2^{\bullet-}$) and hydroxyl ($OH^{\bullet}$) and highly reactive chemical species, such as singlet oxygen ($^1O_2$) and hydrogen peroxide (H_2O_2) (Scandalios, 2005; Soares and Machado, 2007; Goswami et al., 2013). To protect the structure and operation of plant cells from the damaging effects of ROS, a complex antioxidant system is activated. This system consists of three components: (1) lipid soluble and membrane-associated tocopherols; (2) water soluble reducer compounds such as ascorbate (AsA) and glutathione (GSH), and (3) antioxidant enzymes (Cho and Seo, 2005; Morita et al., 2011). The major enzymatic components of the antioxidant defense system in aerobic organisms are superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), glutathione peroxidase (GPx), glutathione reductase (GR), monodehydroascorbate reductase (MDAR**)** and dehydroascorbate reductase (DHAR) (Noctor and Foyer, 1998, Lee and Lee, 2000; Cho and Seo, 2005; Morita et al., 2011). The SOD antioxidant enzyme, which catalyzes the dismutation of the superoxide anion ($O_2^{\bullet-}$) into hydrogen peroxide and molecular oxygen, is one of the most important in the front line of defense against oxidative stress and plays a critical role in the survival of plants under environmental stresses (Gill and Tuteja, 2010; Aydin et al., 2013). This enzyme is ubiquitous in all aerobic organisms and cell compartments prone to oxidative stress. Its groups vary according to their metal cofactor; isoforms are present in chloroplasts (FeSOD, MnSOD, CuZnSOD), mitochondria (MnSOD), peroxisomes (MnSOD and CuZnSOD), the cytosol, and possibly in the extracellular space (CuZnSOD) (Alscher et al., 2002; Jaleel et al., 2009; Gill and Tuteja 2010; Aydin et al., 2013). A significant increase in the activity and/or expression of SOD in plants under drought has been observed in several species, such as the common bean (Zlatev et al., 2006), cowpea (Brou et al., 2007), transgenic rice plants (Prashanth et al., 2008), and sweet potato (Lu et al., 2010). In this context, the activation of the defense mechanisms of the antioxidant system in response to a water deficit supports investigative studies on the development of cultivars of upland rice that are better adapted for cultivation in areas prone to drought. This study was proposed based on the hypothesis that antioxidant enzymes may trigger protective and/or adaptive mechanisms in upland rice plants subjected to drought through changes in gene expression and/or post-transcriptional regulation. Thus, the objective of this study was to evaluate the enzyme SOD, its level of activity, and gene expression in two genotypes of upland rice (*Oryza sativa* ssp. japonica; one drought-tolerant and one drought-sensitive), including analysis of shoot and root tissues, in two stages of plant development (vegetative and reproductive), grown under normal and restricted irrigation.

Results

Identification of SOD genes and PCR amplification

A total of eight genes encoding SOD were identified from the Rice Genome Annotation Project, RGAP database (http://rice.plantbiology.msu.edu/). The genes were distinguished by their subcellular location and the cofactors that bind to their reactive centers; six of these genes represented alternatively spliced forms (Table 1). A total of 24 oligos were designed; 17 of these included an exon-exon junction, with an average of three pairs of oligos for each sequence. The average size of the amplicons was 117 bp, ranging from 100 bp to 123 bp (Table 1). For some gene sequences (*FeSOD2, CuZnSOD1*, *CuZnSOD3* and *MnSOD*), it was not possible to design a primer spanning an exon-exon junction, due to the presence of only two exons in the CDS regions or the different exon sizes. The samples from leaf and root tissues resulted in a good quantity of high-quality total RNA (Supplementary Table 1). Of the set of 24 primer pairs, 20% showed satisfactory qPCR amplification at a 50 nM concentration; the remaining primer pairs had adequate amplification at 25 nM. The Ct values ranged from 24.96 to 37.24, and the ΔRn values ranged from 0.05375 to 0.016769. The efficiency of the qPCR standard curves ranged from 93% to 101% (Table 2). The greatest qPCR efficiency was observed for the *FeSOD2* gene, and the lowest efficiency was obtained for *CuZnSOD3*. The slope values ranged from -3.28 to -3.50, while the coefficient of correlations (R2) ranged from 0.91 to 1 (Table 2). For the three reference genes (ACT, EEF-1α, and GAPDH), the qPCR efficiencies were 96.74%, 99.25%, and 100.16%, respectively. The gene stabilities calculated using the NormFinder algorithm revealed the desired low values of 0.007 for ACT, 0.012 for GAPDH, and 0.006 for EEF-1α.

SOD enzymatic activity profiles under stress

SOD activity was influenced by drought stress (Supplementary Table 2). Among the plants grown under optimal water conditions in the vegetative stage (Fig. 1A, a), the activity of SOD was significantly higher in the leaves of the Primavera cultivar. However, under drought treatment, SOD activity was similar between the two genotypes. SOD activity showed a significant reduction (approximately 29%) in Primavera leaves compared to the control plants, whereas the leaves of the Douradão cultivar had no significant increased SOD activity compared to the control plants. In leaf tissue, drought stress significantly influenced the response of the SOD enzyme in the reproductive stage in both genotypes (Fig. 1B, a). SOD activity in the control treatment was significantly lower in Primavera leaves compared to Douradão leaves under the same conditions. Under water restriction, Primavera cultivar showed a significant increase in SOD activity (213%), whereas the Douradão cultivar showed a reduction (20%). However, even with the decline in

Table 1. Isoforms of SOD used for qPCR analysis in upland rice grown under normal and restrictive irrigation.

Gene ID*	Nomenclature	Cofactor	Alternative *splicing* forms	*Forward* (5'→3')	*Reverse* (5'→3')	*Amplicon size* (bp)
LOC_Os03g11960	Os03g0219200	CuZnSOD1	LOC_Os03g11960.4; LOC_Os03g11960.3; LOC_Os03g11960.2	ACCGGGTATACCGAGGTGA	GTAGAGTTGCAGCCGTTGGT	104
LOC_Os03g22810	Os03g0351500	CuZnSOD2	-	TCAACTGGGCCACACTACAA	CTTCTCCAGCGGTGACATTT	100
LOC_Os04g48410	Os04g0573200	CuZnSOD3	LOC_Os04g48410.2	AAGGGAACCCAAATGATTTTT	GCTTCAACTATAGCCAATTCCA	115
LOC_Os05g25850	Os05g0323900	MnSOD	LOC_Os05g25850.2	ACGTCGCCAACTACAACAAG	GTTGAACTTGATGGCGCTCT	101
LOC_Os06g02500	Os06g0115400	FeSOD1	-	GGATGGGTTTGGCTTTGTTA	AAGAGGATTGATGGCATTCG	123
LOC_Os06g05110	Os06g0143000	FeSOD2	LOC_Os06g05110.3	GCGATATTTTCGCATCCATT	TCCAAAGGCCATTACATTCAT	100
LOC_Os07g46990	Os07g0665200	CuZnSOD4	LOC_Os07g46990.2	CACTTCAATCCTACTGGGAAGG	TAGCAACACCATCTGCTCCA	100
LOC_Os08g44770	Os08g0561700	CuZnSOD5	LOC_Os08g44770.2	ACTTGCATGCGGTGTTGTT	TCAGGCTCGAAGATGACAAA	107

* genes IDs obtained from RGAP – (http://rice.plantbiology.msu.edu/)

Table 2. The SOD isoforms and the corresponding qPCR primer pair concentrations for maximal efficiency of amplification, with the respective coefficient of correlation (R2) and slope values.

Gene	Concentration (nM)		qPCR efficiency	R^2	*Slope*
	forward	*reverse*			
CuZnSOD1	25	25	100%	0,99	-3,3123
CuZnSOD2	25	25	97%	0,99	-3,4
CuZnSOD3	25	25	93%	0,91	-3,5
MnSOD	25	25	98%	0,99	-3,3621
FeSOD1	25	25	94%	0,99	-3,468
FeSOD2	50	50	101%	0,98	-3,285
CuZnSOD4	25	25	99%	1	-3,34
CuZnSOD5	25	25	98%	0,99	-3,3734

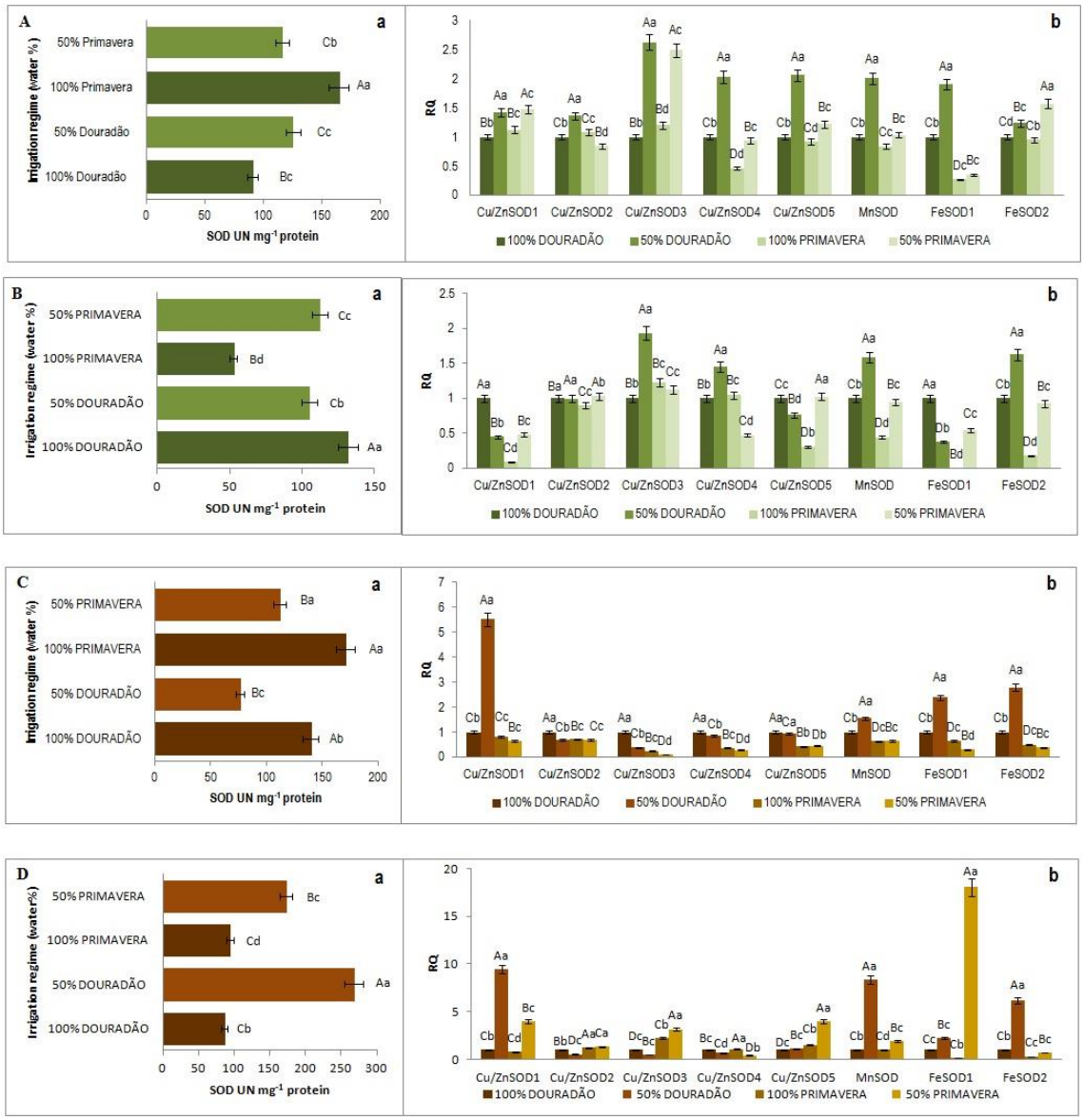

Fig 1. Effects of water deficit on SOD activity (panel a) and the expression of eight SOD isoforms (panel b) in leaf tissues in the vegetative (A) and reproductive stages (B), and in root tissues of upland rice in the vegetative (C) and reproductive stages (D). The different capital letters in the different genotypes under the same water regime indicate statistically significant differences, and the different lowercase letters in the different water regimes for the same genotype indicate statistically significant differences ($p \leq 0.05$).

SOD activity in the Douradão cultivar, the level 104.9 SOD UN mg-1 protein was similar to that in the Primavera (112.1 112.1 SOD UN mg-1 protein) cultivar under drought treatment. The SOD activity was influenced by drought stress in the roots of Douradão and Primavera plants during their early stage of development (vegetative stage) (Fig. 1C, a). In both genotypes, there was no significant difference in SOD activity in the roots of plants grown under optimal water availability, nor in those grown under water restriction. Both Douradão and Primavera had reduced SOD activity after drought treatment compared to the control treatment. In the Douradão cultivar, the reduction was approximately 45%, and in Primavera, the reduction was approximately 34%. The Primavera cultivar, which is sensitive to drought stress, showed a decrease in SOD activity both in the shoots and roots of plants grown under drought in the early stage of plant development. The tolerant Douradão cultivar showed a decrease in SOD activity in the roots and an increase in the shoots. Drought stress significantly influenced the SOD activity levels in the root reproductive phase (Fig. 1D, a). SOD activity was similar in the two genotypes without water restriction, (85.8 SOD UN mg-1 protein and 93.84 SOD UN mg-1 protein in the Primavera and Douradão cultivars, respectively). Under water restriction, the roots of both Douradão and Primavera showed significantly higher SOD activity. The Douradão cultivar showed SOD activity (268.0 SOD UN mg-1 protein) significantly greater than that of the Primavera cultivar (172.6 SOD UN mg-1 protein) in the roots. SOD activity in the Douradão cultivar increased by 212%, whereas the SOD activity in the Primavera cultivar increased by 84%, compared to their respective control plants.

SOD expression profiles under drought stress

In the leaf tissue of the Douradão cultivar in the vegetative stage, the eight SOD genes showed increased expression under drought compared to the control treatment. For the Primavera cultivar, significantly increased expression was

observed for the *CuZnSOD3*, *CuZnSOD4*, *CuZnSOD5* and *FeSOD2* genes, whereas a reduction in *CuZnSOD2* levels was observed under drought treatment. The *CuZnSOD1* and *CuZnSOD3* genes showed increased expression in both genotypes under drought treatment compared to the control. For Douradão, the level of gene expression increased from 1.43 to 2.62 times, and for Primavera, from 1.47 to 2.48 times, with and without water restriction, respectively (Fig. 1A, b). Considering both genotypes under the same water regime (the capital letters in the graphs), five genes showed significantly higher levels of induction of expression ($p \leq 0.05$) in the tolerant Douradão cultivar under drought (*CuZnSOD2*, *CuZnSOD4*, *CuZnSOD5*, *MnSOD*, *FeSOD1*) compared to the sensitive BRS Primavera cultivar. The expression increased from a minimum of 1.36-fold for the *CuZnSOD2* gene to a maximum of 2.05-fold for *CuZnSOD5*, with intermediate values of increased level of 1.90, 2, and 2.02 times for the genes *FeSOD1*, *MnSOD* and *CuZnSOD4*, respectively (Fig. 1A, b). Interestingly, the significantly increased expression of *CuZnSOD2* observed in the Douradão cultivar under drought contrasted with the significantly reduced expression ($p \leq 0.05$) observed in Primavera (0.84 times). Only the *FeSOD2* gene showed increased expression in the sensitive genotype under drought treatment (1.57-fold) (Fig. 1A, a). In the leaf tissue from the reproductive stage, a different pattern of SOD gene expression was observed in the two cultivars grown under normal and drought treatments (Fig. 1B, b). For the tolerant cultivar under both water regimes, four of the eight genes that were assessed showed significantly ($p \leq 0.05$) increased expression (*CuZnSOD3*, *CuZnSOD4*, *MnSOD* and *FeSOD2*), and three had decreased expression (*CuZnSOD1*, *CuZnSOD5*, and *FeSOD1*) in the drought conditions compared to the corresponding controls. For Primavera under drought treatment, the most significantly expressed genes were *CuZnSOD1*, *CuZnSOD2*, *CuZnSOD5*, *MnSOD*, *FeSOD1* and *FeSOD2*, while *CuZnSOD3* and *CuZnSOD4* had reduced expression (Fig. 1B, b). A comparison of the two genotypes under the same irrigation conditions (the capital letters in the graphs) revealed four genes with increased expression under drought in Douradão compared to Primavera (*CuZnSOD3*, *CuZnSOD4*, *MnSOD*, and *FeSOD2*, with expression levels increasing by 1.94-, 1.45-, 1.59-, and 1.62 fold, respectively). *CuZnSOD1* and *FeSOD1* were down-regulated in the Douradão cultivar compared to the Primavera cultivar, both under drought treatment ($p \leq 0.05$). Only *CuZnSOD5* showed increased expression in the susceptible genotype under drought treatment (1.02-fold), whereas the expression level decreased in the tolerant cultivar (Fig. 1B, b). For root tissue in the vegetative stage, *CuZnSOD1* showed the highest level of expression, increasing 5.51-fold in the Douradão cultivar under drought treatment in comparison to the control. In contrast, Primavera showed the lowest expression, with a decrease of 0.64-fold under drought in relation to the control treatment. Similarly, the *FeSOD1*, *FeSOD2*, and *MnSOD* genes had higher expression in the Douradão cultivar under drought conditions ($p \leq 0.05$), showing an increase in relation to the control of 2.38, 2.78, and 1.54-fold, respectively. In Primavera, the level of expression decreased to 0.30, 0.39, and 0.66 times that of control, respectively (Fig. 1C, b). Comparing gene expression between the Douradão and Primavera cultivars in the drought treatment (the capital letters in the graphs), the genes *CuZnSOD1*, *MnSOD*, *FeSOD1*, and *FeSOD2* had higher expression in the tolerant genotype. All other genes (*CuZnSOD2*, *CuZnSOD3*, *CuZnSOD4*, and *CuZnSOD5*) were down-regulated in both genotypes under water stress ($p \leq 0.05$) (Fig. 1C, b). Analysis of root in the reproductive stage revealed that in the presence of drought stress, *CuZnSOD1*, *MnSOD* and *FeSOD2* expression was increased in the Douradão cultivar compared to control plants ($p \leq 0.05$). In the Primavera cultivar, expression increased for *CuZnSOD1*, *CuZnSOD3*, *CuZnSOD5*, *MnSOD* and *FeSOD1* genes, in comparison to control plants ($p \leq 0.05$). The expression of the *FeSOD2* gene increased only in the tolerant cultivar, while *CuZnSOD3*, *CuZnSOD5*, and *FeSOD1* increased only in the susceptible one. Under drought, the genes *CuZnSOD2* and *CuZnSOD3* were down-regulated in Douradão and up-regulated in Primavera. *CuZnSOD4* was down-regulated for both genotypes under drought conditions (0.67- and 0.41-fold, respectively). Under the same water treatment regimes (the uppercase letters in the graphs, Fig. 1D, b), the *CuZnSOD1*, *MnSOD* and *FeSOD2* genes had greater expression in Douradão compared to Primavera under drought (gene expression increased 9.43, 8.33, and 6.17 times, respectively). However, in the susceptible cultivar, the *FeSOD1*, *CuZnSOD5* and *CuZnSOD3* genes showed greater levels of expression under water deficit, an increase of 18-, 3.99-, and 3.14-fold, respectively, compared to the tolerant genotype under the same moisture conditions.

SOD activity and gene expression level

A similar level of SOD activity in both genotypes under drought stress was observed in leaf tissue in the vegetative stage, during which the increased transcription levels in five genes in the tolerant genotype did not correlate with an increase in the total SOD activity in the presence of water. However, in leaf tissue in the reproductive stage, a significant increase in SOD activity and the expression of six (out of eight tested) genes was observed in Primavera plants under drought. For Douradão, the increased expression of the *CuZnSOD3*, *CuZnSOD4*, *MnSOD* and *FeSOD2* genes did not seem to contribute to maintaining and/or increasing the activity of SOD under drought, because the level of SOD activity did not differ from the sensitive genotype under the same stress conditions. In root tissue in the reproductive stage, the overexpression of the *CuZnSOD1*, *MnSOD*, *FeSOD2*, and *FeSOD1* genes appears to show a negative correlation with SOD activity in the tolerant genotype under drought, while in the susceptible cultivar (Primavera), a reduction or no change in gene expression under drought was observed, in accordance with the reduction in SOD activity. In root tissue in the vegetative stage, the SOD activity increased in both genotypes under water deficit, but it increased at a greater rate for the tolerant genotype ($p \leq 0.05$). This defense mechanism is potentially a response to drought stress during a critical stage of plant development. Two genes (*CuZnSOD1*, *MnSOD*) that were up-regulated in both genotypes under drought stress showed a positive correlation with SOD activity. In addition, the *FeSOD2* gene, whose expression increased only in the Douradão cultivar, may also have contributed to the significantly higher expression of SOD in these plants.

Discussion

The components of the antioxidant defense system operate in such a well-coordinated and active manner in response to stress factors that they in turn promote the production of ROS, causing damage to an organism (Mittler, 2002; Foyer and Noctor, 2005). In this study, we assessed SOD at the level of gene expression and enzymatic activity in order to investigate the mechanisms underlying the transcriptional and

translational levels of drought tolerance in upland rice plants. Because SOD is an enzymatic component of the antioxidant defense system, the induction of this enzyme reflects its important role in the defense mechanism of the plant (Ashraf and Ali, 2008). Several studies have been performed at the level of gene expression and enzyme activity to better characterize the SOD in the presence of abiotic stresses such as drought, salinity, and low and high temperatures (Srivalli et al., 2003; Wang et al., 2005; Thounaojam et al., 2012; Ara et al., 2013; Aydin et al., 2013). Different levels of SOD activity were observed under drought treatment in the leaf tissue of both cultivars in the vegetative and reproductive stages. However, in the vegetative stage, SOD activity decreased in Primavera (drought-sensitive) and remained stable in Douradão (drought-tolerant). In the reproductive stage, SOD activity increased in Primavera and decreased in Douradão in relation to the control treatment. Similar results were observed by Basu et al. (2010) in Indica genotypes when drought was induced by PEG in the vegetative stage. Those authors reported a decrease in SOD activity in leaf tissue in the sensitive genotype, whereas in the tolerant genotype, SOD activity did not change. According to these authors, the maintenance of SOD activity in the tolerant genotype is due to the parallel activation of additional mechanisms of tolerance, especially those related to a rearrangement in the composition of the lipid membrane, changes in the photosynthetic apparatus, and greater induction of non-enzymatic antioxidants, among others, whereas sensitive plants may not have the ability to make all of these cellular adjustments when subjected to severe drought. The up-regulation of some SOD genes was not related to increased SOD activity in the roots of the tolerant cultivar in the vegetative stage, which may suggest that post-transcriptional modifications, such as late activation or inactivation of SOD isoforms, coupled with the activation of other defense mechanisms occur in this plant. In contrast, in the reproductive stage, which is critical for plant development due to grain production, significant increases in SOD activity were observed in the roots of both cultivars (greater for Douradão, $p \leq 0.05$), certainly as a mechanism for triggering additional protection. In a previous experiment analyzing water deficits conducted by Silveira et al. (2015) using the same genotypes as this study, it was shown that despite a reduction in Douradão yield levels when stress was applied in the reproductive stage, this cultivar still produced more grain than Primavera. Altogether, these results show that a drought episode during the reproductive stage is particularly damaging. However, it was observed in the current study that the tolerant genotype tends to greatly increase the production of SOD, most likely as a cellular protection mechanism to minimize losses in grain yield. According Sairam and Saxena (2000), the levels of antioxidant metabolites and enzymes that regulate the cellular redox status increase under various stresses, suggesting that greater antioxidant activity conveys greater tolerance to these plants. In the current study, SOD activity was higher in the root, which was expected because the initial perception of low water availability in the soil occurs in this tissue and because the antioxidative process in leaves may or may not have been triggered, depending on the severity and duration of the drought stress. In the shoot, several mechanisms of adaptation may be part of the morphophysiological defense mechanisms, such as changes in plant structure, stomatal closure, and reduced transpiration and photosynthetic rates, among others (Gloser and Glose, 1996; Yordanov et al., 2000). An increase in SOD activity in the root, but a decrease in leaf tissue was observed in rice plants subjected to aluminum toxicity and phosphorus deficiency in the soil (Tian-rong et al., 2012). From *in silico* analysis, eight SOD genes that are differentially expressed during the development stages of rice plants have been identified so far. Phylogenetic analyses revealed that the enzymes MnSOD, FeSOD1, and FeSOD2 are grouped in a clade, while CuZnSOD is more divergent (another clade) and exhibits a more conserved gene sequence (Nath et al., 2014). According to Fink and Scandalios (2002) the biggest difference between the SOD isoforms is in the regulatory sequences of the genes encoding these proteins, and each protein responds differently to oxidative stress at different levels. For the eight isoforms of SOD evaluated in this study, variations in the pattern of expression and activation of a larger number of genes were observed in the leaf tissue and root tissue for the same genotype under drought treatment. The eight genes were up-regulated in the leaf tissue, with more genes expressed in the vegetative phase (eight genes in Douradão and four in Primavera) compared to the reproductive phase (four in Douradão and six in Primavera). However, the enzymatic activity of SOD increased ($p \leq 0.05$) only in the reproductive stage in the sensitive cultivar and failed to show a direct relationship between gene expression and enzyme activity based on these results. These observations may result from a component of experimental variation or indicate the action of post-transcriptional, translational, and post-translational mechanisms, reducing the activity of SOD (Nelson and Cox, 2013). In this scenario, other mechanisms for the detoxification of ROS, such as the enzymes ascorbate peroxidase, catalase, and glutathione peroxidase, among others, must be performing this protection function (Miller et al., 2008), mainly in the tolerant Douradão cultivar. Increased expression of *CuZnSOD3*, *CuZnSOD4*, *FeSOD2*, and *MnSOD* was observed for the tolerant cultivar in the reproductive stage; in this genotype, all SOD isoforms were activated, which may have helped to maintain a higher level of SOD activity even under drought. For the Primavera genotype, no significant increase in any SOD isoform was observed under the same conditions, compared to Douradão. For the root tissue in the vegetative phase, only four genes were overexpressed; all of these were identified in the tolerant cultivar, whereas in the reproductive stage, six genes were up-regulated, three of which (*CuZnSOD1*, *MnSOD*, *FeSOD2*) showed significantly more expression in the tolerant cultivar, which may be directly related to increased levels ($p \leq 0.05$) of SOD activity in the same genotype. Because the activity of SOD decreased in the vegetative phase and then significant increased in the reproductive phase, this may suggest a positive relationship between the expression levels of the isoforms *CuZnSOD1*, *MnSOD*, and *FeSOD2* and greater tolerance of the plant in the presence of drought. Bhoomika et al. (2013) also reported an increase in expression of the *FeSOD* gene in the root tissue of tolerant rice plants subjected to aluminum stress. According to Raychaudhuri (2000), the FeSOD enzyme is predominantly found in plastids of higher plants, while MnSOD is found in the mitochondrial matrix. Several studies have reported a positive correlation between increased expression of the *MnSOD* gene and increased tolerance of plants to environmental stresses (Bowler et al., 1992; Wu et al., 1999; Wang et al., 2005; Dai et al., 2009; Bai et al., 2009; Que et al., 2012). It was observed that the SOD genes whose active center metal is CuZn had increased expression in both genotypes after drought treatment, predominantly in leaf tissue in the vegetative stage and in the tolerant genotype. This enzyme is found mainly in the chloroplast and cytosol, which explains the increased expression in the shoots. Additionally, the *MnSOD*, *FeSOD1*, and *FeSOD2* genes had

increased expression in all tissues and developmental stages, and *MnSOD* was always increased in the tolerant genotype under drought treatment. Thus, after confirming the positive role of these genes in the trait evaluated, they can be strongly suggested as candidates for indirect selection of drought-resistant plant materials, as suggested by Zaefyzadeh et al. (2009).

Conclusion

Altogether, these results suggest that Douradão plants have greater activation of both the transcription and translation of the antioxidative defense system, the SOD pathway, under conditions of low water availability, and this can best be detected in the root tissue in the reproductive stage. Although the increased activity of antioxidant enzymes under drought stress is extremely variable between different plant species and even between cultivars of the same species (Reddy et al., 2004), certainly, the different patterns of induction in activity level and/or gene expression of SOD, reported in this study in upland rice plants, should be strongly considered in elucidating the cellular mechanisms of drought tolerance. These findings aim to support breeding programs aimed at developing more efficient cultivars that are better suited to areas prone to water stress.

Materials and Methods

Plant materials

Two upland rice cultivars were analyzed in this study, Douradão (drought-tolerant) and Primavera (drought-sensitive). Douradão originated from a cross between the cultivar IAC-25 and the African line 63-83 (Soares et al., 1989) and was released for commercial use by Embrapa Arroz e Feijão (Embrapa Rice and Beans) in 1997. This cultivar has highly important traits for upland rice breeding, such as a short cycle, initial seedling robustness, environmental adaptability, tolerance to drought stress, and long, clear, and translucent grains. Flowering occurs at approximately 80 days after sowing, and seed maturity occurs at 30-40 days after flowering (Soares et al., 1989). The Primavera cultivar was derived from a cross between the line IRAT 10 and LS 85-158 (Soares et al., 2001) and released by Embrapa Rice and Beans in 1987. This cultivar stands out for its long, fine grains, but it is sensitive to herbicides, has low resistance to lodging, and is quite sensitive to rice blast disease and drought. Flowering occurs, on average, 85 days after sowing; maturation occurs approximately 30 days after flowering (Soares et al., 2001).

Greenhouse drought stress experiment

The drought-tolerance experiment was conducted in a greenhouse at Embrapa in the 2011/2012 growing season (November to March). Periods of water restriction were imposed according to the phenological phases of each genotype. In both genotypes, the first period occurred between 17 and 31 days after emergence (DAE), and the second took place between 60 and 72 DAE for Douradão and between 67 and 77 DAE for Primavera. Two irrigation groups were used: Group A (control group) had its water availability established at field capacity (FC), and Group B was exposed to 50% of FC. Irrigation was provided normally for the set of plants exposed to the second period of water deficiency until more than 50% of the plants in this set of pots reached reproductive stage. The level of drought stress to the plants was maintained by replacing the water lost through evapotranspiration daily. A split-plot randomized block experimental design was used. Six replicates were used for each cultivar and for each cycle of water deficiency. A larger reduction in the yield index was observed in plants subjected to water restriction during the reproductive phase when compared to control plants, indicating that response mechanisms, of greater or lesser intensity, were triggered in both genotypes (Lanna et al., 2013). To evaluate the biochemical and molecular properties of the plant material, the leaves and roots were collected on the last day of water restriction, in each period of water stress.

SOD enzymatic activity determination

Leaf and root samples (1 g) were ground in liquid nitrogen and homogenized in buffer consisting of 100 mM potassium phosphate buffer, pH 7.8, containing 0.1 mM EDTA, 1% (w/v) polyvinylpyrrolidone (PVP) and 0.5% (v/v) Triton X-100. The homogenate was centrifuged and the supernatant was used to determine the protein content (Bradford, 1976) and SOD activity (Del Longo et al., 1993), with modifications. The method is based on the ability of SOD to inhibit the photochemical reduction of nitroblue tetrazolium (NBT). According to Corte et al. (2010) one unit of SOD is defined as the amount of enzyme that causes a 50% decrease in the SOD-inhibitable NBT reduction. A calibration curve was then obtained by plotting the percentage inhibition versus the crude extract volume (leaf and root). All treatments were performed in triplicate, and a hyperbolic curve was generated by a linear double-reciprocal plot to obtain the extract volume corresponding to 50% inhibition of NBT photoreduction. The specific activity of SOD (SOD UN mg^{-1} protein) was defined as the ratio between the activity of SOD (50% inhibition of the NBT photoreduction obtained in a given volume of crude leaf and root extract) and the amount of total soluble protein contained in this volume (Supplementary Table 2).

Extraction of total RNA and cDNA synthesis

Total RNA was extracted from the leaf and root tissue samples collected from the drought experiments in triplicate. The tissues were immediately frozen in liquid nitrogen and stored at -80ºC. The RNA extraction protocol used strong denaturing agents such as phenol and guanidine salts, according to the method described by Chomczynski and Sacchi (1987). RNA samples were treated with DNase (Deoxyribonuclease I, Invitrogen) to remove residual genomic DNA. Finally, the total RNA was resuspended in RNase-free water and stored at -80ºC. The quantity and quality of the RNA was assessed in denaturing 1% agarose gel in TAE RNase free (Tris-Acetate EDTA) using a NanoVueTM (GE Healthcare) spectrophotometer and a 2100 BioAnalyzer (Agilent) with RIN $\geq$ 6.0.

Complementary DNA (cDNA) was reverse transcribed from RNA using random primers and the Superscript II Reverse Transcriptase kit (Invitrogen) according to the manufacturer's instructions. The cDNA was then quantified using a Qubit Fluorometer ® 2.0 (Life Technologies) and the Qubit ® ssDNA Assay Kit, adjusted to a concentration of 12.5 ng μL^{-1}, and stored at -20°C. A control PCR amplifying the elongation factor 1-alpha 1 (EEF-1α) gene was conducted using the synthesized cDNA as template to ensure the absence of contaminating DNA; amplicons of different sizes are generated due to the presence of the intronic region in the DNA and its absence on the cDNA.

SOD genes identification and primer design

Searches of the NCBI (http://www.ncbi.nlm.nih.gov/), Rice Genome Annotation Project (http://rice.plantbiology.msu.edu) and UniProt (http://Web.expasy.org/docs/swiss-prot_guideline.html) public databases were performed to identify genes encoding the SOD enzyme and its isoforms. The genomic sequences and CDS (coding sequence of DNA) were aligned using the ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/) and EMBOSS (https://www.ebi.ac.uk/Tools/PSA/emboss_water/nucleotide.html) programs, with an emphasis on identifying of the exon/intron consensus sequences to develop common primers between sequences.
Primer pairs were developed using OligoPerfect™ Designer software (Invitrogen; available at [http://tools.invitrogen.com/content.cfm?pageid=9716]) with the default parameters; the primers were primarily targeted to the junction of adjacent exons to ensure the amplification of the target transcript. To ensure the adequate amplification of at least one fragment using the SYBR® Green system, three pairs of primers were derived per sequence (Supplementary Table 3).

Differential gene expression

All experiments were performed in triplicate using standard reagents for real-time quantitative PCR (qPCR). Three reference genes were used: eukaryotic elongation factor-1α (EEF-1α) (GenBank™ ID AK061464), actin (ACT) (GenBank™ ID X15865), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (GenBank™ ID AK064960). The amplification reactions were performed according to manufacturer's instructions with Platinum® SYBR Green qPCR SuperMix UDG (Uracil DNA Glycosylase) and 25 ng of cDNA in a final volume of 20 µL. The amplification and readings were performed on an Applied Biosystems® 7500 Real-Time PCR System (Applied Biosystems®) and analyzed using the Sequence Detection program (SDS) v2.0.5 (Applied Biosystems®). The thermal cycling conditions consisted of a step at 50°C for 2 minutes (UDG incubation), 95°C for 2 min (denaturation), 40 cycles of 95°C for 15 s and 60°C for 30 seconds (annealing and extension). The amplification reactions, conducted in duplicate, were analyzed through their dissociation curves, whose melting temperatures ranged from 60°C to 95°C. The concentrations of the oligos were selected such that, when combined, the products were amplified with specificity (i.e., without dimer formation and with a lower Ct).
An estimated efficiency of the primers for the target genes and reference genes was obtained as a percentage using the equation $E = \left(10^{\frac{-1}{slope}} - 1\right) \times 100$ (Bustin et al., 2009). For analysis of gene expression, the comparative method was used (ΔΔCT) via DataAssist version 3.01 (Life Technologies). The stability of the reference genes was estimated using NormFinder software (Andersen et al., 2004).

Statistical analysis

All analyses were performed in biological and technical (repetition in laboratory) triplicate. The data were statistically analyzed using the RV 3.0.2 software at a *p-value* ≤ 0.05 (available at CRAN, [http://cran.r-project.org/]). Analysis of variance (ANOVA) was performed to assess: 1) the variations observed for the same genotype subjected to the control treatment (100% irrigation) and water restriction (maintenance of 50% water in the pots), and 2) the variations observed between contrasting genotypes (tolerant and sensitive) subjected to the same drought conditions. Tukey's test was used to evaluate the significance of changes in conditions 1 and 2 at the $p \leq 0.05$ level of significance (Supplementary Table 4).

Acknowledgments

We thank the National Council for Scientific and Technological Development (CNPq) for the grants to CB and RPV; the Office for Improvement of Higher-Education Personnel/Ministry of Education (CAPES/MEC) for the grants to KED and FRMA, and the Brazilian Agricultural Research Corporation (EMBRAPA/MP2) for financial support for this study and the research grant to WJP.

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