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Rice genotypes for drought tolerance: morphological and transcriptional evaluation of auxin-related genes

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ABSTRACT: Drought stress affects crop quality and productivity. The challenge of increasing food availability for a growing worldwide demand relies on the development of tolerant cultivars that will need to be adapted to arid and semi-arid areas. In order to help the understanding of rice response to stress, the phenotypic response of 6 Brazilian rice cultivars and 2 different crosses between them were characterized under drought conditions. Since gene regulation is an important part of root morphological responses to stressful conditions, 4 genes related to auxin response and root modifications (*OsGNOM1/CRL4, OsIAA1*, *OsCAND1* and *OsRAA1*) were evaluated. The expression of these genes was analyzed in stressed rice using public available microarray data and then through real-time quantitative polymerase chain reaction (RT-qPCR), in the 6 phenotypically evaluated Brazilian genotypes under standard conditions (absence of stress). Our results show that all genotypes lengthened its roots in response to drought, specially the 2 hybrids. The expression of these genes is modified in response to stress, and *OsRAA1* has a very special behavior, constituting a target for future studies. Further steps include the study of polymorphisms in these genotypes in order to understand if differences in these genes or in regulatory regions can be associated with differences in root system architecture and/or stress tolerance.

KEY WORDS: *Oryza sativa*, RT-qPCR, root, abiotic stress.

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INTRODUCTION

Rice (*Oryza sativa* L.) is the second most cultivated cereal in the world and a primary source of food for about two-thirds of the world's population (Pirdashti et al. 2009). The cultivation of rice faces many different challenges, according to region, climate and cultivation system, i.e. upland or lowland. In order to be successful, plants have multiple mechanisms to respond and adapt to adverse environmental conditions.

Drought stress is a problem in approximately 45% of agricultural areas and the largest global constraint to productivity, becoming a major issue in scientific reports (Ahmadi et al. 2012; Ambavaram et al. 2014; Heinemann et al. 2015; Todaka et al. 2015). In rainfed conditions, water deficit can become a primary limiting factor for productivity and its importance is increasing due to climate changes (Datta et al. 2012). In Asia, rice cultivation areas go through dry periods of varying intensity affecting different stages of the crop cycle (Dixit et al. 2012).

In this difficult scenario in which climate is currently changing, world's demand for rice continues to grow, and the use of dry areas in arid and semi-arid tropical regions is both an opportunity and a problem (Sheshshayee et al. 2011). Drought tolerance is a complex quantitatively inherited character that refers to the plant ability to live, grow and reproduce satisfactorily with limited water supply, being able to endure periods of water deficiency (Fleury et al. 2010).

Efforts to improve plant tolerance to drought over time across different regions have resulted in a range of variability for drought tolerance (Degenkolbe et al. 2009). However, to obtain high yields, conventional breeding programs have had limitations in generating new drought tolerant cultivars (Ramya et al. 2010; Serraj et al. 2011).

Current studies have tried to identify factors that influence the level of tolerance of a given species or cultivar through the evaluation of the transcriptional expression of different genes. Thus, assessments on the genetic basis of stress tolerance in plants have been performed (Lasanthi-Kudahettige et al. 2007; Santos et al. 2013).

This study aimed to select and assess the transcriptional expression of 4 genes related to auxin and root development in different stresses. Morphological responses of 8 genotypes to drought were also evaluated. Both informations are crossed in order to detect possible differential stress responses, which can assist the breeding of more resilient crop varieties.

MATERIAL AND METHODS

Phenotypic analysis of 6 cultivars and 2 hybrids under polyethylene glycol 6,000 stress

Six genotypes, with contrasting phenotypes according to the results previously obtained at our laboratory (Mistura et al. unpublished data), were selected: 3 with well-developed root systems ("Arroz de Sequeiro", Ligeirinho, BRS Colosso) and 3 with poorly developed root systems (SCS BRS 112, BRS Vencedora, Farroupilha). In addition, 2 hybrids (H.5×2 and H.6×5) had their morphological response to drought assessed. H.5×2 was obtained from the cross Farroupilha × "Arroz de Sequeiro", while H.6×5 was obtained from the cross BRS Colosso x Farroupilha.

Rice seedlings (14 days of age) were stressed in nutrient solution (Camargo and Oliveira 1981) containing 10% polyethylene glycol (PEG) 6,000 (−1.48 MPa of osmotic pressure). A control was used, consisting of seedlings kept in standard plant nutrient solution for a period of 2 weeks. After that, plants proceeded to the evaluation of morphological traits: shoot length (SL), root length (RL), root dry weight (RDW) and shoot dry weight (SDW).

The analysis of variance (ANOVA) was performed in a completely randomized design and the Tukey's honest significant difference (HSD) test was used for multiple comparisons between pairs at a 95% confidence interval ($p < 0.05$). These analyses were made using the R package "Agricolae" (R Core Team 2013), and the biological material was composed by 3 replicates of 4 plants.

Transcriptional analysis using public database

The transcriptional analysis of genes related to auxin response, which has been preselected in literature (Table 1), was first performed using Genevestigator (Zimmermann et al. 2008) with data from rice seedlings of a drought-sensitive cultivar (IR64) subjected to drought, salt and cold conditions (Jain et al. 2007). Euclidean distance was used to measure the distance for hierarchical clustering analysis, and optimal leaf-ordering was applied to keep similar vectors positioned close to each other. The differential expression under drought conditions considered filtered results with p-values under 0.05 and 0.001.

Table 1. Four genes related to auxin response and root modifications evaluated in this study.

Generic name	ID	References	Probe name in genevestigator analysis	Forward primer for RT-qPCR analysis	Reverse primer for RT-qPCR analysis
OsGNOM1/CRL4	Os03g0666100	Kitomi et al. (2008); Liu et al. (2009)	Os.19756.1.S1_at	TGCTCGCCATGAAACTAGTCG	GCCACGGACCTTGATCTTGAT
OsIAA1	Os01g0178500	Song et al. (2009); Thakur et al. (2001)	Os.9069.1.S1_at	ACCATATACGCAAAAAAACCGATG	CTGACGACACGCGAGCC
OsCAND1	Os02g0712000	Wang et al. (2011)	Os.5788.1.S1_at	ACTTGGTTTTTTGCCCGAAG	TCCACTATCCACTGCAGAGCAT
OsRAA1	Os01g0257300	Ge et al. (2004); Han et al. (2005)	Os.487.1.S1_at	CTCTTCCAGTTCCACAAGCGT	AAGGAGTCGCGGTTCTTGA

Real-time quantitative polymerase chain reaction for auxin-related genes in different genotypes

Seedlings (21 days of age) of rice genotypes SCS BRS 112, "Arroz de Sequeiro", BRS Ligeirinho, BRS Vencedora, Farroupilha and BRS Colosso grown in nutrient solution (Camargo and Oliveira 1981) were used in this experiment. As the production of hybrid seeds was enough to cover only the morphological analyses, these genotypes were not included in transcriptional analysis.

Total RNA was extracted from 0.1 g of root tissue from rice seedlings following the protocol described by PureLink™ reagent (Invitrogen™). Samples were treated with DNAse I (Invitrogen™). The quantity of RNA was assessed, and each sample was reverse-transcribed into cDNA using the commercial kit SuperScript® III First-Strand System for real-time quantitative polymerase chain reaction (RT-qPCR) (Invitrogen™). Samples consisted of cDNA produced from mRNA obtained from 3 different biological replicates. These replicates were composed by bulks of 4 plants each.

Primers for 4 genes related to auxin response and root development were designed according to Applied Biosystems® recommendations (Table 1) and evaluated for amplification efficiency above 90%. Also, the dissociation curves of each pair of primers contained a single peak, and the agarose gels revealed single bands corresponding to the predicted amplicon length.

The RT-qPCR was performed in a 7500 Real-Time PCR System (Applied Biosystems®) using SYBR® Green (Invitrogen™) dye. Relative quantification of each single gene expression was performed using the comparative threshold cycle method (Livak and Schmittgen 2001), and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) was used as a reference gene to quantify transcript abundance of each gene (Iskandar et al. 2004; Lasanthi-Kudahettige et al. 2007). Gene expression data was displayed as a heat map graphic, through Multi Experiment Viewer (MeV), EASE Expression Analysis Systematic Explorer version 4.6 (Saeed et al. 2003), where the cultivar SCS BRS 112 was used as calibrator.

RESULTS AND DISCUSSION

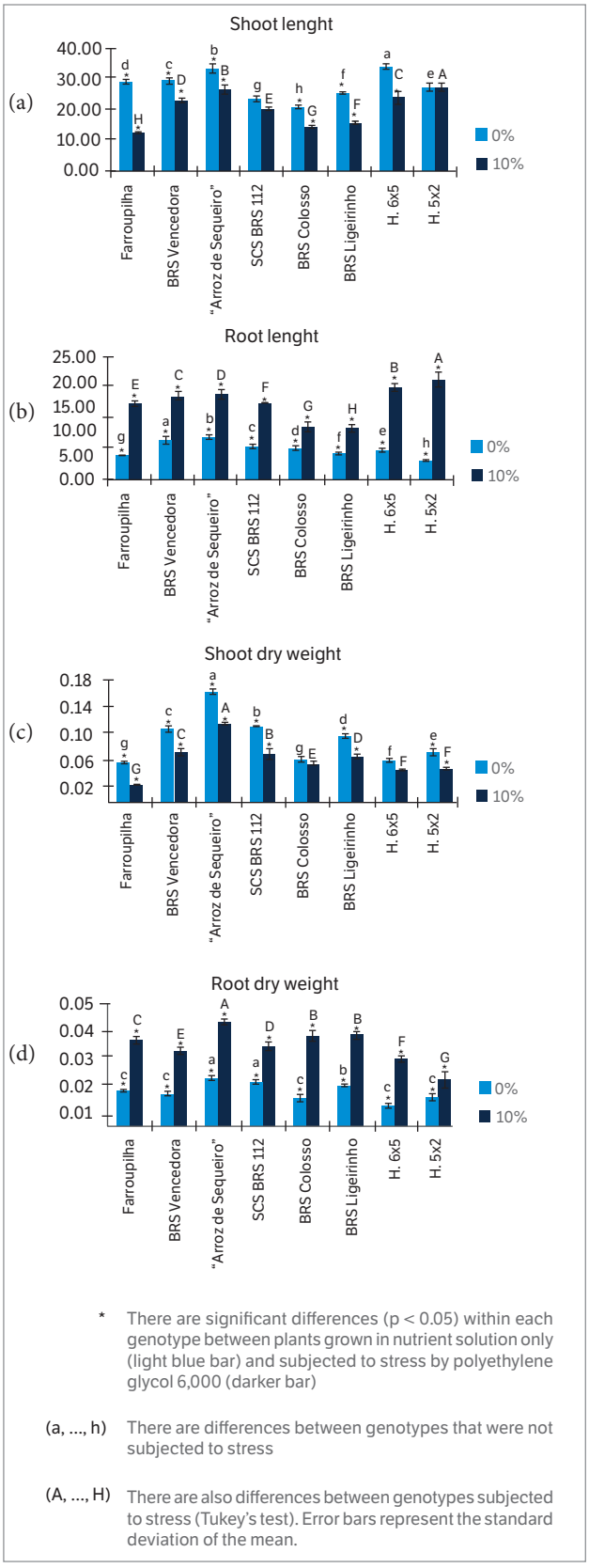
Effect of stress on morphology PEG 6000

Significant differences ($p < 0.05$) between genotypes were observed for SL and RL, as well as for SDW and RDW, under control and stressful conditions (Figure 1).

For RL (Figure 1b), both hybrids presented very high values when subjected to stress. This response becomes especially important when it is observed that the values found for this variable are quite low when the plants are in control conditions, indicating that indeed the hybrids provided an intense response.

The genotypes characterized as having well-developed root systems did not show the highest values for RL as a response to stress. However, when analyzing RDW (Figure 1d), genotypes with well-developed root systems showed the highest values under stress, along with BRS Ligeirinho.

Since small changes in the root system can have significant effects on productivity, the importance of root growth to maintain crop productivity is being increasingly recognized by plant breeders (Meng et al. 2010; Bengough et al. 2011). The transfer of carbohydrates from leaves to roots and the consequent increase in root growth is a response that favors its uptake capacity (Davatgar et al. 2009). In this study, plants subjected to water deficit condition increased root development, something that can be seen through the increase in RL and RDW.



* There are significant differences ($p < 0.05$) within each genotype between plants grown in nutrient solution only (light blue bar) and subjected to stress by polyethylene glycol 6,000 (darker bar)

(a, ..., h) There are differences between genotypes that were not subjected to stress

(A, ..., H) There are also differences between genotypes subjected to stress (Tukey's test). Error bars represent the standard deviation of the mean.

Figure 1. Comparison of (a) Shoot length; (b) Root length; (c) Shoot dry weight; (d) Root dry weight.

The indirect effect of drought, simulated by PEG 6,000, can be observed through the reduction of SDW for all genotypes (Figure 1c). However, genotypes characterized as having less developed root system presented longer shoots in stress conditions, except for "Arroz de Sequeiro". As expected, this response was similar to what happened to SL (Figure 1a), where the main differences were in hybrids, which had SL less affected than SDW.

Transcriptional expression

A small transcriptional change in genes *OsGNOM1/CRL4*, *OsCAND1* and *OsIAA1*, which are all repressed under the 3 different analysed stresses, was seen in the Genevestigator platform (Figure 2a). For *OsRAA1*, however, an increase in expression was observed (Jain et al. 2007). It is known that drought, high salinity, and low temperature induce the expression of a large number of genes (Todaka et al. 2015). These stresses are very complex stimuli that possess many different yet related attributes (Xiong et al. 2002). It is interesting to point out that this gene had a very distinct position in the clustering analysis.

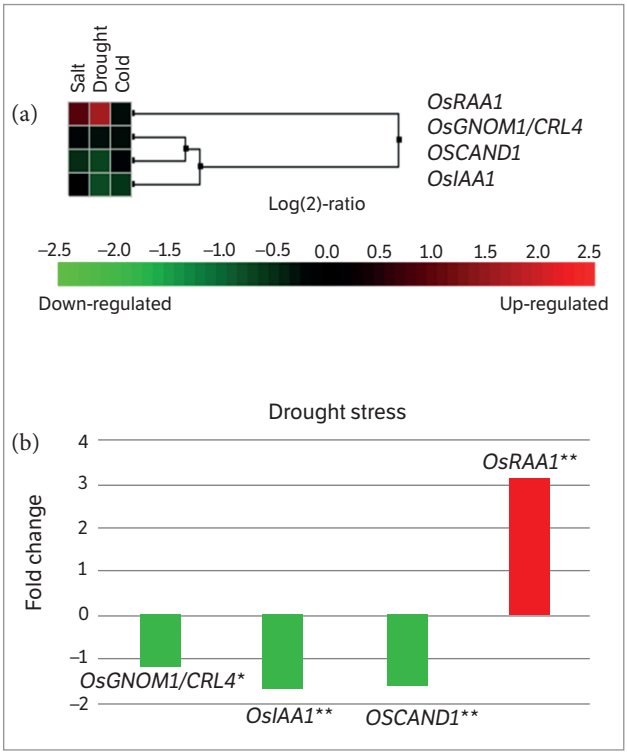


Figure 2. Expression of genes related to auxin response and root development. (a) Hierarchical clustering grouping genes that have similar profiles under cold, salt and drought stresses; (b) Differential expression under drought conditions (*$p < 0.05$; **$p < 0.001$).

The transcriptional change of these genes under drought stress can be seen in Figure 2b. Interestingly, *OsRAA1* shows a positive 3.13-fold change ($p < 0.001$) in IR64, but different results were found when analyzing the other cultivars with PEG 6,000. Since overexpression of *OsRAA1* results in reduced growth of primary roots (Han et al. 2005), this may suggest that, in the experiment with the genotype IR64 (sensitive), the upregulation of this gene can be one of the reasons that make this cultivar more susceptible to drought. This is supported by opposite results we found for the cultivars analyzed under stress by PEG 6,000 in the experiment described here. This gene, that is transcriptionally responsive to stresses and has a proven impact on root morphology, is still poorly studied, and greater efforts to better understand its role in rice under stressful conditions are needed (Ge et al. 2004; Han et al. 2005; Wu and Cheng 2014).

The transcriptional expression of these genes in each of the 6 rice genotypes can be seen in Figure 3. Low expression was observed for all studied genes in BRS Ligeirinho and BRS Vencedora. Auxin plays a crucial role in regulation of various plant responses, such as cell elongation, cell division, photoperiodism, gravitropism,

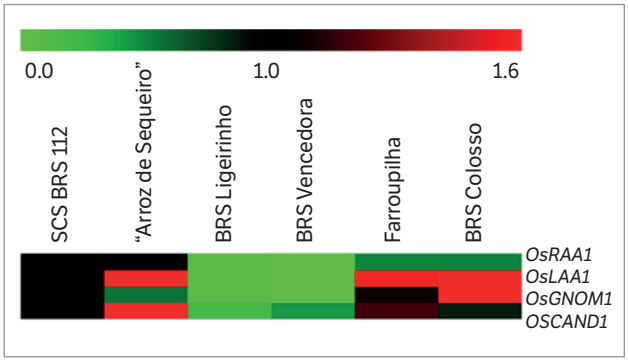


Figure 3. Heat map graphic of the relative expression of the 4 genes involved in root development in rice, where the genotype SCS BRS 112 was used as control. The data are presented as means.

apical dominance and root initiation (Du et al. 2011), and is also important in root development and architecture (Wang et al. 2011). Differences in cycle length between these cultivars possibly have harmed the comparison of transcriptional activity of the studied genes, since the metabolism of these plants operates on quite different rates and may vary even with very small differences in developmental stages.

In general, plants subjected to drought stress had reduced shoot growth and number of roots. An increase in root lenght and, consequently, an increase in its dry weight were also observed. The ratio of dry weight of roots and shoots also registered a significant increase due to stress.

CONCLUSION

From the results presented here, it was possible to confirm that these genotypes respond differently to drought stress. However, even though these genes are transcriptionally responsive to different stresses, their transcriptional levels do not explain root morphological differences between the tested genotypes in the absence of stress. Of the different assessed phenotypic parameters, the one that showed best results was RDW, which was more responsive to drought stress in genotypes with well-developed root systems.

Further research using plants adapted to drought (xerophytes), assisted by bioinformatics and molecular biology, can provide us information about relevant differences found in these genes when compared to crops. The identification of polymorphisms and the pattern of regulation of homologs of these genes in these species may be valuable, but the conservation of its function will have to be tested in different crops, including rice.

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