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BRIEF COMMUNICATION

Chloroplastic and mitochondrial *GPX* genes play a critical role in rice development

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Abstract

Plant glutathione peroxidases (GPX) catalyze the reduction of H_2O_2 or organic hydroperoxides to water, mitigating the toxicity of these compounds to cells. In rice plants, the *GPX* gene family is composed of five members that are distributed in a range of sub-cellular compartments including cytosol, mitochondria, chloroplasts, or endoplasmic reticulum. Of these, *OsGPX1* and *OsGPX4* are located in mitochondria and chloroplasts, respectively. To understand the role of these *GPX* in rice, the effect of knockdown of *OsGPX1* and *OsGPX4* in rice plants was evaluated. Our data show that *OsGPX4* was essential for *in vitro* rice regeneration because no plants were obtained from calli carrying a hairpin construct against *OsGPX4*. Although the knockdown of *OsGPX1* did not impair plant regeneration, the plants with silenced *OsGPX1* (*GPX1s* plants) showed reduced shoot length and a reduced number of seeds compared to the non-transformed rice plants. These results indicate that OsGPX1 and OsGPX4 are essential for redox homeostasis which leads to normal growth and development of rice.

Additional key words: glutathione peroxidase, hairpin construct, *in vitro* regeneration, *Oryza sativa*, oxidative stress, seed production, transgenic plants.

Reactive oxygen species (ROS), which are generated during normal plant development, act as signaling molecules and regulate essential processes. They are generated in toxic concentrations during biotic and abiotic stresses (Halliwell *et al*. 2006, Faltin *et al.* 2010). To maintain redox homeostasis, ROS-scavenging systems use thiol-containing proteins as redox transducers (Foyer and Noctor 2005) as well as ascobate peroxidase (APX), catalase (CAT), superoxide dimustase (SOD), and non-enzymatic compounds ascorbate (Asc), glutathione (GSH), tocopherols, and carotenoids (Koh *et al*. 2007). Plant glutathione peroxidases (GPX) preferentially use thioredoxin as electron donor to catalyze the reduction of H_2O_2 or organic hydroperoxides to water or corresponding alcohols (Herbette *et al.* 2007). Contrary to most plant GPX, mammalian isoforms carry seleno-cysteine in their active site, allowing them to preferentially use glutathione as electron donor (Herbette *et al.* 2007). In plants, GPXs are involved in biotic and abiotic stress responses (Milla *et al.* 2003, Navrot *et al.* 2006, Xu *et al.* 2012, Zhang *et al.* 2012). For example, in *Arabidopsis thaliana*, a GPX loss or gain of function mutants demonstrated the role of these enzymes in H_2O_2 scavenging, signal transduction, photo-oxidative stress tolerance, and nuclear DNA damage protection (Miao *et al.* 2006, Chang *et al.* 2009, Gaber *et al.* 2012). To understand the role of GPX in rice, we tried to obtain transgenic rice plants that were silenced for the whole *GPX* family. In the current study, we present the characteristics of *OsGPX1* or *OsGPX4* rice knock-down transgenic plants. The transformation of rice calli with a hairpin construct targeted to chloroplastic *OsGPX4*

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Abbreviations: GPX - glutathione peroxidase; qPCR - quantitative polymerase chain reaction.
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failed to regenerate plants; this highlighted the importance of OsGPX4 during *in vitro* regeneration. Additionally, mutants deficient in the mitochondrial *OsGPX1* gene allowed the formation of fertile plants. However, these plants (*GPX1s* plants) had shorter shoots, a lower biomass, and mostly a lower number of spikelets and seeds than the non-transformed (NT) plants.

Target gene sequences from *OsGPX1* (LOC_Os04g46960) and *OsGPX4* (LOC_Os06g08670) of 115 and 197 bp, respectively, were inserted into the pANDA RNAi vector (Miki and Shimamoto 2004) by the gateway LR reaction, using cDNAs as template. Primer pairs used to amplify the referred sequences are listed in Table 1. The plasmids were introduced into rice calli *via Agrobacterium tumefaciens* mediated transformation, as described by Upadhyaya (2002). T1 rice seeds from the NT and transgenic plants were germinated on MS medium (*Sigma-Aldrich*, São Paulo, Brazil) supplemented with hygromycin, under a 12-h photoperiod, photosynthetic photon flux density (PPFD) of 150 μmol m^{-2} s^{-1}, temperature of 25 °C, and relative humidity of 80 %. cDNA and real-time qPCR assays were performed as described by Rosa *et al*. 2010. Primers were designed to produce DNA fragments ranging from 180 to 250 bp. PCR amplifications were performed using the specific primers (Table 1). All reactions were repeated 4 times and expression data analyses were performed after comparative quantification of amplified products using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001, Schmittgen and Livak 2008). The rice NT and *GPX1s* plants were grown in a greenhouse at mean day/night temperatures of 29/24 °C, mean relative humidity of 61 %, a 12-h photoperiod, and maximum PPFD of 850 μmol m^{-2} s^{-1} until they reached the reproductive phase. Statistical analyses were performed using the Student's *t*-test to compare the mean gene expression values at a confidence level of 0.05. For phenotypic comparisons, data were analyzed using the analysis of variance (*ANOVA*), and the mean values were compared using the Tukey's test at a confidence level of 0.05.

Table 1. Sequences of the primers used for the RNAi analysis and qPCR.

Primer name	Forward	Reverse
RNAi*OsGPX1*	CACCGTCCTCGTCTCCACGCTACC	AAGTCGTGGACGGAGGTG
RNAi*Os*GPX4	CACCCTGTTGCAGCATCCTTTTTG	CTGACGACACCCACAACAAC
qRT*OsGPX1*	AGCAACCTGCACTTATGCACT	CAGCAAGGAAATTTATTGACATGA
qRT*OsGPX2*	CTGGTTGGGTAGGCACTGTT	TGCAAACACAAACCTTACGCTAC
qRT*OsGPX3*	TTGCATTGAGCACTTGGAAC	AGGGGCAAAGTGATGCAGTA
qRT*OsGPX4*	CTGTACATATGCCTTGCCTCA	GTTACAGGGGCCAGATAAGC
qRT*OsGPX5*	AAGATTGAGAATGATATCCAGAAGC	GCAAACCACATTCTTACGAACA
qRT*OsActina2*	GGACGTACAACTGGTATCGTGTT	GTTCAGCAGTGGTAGTGAAGGAG
qRT*OsFdh3*	TTCCAATGCATTCAAAGCTG	CAAAATCAGCTGGTGCTTCTC

Table 2. The quantitative determination of *GPX* mRNAs in *GPX1s* plants and in *GPX4s* calli in comparison with NT plants/calli. Values represent the means of the lines 9 and 21 ± SD, n = 21 (*GPX1s*), n = 9 (*GPX4s*). Significantly different *GPX* expressions between the NT and transgenic plants/calli were marked with different letters.

Sample	*GPX1*	*GPX2*	*GPX3*	*GPX4*	*GPX5*
NT plants	1.001±0.06a	1.008±0.15a	0.780±0.12a	1.011±0.18a	1.005±0.12a
GPX1s plants	0.409±0.03b	0.861±0.26a	0.923±0.26a	1.217±0.49a	0.684±0.22b
NT calli	1.000a	1.000a	1.000a	1.000a	1.000a
GPX4s calli	1.279±0.23a	0.755±0.20a	0.978±0.21a	0.244±0.02b	0.582±0.12b

Whereas seven lines carrying the hairpin construct for *OsGPX1* were recovered (4, 17, 29, 30, 36, 40, and 45; Fig.1*A*), all attempts to obtain regenerated plants expressing an RNAi*OsGPX4* construct failed. For this construct, the calli turned green but did not evolve into plants (Fig. 1*B*,*C*). The RT-qPCR analysis of the *GPX4s* calli showed that the *OsGPX4* mRNA level was reduced compared to the NT calli (Table 2). To investigate whether the other *OsGPX* genes could be differently modulated in the *OsGPX4s* calli, we analyzed the amount of transcripts of the other *OsGPX* genes. The expression of the cytosolic *OsGPX*5 gene was reduced to 42 %, whereas the expressions of the other members of the gene family did not change in the *GPX4s* calli (Table 2). On the other hand, the knockdown of *OsGPX1* produced plants with shorter shoots compared to the NT plants (Table 3). RT-qPCR analysis showed that *OsGPX1* mRNA was reduced to 40 % compared to the NT plants (Table 2). The evaluation of the other members of the family in the *GPX1s* transgenic plants showed a reduction in the *OsGPX5* gene expression to about 70 % of the NT plants, whereas the expression of the other genes did not change (Table 2).

Most of the *GPX1s* plants had shorter shoots

compared to the NT plants (Table 3). Although some lines had a higher spikelet number compared to the NT plants, the number of seeds per spikelet was lower than in the NT plants. A similar trend was observed with the production of viable seeds: the *GPX1s* plants had a lower number of viable seeds in the majority of the lines analyzed. The viability index of seeds (a number of viable seeds over the total number of seeds) followed the same pattern, whereas the seed mass mostly decreased (Table 3).

Our efforts to regenerate the knockdown *GPX4s* rice plants failed on all attempts, although about 20 % of the *OsGPX4* expression was retained. It has been reported that the overexpression of *cit-PHGPX* (*Citrus sinensis*) prevents the regeneration of tobacco, potato, and tomato (Faltin *et al.* 2010). However, the overexpression of *cit-PHGPX* is achieved when tobacco and potato cell cultures are used or the floral-dip method is employed in *Arabidopsis* plants (Faltin *et al.* 2010). These data suggest that the equilibrium in the H_2O_2 content, not merely the presence or absence of H_2O_2, is crucial for *in vitro* regeneration of plants. Additionally, the GPX isoenzymes interfere with shoot organogenesis during *in vitro* cultures of different plant species (Faltin *et al.* 2010). These data strongly suggest that GPX4 is essential for rice development. It is also possible that the considerable decrease in the expression of *OsGPX4* together with decrease in *OsGPX5*, was too drastic for the cells and thus resulted in impaired regeneration. However, the expression of only 40 % of *OsGPX1* (Table 2) and 5 % of *OsGPX3* (Passaia *et al.* 2013), both mitochondrial GPX isoforms, did not affect *in vitro* regeneration indicating that the reduced expression of mitochondrial GPX does not impair *in vitro* regeneration, but that of chloroplastic GPX4 does.

The *GPX1s* lines expressing only 40 % of *OsGPX1* had shorter shoots than the NT plants, and their seed production was affected as well. The knockdown of different antioxidant enzymes in rice produces numerous phenotypic alterations, but the reduction in seed production has not yet been described. Previously, our group demonstrated that the cytosolic *APX* knockdown in rice plants exhibits a normal phenotype, and its photosynthesis parameters are not affected (Rosa *et al.* 2010, Ribeiro *et al.* 2012). Moreover, the reduction in the expression of chloroplastic APX in rice does not result in

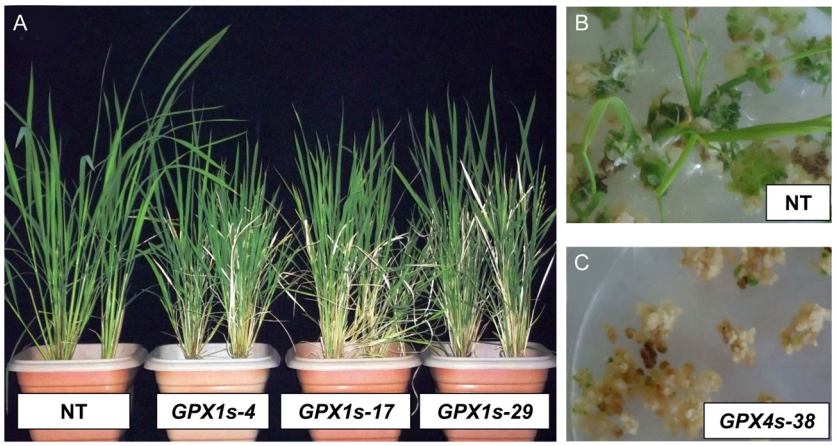

Fig. 1. *GPX1s* plants and calli during *in vitro* regeneration: *A* - non-transformed (NT) plants and three independent lines of *GPX1* plants grown under controlled conditions; *B* - NT and *C* - GPX4s calli grown *in vitro*.

Table 3. Shoot length [cm], panicle number [plant^{-1}], fertile panicle number [plant^{-1}], number of viable seeds [plant^{-1}], seed viability index (viable seeds/total seeds ratio), seed mass [mg seed^{-1}], shoot biomass [g plant^{-1}], and total seed mass [g plant^{-1}] of *GPX1s* knockdown mutants and non-transformed rice plants (NT) at late reproductive stage. Data are means of four replicates ± SD. Different letters represent significant differences ($P \leq 0.05$) among the different rice lines according to the Tukey's test.

Parameter	NT	GPX1-L4	GPX1-L17	GPX1-L29	GPX1-L30	GPX1-L36	GPX1-L40	GPX1-L45
Shoot length	82.33±2.1a	39.51±13.8b	52.05±8.1b	56.01±7.0b	58.33±2.1b	55.67±4.0b	67.01±10.6b	70.01±9.8ab
Panicle number	7.67±2.5b	13.01±1.1a	-	10.01±6.2ab	4.67±2.8b	9.33±1.7b	8.01±1.7b	15.02±1.7a
Fertile panicles	69.01±12.8a	35.02±7.6b	-	43.33±14.8ab	61.02±16.6ab	45.02±13.2ab	42.33±8.7b	42.03±7.0b
Viable seeds	480.67±142a	189.03±21.5b	-	43.51±28.5d	184.68±4.7b	49.33±10.1d	94.98±10.5c	465.01±126.5a
Viability index	92.58±3.7a	50.75±7.0c	-	19.07±9.0d	77.29±6.0b	12.94±5.5d	21.96±9.5d	91.09±2.2a
Seed mass	23.30±4.0a	16.30±2.0c	-	18.00±2.0bc	18.10±1.0bc	21.00±0.0ab	25.40±2.0a	24.20±2.0a
Shoot biomass	64.84±14.4a	38.52±2.9b	32.79±14.7bc	47.57±11.6ab	19.71±3.8c	28.83±4.5c	32.98±13.6bc	64.02±6.8a
Total seed mass	10.84±2.4b	4.52±0.9c	-	4.84±2.2c	4.71±0.8c	3.51±0.6c	4.31±2.6c	18.02±2.1a

any phenotypic alteration, however, photosynthesis parameters are affected (Caverzan *et al*. 2014).

Additionally, a grass pea ascorbate deficient mutant showed development alterations (Talukdar *et al.* 2012). Taken together, our data demonstrate that the reduced expression of the mitochondrial *OsGPX1* and chloroplastic *OsGPX4* genes in rice affected important processes of rice development indicating that these enzymes play essential roles in the interaction between redox homeostasis and normal plant development.

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