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The knockdown of chloroplastic ascorbate peroxidases reveals its regulatory role in the photosynthesis and protection under photo-oxidative stress in rice

Andréia Caverzan[a,b], Aurenivia Bonifacio[d], Fabricio E.L. Carvalho[d], Claudia M.B. Andrade[b,1], Gisele Passaia[a], Mariana Schünemann[a], Felipe dos Santos Maraschin[c], Marcio O. Martins[d], Felipe K. Teixeira[a], Rafael Rauber[b], Rogério Margis[b,e], Joaquim Albenisio Gomes Silveira[d], Márcia Margis-Pinheiro[a,*]

[a] Department of Genetics, Federal University of Rio Grande do Sul, Brazil
[b] Biotechnology Center, Federal University of Rio Grande do Sul, Brazil
[c] Department of Botany, Federal University of Rio Grande do Sul, Brazil
[d] Department of Biochemistry and Molecular Biology, Federal University of Ceará, Brazil
[e] Department of Biophysics, Federal University of Rio Grande do Sul, Brazil

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ABSTRACT

The inactivation of the chloroplast ascorbate peroxidases (chlAPXs) has been thought to limit the efficiency of the water–water cycle and photo-oxidative protection under stress conditions. In this study, we have generated double knockdown rice (*Oryza sativa* L.) plants in both *OsAPX7* (sAPX) and *OsAPX8* (tAPX) genes, which encode chloroplastic APXs (chlAPXs). By employing an integrated approach involving gene expression, proteomics, biochemical and physiological analyses of photosynthesis, we have assessed the role of chlAPXs in the regulation of the protection of the photosystem II (PSII) activity and CO_2 assimilation in rice plants exposed to high light (HL) and methyl viologen (MV). The chlAPX knockdown plants were affected more severely than the non-transformed (NT) plants in the activity and structure of PSII and CO_2 assimilation in the presence of MV. Although MV induced significant increases in pigment content in the knockdown plants, the increases were apparently not sufficient for protection. Treatment with HL also caused generalized damage in PSII in both types of plants. The knockdown and NT plants exhibited differences in photosynthetic parameters related to efficiency of utilization of light and CO_2. The knockdown plants overexpressed other antioxidant enzymes in response to the stresses and increased the GPX activity in the chloroplast-enriched fraction. Our data suggest that a partial deficiency of chlAPX expression modulate the PSII activity and integrity, reflecting the overall photosynthesis when rice plants are subjected to acute oxidative stress. However, under normal growth conditions, the knockdown plants exhibit normal phenotype, biochemical and physiological performance.

Abbreviations: APX, ascorbate peroxidase; AsA, ascorbate; cAPX, cytosol APX; CAT, catalase; chlAPX, chloroplastic APX; Ci, intercellular concentration of CO_2; E, transpiration; ETR, apparent electron transport rate; EXC, energy excess; F_v/F_m, potential quantum yield of photosystem II; GPX, glutathione peroxidase; g_s, stomatal conductance; H_2O_2, hydrogen peroxide; HL, high light; J_{max}, maximum photosynthetic electron transport; mAPX, peroxisome/glyoxysomes APX; miAPX, mitochondria APX; MV, methyl viologen; NPQ, non-photochemical quenching; NT, non-transformed; PET, photosynthetic electron transport; P_N–Ci, photosynthesis depending on the intercellular concentration of CO_2; P_N–PPFD, photosynthesis depending on light intensity; P_N, net CO_2 assimilation; PPFD, photosynthetic photon flux density; PQ, plastoquinone pool; P_r, photorespiration; PSI, photosystem I; PSII, photosystem II; qP, photochemical quenching; R_d, light respiration; R_n, dark respiration; ROS, reactive oxygen species; RT-qPCR, quantitative real-time PCR; sAPX, stroma APX; SOD, superoxide dismutase; tAPX, thylakoid APX; TBARS, thiobarbituric acid-reactive substances; V_{cmax}, maximum Rubisco carboxylation rate; $\Delta F_v/F'_m$, actual quantum yield of photosystem II.

* Corresponding author at: Universidade Federal do Rio Grande do Sul, Avenida Bento Gonçalves, 9500, CEP: 915910-970, Porto Alegre, RS, Brazil. Tel.: +55 51 3308 9814.
E-mail addresses: marcia.margis@ufrgs.br, marcia.p.margis@gmail.com (M. Margis-Pinheiro).
[1] Present address: Department of Chemistry, Federal University of Mato Grosso, Brazil.

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1. Introduction

Ascorbate peroxidases (APXs) are heme-binding enzymes that reduce hydrogen peroxide (H_2O_2) to water using ascorbate (AsA) as an electron donor [1]. In rice plants, a small gene family encodes APX isoforms which are targeted to distinct subcellular compartments: mitochondria (miAPX), cytosol (cAPX), peroxisome/glyoxysomes (mAPX), thylakoid membranes (tAPX) and stroma (sAPX) in chloroplasts [2]. The APX isoenzymes, especially the chloroplastic APXs (chlAPXs), are unstable in low concentrations of AsA and in the presence of H_2O_2 [3,4]. Decreases in AsA levels and inactivation of APXs in chloroplasts have been considered to be limiting factors in the efficiency of the water–water cycle and photo-oxidative protection under stress conditions [4]. The coordinate action of this cycle is essential to dissipate excess energy from photosystem I and II (PSI and PSII) and avoid impairment in photosynthesis [1].

The water–water cycle directly involves the chloroplast superoxide dismutase (Cu-ZnSOD isoform) and tAPX activities, which operates in association to eliminate superoxide radical and H_2O_2 contributing to avoid oxidative stress [5]. Under oxidative stress generated by methyl viologen, the water–water cycle is unable to eliminate great amounts of superoxide and H_2O_2 [1]. These reactive oxygen species (ROS) may be also eliminated by other SOD isoforms and sAPXs localized in the stroma in association with other enzymes involved with the ascorbate–glutathione cycle [6]. Thus, both chloroplast APXs might protect the photochemistry machinery against photo-oxidative damage and photoinhibition by consuming excess electrons in the photosynthetic electron transport (PET) chain [7]. To minimize the excess of electrons and to eliminate H_2O_2 in chloroplasts, other peroxidases such as peroxiredoxins and glutathione peroxidase (GPX) may be also important [8,9]. The effectiveness of these peroxidases in compensating for APX deficiency is still in debate [10].

Paradoxically, as the chlAPXs are immensely sensitive to H_2O_2 they are among the first molecules attacked by ROS in chloroplasts. However, the role of chlAPXs in the photo-oxidative protection of chloroplasts is still a matter under discussion, which highlights the following question: if the chlAPXs are essential for oxidative defense, why a variety of studies showed contradictory results, often indicating little importance of these enzymes? A study with single and double mutants of *Arabidopsis* lacking cAPX and tAPX suggested that the current paradigm of the central pathways for ROS elimination involving APXs should be reviewed [11].

Recently, we demonstrated that rice plants deficient in both cytosolic APXs exhibit a compensatory mechanism employing other peroxidases, a mechanism that apparently does not occur in *Arabidopsis* [10]. Most of the studies of the effect of the lack or deficiency of chlAPXs have been carried out with *Arabidopsis*; however, several studies have shown that plants differ widely in their mechanisms of oxidative response, especially with regard to networks associated with perception, signaling, gene expression and protective physiological strategies [12,13]. This variability is based in part on the large antioxidant metabolism redundancy and plasticity in higher plants [11]. Thus, it is plausible that a plant such as rice, with a more complex genome than *Arabidopsis*, could support more complex gene networks and different metabolic and physiological mechanisms to cope with abiotic stress induced by oxidative damage [10].

Thus, a better understanding of the role played by chlAPXs in monocot crop models such as rice could contribute to a more complete understanding of the molecular, biochemical and physiological mechanisms that might control plant tolerance to abiotic stress. We have previously shown that rice *OsAPX* genes are modulated by different stresses such as salt, drought and exogenous H_2O_2 [2,14]. We also studied the functional role of the cytosolic APXs in rice and showed the importance of these enzymes for the ROS compensatory metabolism in response to several abiotic stresses [10,14]. We demonstrated that, in contrast to *Arabidopsis*, rice plants silenced for both cytosolic APXs exhibited a compensatory mechanism involving the expression and activity of other peroxidases, especially isoforms of catalase (CAT) and GPX.

In the current study rice plants with knockdowns in both chloroplastic ascorbate peroxidases (*OsAPX7* and *OsAPX8*) were exposed to oxidative stress (MV) and photo-oxidative (high light) to assess the role of these proteins in photochemical activity, CO_2 assimilation and antioxidant response. The study was performed using an integrated approach involving gene expression, proteomics, enzymatic activities and *in vivo* photosynthesis measurements (photochemical and Calvin cycle). Our data reveal that under acute stress imposed by MV but not by HL, the chlAPXs are crucial to oxidative protection, photodamage and photoinhibition in the PSII. Apparently chlAPXs were not important to photo-protection in presence of high light. In addition, our results strongly suggest that these enzymes are more important to PSII protection against MV-induced oxidative stress than to HL-induced photo-oxidation. The physiological significance of the deficiency of both chlAPXs isoforms in overall photosynthesis and oxidative response is discussed.

2. Materials and methods

2.1. Construction of the plant vector and plant transformation

A chimeric gene producing mRNA with a hairpin structure (hpRNA) was constructed based on the sequence of the *OsAPX7* (LOC_Os04g35520) and *OsAPX8* (LOC_Os02g34810) genes. The following primer pairs were used to amplify a 238-bp RNAi*OsAPX7/8* sequence: 5′-CACCCTCTAAAGCTTGTCCAAC-3′ and 5′-TCAAGACCCATCCTGTAA-3′. PCR products were cloned into the Gateway vector pANDA, in which hairpin RNA is driven by the maize ubiquitin promoter and an intron placed upstream of the inverted repeats [15]. *Agrobacterium tumefaciens*-mediated transformation was performed as described previously [16].

2.2. Plant material and growth conditions

The rice plants knockdowns for both *OsAPX7* and *OsAPX8* were obtained by *A. tumefaciens*-mediated transformation of rice embryogenic calli (*Oryza sativa* L. ssp. *japonica* cv. Nipponbare) induced from seeds and cultivated in NB medium [16] at 28 °C in the dark. Rice seeds from the T1 generation of non-transformed (NT) and transgenic lines, in which the chloroplastic *OsAPX7* and *OsAPX8* genes (Apx7/8s) had been silenced, were germinated in MS medium (Sigma–Aldrich) supplemented with hygromycin under controlled conditions (150 $\mu mol\ m^{-2}\ s^{-1}$ photosynthetic photon flux density (PPFD), 25 °C, 80% relative humidity and a 12 h photoperiod). Two weeks after being sown, the rice seedlings were transferred to plastic 2-L pots (three seedlings per pot) filled with ¼ strength Hoagland-Arnon's nutritive solution [17]. The seedlings were grown for 2 months in a greenhouse (29 °C mean temperature, 68% mean relative humidity, average PPFD of 550 $\mu mol\ m^{-2}\ s^{-1}$ and a 12-h photoperiod).

2.3. Methyl viologen (MV) and high light (HL) treatments

The 2-month-old transformed and NT plants were grown as described previously. For the MV treatment, a group of plants were transferred to a growth chamber at 27 °C/24 °C (day/night) and 70% humidity with a PPFD of 500 $\mu mol\ m^{-2}\ s^{-1}$. The plants were then acclimated for 24 h. The MV was dissolved in 0.1% Triton-X-100 at