Journal of Plant Physiology 201 (2016) 17–27

Contents lists available at ScienceDirect

Journal of Plant Physiology

journal homepage: www.elsevier.com/locate/jplph

Silenced rice in both cytosolic ascorbate peroxidases displays pre-acclimation to cope with oxidative stress induced by 3-aminotriazole-inhibited catalase

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ARTICLE INFO

Article history:
Received 15 January 2016
Received in revised form 23 June 2016
Accepted 24 June 2016
Available online 25 June 2016

Keywords:
Ascorbate peroxidase
Hydrogen peroxide
Oryza sativa
Photorespiration
Photosynthesis
Redox metabolism

ABSTRACT

The maintenance of H_2O_2 homeostasis and signaling mechanisms in plant subcellular compartments is greatly dependent on cytosolic ascorbate peroxidases (APX1 and APX2) and peroxisomal catalase (CAT) activities. APX1/2 knockdown plants were utilized in this study to clarify the role of increased cytosolic H_2O_2 levels as a signal to trigger the antioxidant defense system against oxidative stress generated in peroxisomes after 3-aminotriazole-inhibited catalase (CAT). Before supplying 3-AT, silenced APX1/2 plants showed marked changes in their oxidative and antioxidant profiles in comparison to NT plants. After supplying 3-AT, APX1/2 plants triggered up-expression of genes belonging to *APX* (*OsAPX7* and *OsAPX8*) and *GPX* families (*OsGPX1*, *OsGPX2*, *OsGPX3* and *OsGPX5*), but to a lower extent than in NT plants. In addition, APX1/2 exhibited lower glycolate oxidase (GO) activity, higher CO_2 assimilation, higher cellular integrity and higher oxidation of GSH, whereas the H_2O_2 and lipid peroxidation levels remained unchanged. This evidence indicates that redox pre-acclimation displayed by silenced rice contributed to coping with oxidative stress generated by 3-AT. We suggest that APX1/2 plants were able to trigger alternative oxidative and antioxidant mechanisms involving signaling by H_2O_2, allowing these plants to display effective physiological responses for protection against oxidative damage generated by 3-AT, compared to non-transformed plants.

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1. Introduction

The maintenance of an adequate cellular redox homeostasis is essential for plant growth, especially under adverse environmental conditions (Foyer and Noctor, 2015). This metabolic state involves redox networks that operate in coordination to facilitate plant protection and development (Baxter et al., 2014; Gilroy et al., 2016; Munné-bosch et al., 2013). For this purpose, several redox reactions must occur in virtually all cell compartments, involving a robust communication system to confer efficient energy use (Gilroy et al., 2016; Munné-bosch et al., 2013). The comprehension of signaling mechanisms and integration between complex processes has been a major issue in redox modulation that has emerged in recent years (Munné-bosch et al., 2013; Suzuki et al., 2013a). This holistic view could contribute to understanding some complex results involving transformed plants deficient in APX and/or CAT isoforms (Davletova et al., 2005; Han et al., 2013; Suzuki et al.,

Abbreviations: AO, ascorbate oxidase; APX, ascorbate peroxidase; APX1/2, rice cytosolic ascorbate peroxidases 1 and 2; ASC, reduced ascorbate; AT, 3-aminotriazole; cAPX, cytosolic ascorbate peroxidases; CAT, catalase; DHA, dehydroascorbate; Fv/Fm, photosystem II maximum quantum efficiency; GO, glyoxylate oxidase; GPOD, guaiacol peroxidase; GPX, glutathione peroxidase; GR, glutathione reductase; Gs, stomatal conductance; GST, glutathione-S-transferase; KO, knockout; NT, non- transformed rice plants; phGPX, phospholipid hydroperoxide glutathione peroxidase; Pn, net CO_2 assimilation rate; PPFD, photosynthetic photon flux density; ROS, reactive oxygen species; SOD, superoxide dismutase; TBARS, thiobarbituric acid reactive substances.

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http://dx.doi.org/10.1016/j.jplph.2016.06.015
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2013b; Willekens et al., 1997). Indeed, in general, these mutants have displayed unexpected and unexplained responses, particularly in a physiological context.

Cytosolic APXs are considered the most important peroxidases to scavenging and maintaining H_2O_2 homeostasis in cytosol (Davletova et al., 2005; Koussevitzky et al., 2008; Maruta et al., 2012; Pnueli et al., 2003; Suzuki et al., 2013b). These enzymes are present in large amounts, exhibit high activity, and the expression of their genes is strongly responsive to abiotic and biotic stresses (Shigeoka and Maruta, 2014). Moreover, experimental evidence obtained from *Arabidopsis* has suggested that these enzymes are crucial for cell signaling, antioxidant protection and photosynthesis (Davletova et al., 2005; Koussevitzky et al., 2008; Maruta et al., 2012; Pnueli et al., 2003; Suzuki et al., 2013b). Our group has employed double and single knockdowns in both cytosolic APX genes in rice plants as a model. Interestingly, single and double silencing of APX1 and APX2 trigger strong changes in redox metabolism, drastically altering the growth and development (Rosa et al., 2010). The up-expression of other peroxidases and photosynthetic proteins is able to compensate for deficiency in these enzymes (Bonifacio et al., 2011; Carvalho et al., 2014). The cytosolic APX knockdown in these plants is closely related to an increased H_2O_2 concentration, which is able to trigger significant changes at the transcriptomics, proteomics and metabolic levels (Ribeiro et al., 2012).

H_2O_2 is widely known as an important ROS and a powerful signaling molecule for several biological processes, such as development and programmed cell death (Dat et al., 2003; Mullineaux et al., 2006; Suzuki et al., 2013a). In leaves of C3 plants, peroxisomes are the major site of H_2O_2 production, followed by chloroplasts during photosynthesis (Corpas, 2015; Foyer and Noctor, 2003). H_2O_2 is distributed in virtually all cell compartments and might cross subcellular membranes (Mubarakshina et al., 2010; Sewelam et al., 2014). Currently there is a relative consensus that APX isoforms, together with catalase (CAT), are the most important peroxidases for scavenging and maintaining H_2O_2 homeostasis in plant cells, especially in cytosol (Shigeoka and Maruta, 2014) and peroxisomes during high photorespiratory conditions (Mittova et al., 2003; Wang et al., 1999). Nevertheless, some studies have questioned the claimed importance of APXs as the most important H_2O_2 scavengers in plant cell compartments (Bonifacio et al., 2011; Caverzan et al., 2014; Miller et al., 2007; Narendra et al., 2006; Sousa et al., 2015). Recent evidence has suggested that, at least in chloroplasts, thioredoxin-dependent peroxiredoxins and thylakoid-bound have overlapping ascorbate peroxidases in antioxidant function (Awad et al., 2015).

CAT is a crucial enzyme for peroxisomal H_2O_2 scavenging, especially during high photorespiration conditions (Chamnongpol et al., 1996; Kendall et al., 1983; Queval et al., 2007; Vanderauwera et al., 2011). The elevated maximum catalytic velocity exhibited by CAT is essential to eliminate high H_2O_2 amounts produced by glycolate oxidase (GO) in peroxisomes (Corpas, 2015; Mhamdi et al., 2012). Plants lacking CAT are very sensitive to normal air CO_2 concentrations and/or moderate and high concentrations (Chamnongpol et al., 1996; Kendall et al., 1983; Queval et al., 2007; Vanderauwera et al., 2011). These plants also present strong alterations in their GSH redox homeostasis, which are frequently associated with oxidative stress (Gao et al., 2014; Han et al., 2013; Queval et al., 2011). CAT deficiency might induce overexpression of APX isoforms. However, a lack of catalase is not frequently compensated by APX activity (Rizhsky et al., 2002; Sousa et al., 2015). Recently, our group showed that deficient rice plants in both peroxisomal APX exhibited better acclimation to oxidative stress induced by CAT inhibition, employing the 3-AT pharmacological inhibitor (Sousa et al., 2015).

The complexity of the relationships between APX and CAT metabolism associated with H_2O_2 homeostasis is an old and a still unsolved question. Rizhsky et al. (2002) found paradoxical results working with tobacco single and double KOs (knockouts) for CAT2 and APX1. Unexpectedly, double KO plants were better acclimated to oxidative stress than plants lacking CAT or APX alone. The authors suggested that unknown compensatory antioxidant pathways could be involved in these intriguing responses. Vanderauwera et al. (2011) reported similar conclusions working with *Arabidopsis* lacking (KO) both APX1 and CAT2. The authors highlighted that double knockout plants triggered signaling involving peroxisomes and the nucleus. They found that in the apax1 cat2 double mutant, the DNA anti-damage response is highly and specifically activated, which alleviates oxidative stress sensitivity of the mutant.

Plants display high redundancy and phenotypic plasticity (Silveira and Carvalho, 2016; Souza and Lüttge, 2015), especially in terms of antioxidant metabolism and the gene network (Gilroy et al., 2016; Mittler et al., 2011; Suzuki et al., 2013a). Indeed, the simultaneous deficiency of essential enzymes such as cytosolic APX1 and APX2 in rice might trigger an antioxidant pre-acclimation and a priming effect involving increased H_2O_2 levels. In this study, we tested the hypothesis that double cytosolic APX1/2 knockdown rice plants are able to trigger a pre-antioxidant acclimation followed by priming against oxidative stress generated by supplying 3-AT. The obtained data corroborate the idea that these plants were able to cope with excess H_2O_2 and oxidative stress induced by 3-AT, including full CAT inhibition for approximately three days. APX1/2 silenced plants displayed different antioxidant and physiological mechanisms compared to non-transformed plants. The importance of these alternative strategies to oxidative stress resistance is discussed.

2. Materials and methods

2.1. Constructing of plant vector, plant transformation and growth conditions

The background of rice (*Oryza sativa* L. cv. Nipponbare) plants employed for double silencing of cytosolic ascorbate peroxidase (APX1/2) was previously described by Rosa et al. (2010). A chimeric gene producing mRNA with a hairpin structure (hpRNA) was constructed based on the sequences of the *OsAPX1* (LOC_Os03g17690) and *OsAPX2* (LOC_Os07g49400) genes. The following primer pair was used: CGCCGCCAACGCCGGCCTCGA and CACTCAAACCCATCTGCGCA (*OsAPX1*/2RNAi). The PCR products were cloned into a Gateway vector (pANDA) in which hairpin RNA is driven by a maize ubiquitin promoter, and an intron is placed 50 bp upstream of inverted repeats (Miki and Shimamoto, 2004). Agrobacterium-mediated transformation was performed as described previously (Upadhyaya et al., 2000). Regenerated seedlings were grown at 28 °C in MS medium with a photoperiod of 12 h and 150 $\mu mol\ m^{-2}\ s^{-1}$ of photosynthetic photon flux density (PPFD) in a growth chamber for seven days. In this study, we used the same silenced line at the F3 generation as previously utilized (Bonifacio et al., 2011; Carvalho et al., 2014; Ribeiro et al., 2012). These plants have exhibited similar cytosolic *OsAPX1* and *OsAPX2* transcript amounts and APX activity in the F1, F2 and F3 generations (Bonifacio et al., 2011). APX1/2 and NT plants were transferred to 3 L plastic pots filled with half-strength Hoagland-Arnon's nutritive solution. The pH was adjusted to 6.0 ± 0.5 every two days, and the nutrient solution was changed weekly. Subsequently, the seedlings were grown for 45 days in a greenhouse under natural conditions: day/night mean temperature of 29/24 °C, mean relative humidity of 68%, and a photoperiod of 12 h. The light intensity inside the

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