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NADPH oxidase-dependent H_2O_2 production is required for salt-induced antioxidant defense in *Arabidopsis thaliana*

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ABSTRACT

The involvement of hydrogen peroxide (H_2O_2) generated by nicotinamide adenine dinucleotide phosphate-oxidase (NADPH oxidase) in the antioxidant defense system was assessed in salt-challenged *Arabidopsis thaliana* seedlings. In the wild-type, short-term salt exposure led to a transient and significant increase of H_2O_2 concentration, followed by a marked increase in catalase (CAT, EC 1.11.16), ascorbate peroxidase (APX, EC 1.11.1.11) and glutathione reductase (GR, EC 1.6.4.2) activities. Pre-treatment with either a chemical trap for H_2O_2 (dimethylthiourea) or two widely used NADPH oxidase inhibitors (imidazol and diphenylene iodonium) significantly decreased the above-mentioned enzyme activities under salinity. Double mutant *atrbohd/f* plants failed to induce the antioxidant response under the culture conditions. Under long-term salinity, the wild-type was more salt-tolerant than the mutant based on the plant biomass production. The better performance of the wild-type was related to a significantly higher photosynthetic activity, a more efficient K^+ selective uptake, and to the plants' ability to deal with the salt-induced oxidative stress as compared to *atrbohd/f*. Altogether, these data suggest that the early H_2O_2 generation by NADPH oxidase under salt stress could be the beginning of a reaction cascade that triggers the antioxidant response in *A. thaliana* in order to overcome the subsequent reactive oxygen species (ROS) production, thereby mitigating the salt stress-derived injuries.

Introduction

Drought and salt are among the most limiting abiotic stresses to crop productivity and yield. Generally, salinity reduces the plant growth and/or damages the plant through its: (i) osmotic effect (causing water deficit), (ii) ion-related toxic effect, and (iii) impact on the uptake of essential nutriments. As a consequence of these primary effects, secondary stresses such as oxidative damage often occur (Zhu, 2001) due to the excessive accumulation of deleterious chemical compounds called reactive oxygen species (ROS), including hydrogen peroxide (H_2O_2), superoxide anion ($O_2^{\bullet -}$), and hydroxyl radicals ($OH^{\bullet}$), which induce cellular damage by protein degradation, enzyme inactivation, alterations in the gene and interference in various pathways of metabolic significance (Choudhury et al., 2013). Still, there is increasing evidence of ROS involvement as signaling molecules in plant responses to abiotic and/or biotic stresses. Indeed, at low concentrations, ROS operate as messengers for the activation of defense genes (Foyer and Noctor, 2009).

Abbreviations: ABA, abscisic acid; APX, ascorbate peroxidase; C, control; CAT, catalase; Chl, chlorophyll; DAB, 3,3′diaminobenzidine; DMTU, dimethylthiourea; DPI, diphenylene iodonium; DW, dry weight; F_0, minimal fluorescence; F_m, maximal fluorescence; F_v/F_m, maximum quantum efficiency of PSII photochemistry; FW, fresh weight; GR, glutathione reductase; H_2DCFDA, 2′,7′-dichlorofluorescin diacetate; H_2O_2, hydrogen peroxide; MAPK, mitogen-activated protein kinase; MDA, malonyldialdehyde; NADPH oxidase, nicotinamide adenine dinucleotide phosphate-oxidase; NBT, nitroblue tetrazolium; NO, nitric oxide; $O_2^{\bullet -}$, superoxide anion; $OH^{\bullet}$, hydroxyl radicals; PSII, photosystem II; Rboh, respiratory burst oxidase homologues; ROS, reactive oxygen species; S, salt stress; SOD, superoxide dismutase.

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Addressing ROS homeostasis is crucial both for mitigating ROS toxicity and to determine their likely role in signaling pathways. Cellular ROS homeostasis depends on a balance between production and degradation of the free radicals. In plants, ROS can be produced by chloroplasts, mitochondria and peroxisomes or by apoplastic cell wall peroxidases, amine oxidases and plasma membrane nicotinamide adenine dinucleotide phosphate-oxidase (NADPH oxidase) (Gill and Tuteja, 2010). In order to reduce ROS-related damages, plants have evolved a complex antioxidant defense system that scavenges excessively accumulated ROS under stress conditions. This system includes enzymatic (CAT, ascorbate peroxidase (APX), glutathione reductase (GR), and superoxide dismutase, SOD) and non-enzymatic (low molecular weight antioxidant compounds) components (Li et al., 2010).

Pharmacological and genetic data indicate that the enzymes responsible for most of the ROS generated during biotic interactions and in early response to abiotic stresses are the plasma membrane-localized NADPH oxidases, known as respiratory burst oxidase homologues (Rbohs) (Wu et al., 2013). The rapid generation of ROS during the early stages of plant defense signaling is known as oxidative burst (Hao et al., 2008). Because H_2O_2 is relatively stable and diffusible through membrane, it is now considered the main ROS that triggers defense mechanisms in plant cells. H_2O_2 has been shown to play many critical roles in signaling and in several aspects of plant development, including in plant defense, root hair development, stomatal closure, and early responses to salt stress (salt stress) (Torres et al., 2002; Foreman et al., 2003; Kwak et al., 2003; Leshem et al., 2007).

The *Arabidopsis* genome contains 10 NADPH oxidase-encoding genes designated as *AtRbohA* to *J* exhibiting different patterns of expression throughout plant development and in response to environmental factors (Fluhr, 2009). Moreover, H_2O_2 produced by NADPH oxidase has been demonstrated to mediate rapid systemic signaling triggered by multiple abiotic stresses (Miller et al., 2009). A transient increase in the endogenous levels of H_2O_2 obtained by exogenous application of salicylic acid or heat can lead to subsequent thermotolerance in mustard seedlings (Dat et al., 1998). In maize, protection against chilling injury can be achieved by a transient increase in endogenous H_2O_2 levels during low-temperature acclimation (Prasad et al., 1994). Alternatively, direct exposure of plant tissues to H_2O_2 has been shown to activate antioxidant enzymes as well as the expression of antioxidant enzyme-encoding genes (Mylona and Polidoros, 2010). Both *AtRbohD* and *AtRbohF* have been identified as main isoforms, which are highly expressed under salt stress (Ma et al., 2012; Xie et al., 2011). Leshem et al. (2007) have described that H_2O_2 produced by NADPH oxidase are coordinated by phospholipid-regulated signaling pathways and act in the signal transduction of salt stress responses in *Arabidopsis*, and to be required for haem oxygenase mediated salt acclimation signaling in *Arabidopsis* (Xie et al., 2011).

The present study aims at better understanding the relationship between the early production of H_2O_2 by the NADPH oxidase and the antioxidant response of *Arabidopsis thaliana* seedlings exposed to short and long salt treatments. The function and the implication of H_2O_2 as a stressor or as a signaling molecule were also studied. First, the effect of short-term salinity on the H_2O_2 production and the antioxidants enzyme activities (SOD, CAT, APX and GR) was investigated. Then, the effect of pre-treatment with dimethylthiourea (DMTU), a chemical trap for H_2O_2 and diphenylene iodonium (DPI) and imidazol, an NADPH oxidase inhibitor on the activities of the salt stress-induced antioxidant enzymes were examined. Moreover, the antioxidant response was assessed in *atrbohd/f* double mutant. Finally, the comparative responses of *atrbohd/f* plants and the wild-type to long-term salt-stress were addressed. Several parameters were considered including the plant growth activity, the photosynthetic activity, leaf Na^+/K^+ selectivity, and the oxidative stress leaf responses.

Materials and methods

Plant material and culture conditions

Arabidopsis thaliana transposon insertion mutant lines atrbohd-3 (European Arabidopsis Stock Centre code N9555), *atrbohf-3* (European Arabidopsis Stock Centre code N9557) and double mutant *atrbohd/f* (European Arabidopsis Stock Centre code N9558) (Torres et al., 2002) were ordered from the European *Arabidopsis* Stock Centre.

In a first experiment, the implication of hydrogen peroxide (H_2O_2) in the induction of antioxidant response to short-term salt stress was assessed. Surface-sterilized seeds of wild-type (ecotype Columbia (Col-0)) and *Arabidopsis* mutant plants were sown in square Petri dishes on half-strength agar-solidified Murashige and Skoog (MS) medium according to Parre et al. (2007). After 24 h at 4 °C to break dormancy, seedlings were grown at 22 °C under continuous light (90 μmol photons m^{-2} s^{-1}). Twelve day-old *Arabidopsis* seedlings were transferred to liquid MS/2 medium supplemented or not with 200 mM NaCl and harvested at 3, 6, 9 and 24 h. In order to study the effects of scavengers and inhibitors, the seedlings were pre-incubated or not for 4 h with either 40 mM dimethylthiourea (DMTU, a chemical trap for H_2O_2) or 20 μM diphenylene iodonium (DPI) or 10 mM imidazol, two nicotinamide adenine dinucleotide phosphate-oxidase (NADPH oxidase) inhibitors. They were then grown in liquid MS/2 medium at 200 mM NaCl for 24 h under the same conditions as described above. All experiments were performed with three independent biological and three technical repetitions.

In a second experiment, seeds of the wild-type (Col-0) and the mutant *atrbohd/f* were sown in pots containing a mixture of vermiculite:sand (1:3). Seedlings were irrigated with one-quarter-strength (Hewitt, 1960) nutrient solution under greenhouse conditions (300 μmol m^{-2} s^{-1} photosynthetic active radiation (PAR), 25 ± 5 °C temperature, and 60 ± 10% relative humidity). Four week-old plants at rosette stage were exposed to 100 mM NaCl for 7 d. To reduce osmotic shock on plants, salt treatments were daily increased by 50 mM NaCl. Pots were irrigated every 2 d.

Chlorophyll fluorescence measurements, plant growth, leaf chlorophyll and ion concentrations

Chlorophyll (Chl) fluorescence was measured using a modulated chlorophyll fluorimeter (OS1-FL) following the procedure described by Genty et al. (1989). The minimal (F_0) and maximal (F_m) Chl a fluorescence were assessed in leaves after 20 min of dark adaptation. The maximum quantum efficiency of Photosystem II (PSII) photochemistry was calculated as $F_v/F_m = (F_m - F_0)/F_m$.

At the harvest, rosette fresh weight (FW) was immediately estimated and dry weight (DW) was determined after having being dried at 60 °C until constant weight. Chl was extracted from 100 mg leaf FW in 5 ml 100% acetone. After centrifugation for 5 min at 500 × g, supernatants were measured at 470, 645, and 662 nm. Concentrations of total Chl were calculated as given by Lichtenthaler (1987). After ion extraction from dried ground leaf in 0.5% HNO_3, Na^+ and K^+ was assayed by flame emission photometry (Corning, UK).

H_2O_2 concentration

H_2O_2 concentration was determined spectrophotometrically as described by Oracz et al. (2009). 300 mg FW plant materials were ground in a mortar on ice in 1 ml perchloric acid