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Induction of reactive oxygen species and necrotic death-like destruction in strawberry leaves by salinity

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ABSTRACT

The effect of salinity (200 mM NaCl, 7 d) on cellular oxidative metabolism and necrotic lesion formation were analyzed in strawberry (*Fragaria* × *ananassa* Duch., cv. Selva) leaves. It was found that NaCl-induced oxidative stress in strawberry leaves, as evidence by an $H_2O_2/O_2^{\bullet-}$ accumulation, an increase in lipid peroxidation and carbonyl-groups content. Salinity visible symptoms, $H_2O_2/O_2^{\bullet-}$ generation and cell death lesions formation co-occurred mainly in the rim of the leaf surface. However, DNA laddering was not evident in the leaves exposed to salinity. Leaf extracts from plants exposed to NaCl were able to reduce Fe^{3+} but not to chelate Fe^{2+}, as judged by their promoting effect on deoxy-D-ribose oxidation system. Also, NaCl-treated leaf extracts were ineffective at protecting against plasmid DNA strand breakage induced by $^{\bullet}OH$ in a Fenton-type system. NaCl caused an accumulation in putrescine and spermidine, an oxidation of ascorbate and glutathione redox pairs and an inhibition in the activities of some ROS-metabolizing enzymes (e.g., catalase, ascorbate peroxidase, glutathione reductase). Experiments employing pharmacological agents suggested that NaCl-induced production of H_2O_2 was likely linked to NAD(P)H-oxidase and amine oxidase regulation and was signalled by nitric oxide (NO), salicylic acid (SA), protein kinase and Ca^{2+} channel activity. Further, a conceptual model for the action of NaCl-driven oxidative stress on necrotic death-like destruction is proposed.

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1. Introduction

From a general point of view, high salinity, most commonly mediated by NaCl, in soil or irrigation water is one of the major abiotic stresses globally. The phytotoxicity of NaCl is likely due to its ability to generate reactive oxygen species (ROS) represented predominantly by superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($^{\bullet}OH$) (Hernández et al., 2000, 2001; Borsani et al., 2001; Mazel et al., 2004; Mittova et al., 2004; Huang et al., 2005; Verma and Mishra, 2005; Yadav et al., 2005; Tuteja, 2007; Valderrama et al., 2007). Multiple enzymatic sources including oxalate oxidase, amine oxidase, pH-dependent cell-wall peroxidases and membrane-bound NAD(P)H-oxidases have been proposed as being responsible for $O_2^{\bullet-}/H_2O_2$ generation in response to salinity (Hernández et al., 2001). A sudden and dramatic increase in cellular ROS production leads to protein and lipid oxidation and DNA single- and double-strand breakage (Miller et al., 2008), thus upsetting the homeostasis of the cell. In parallel, however, H_2O_2 has now been shown as stress signal in plants (Houot et al., 2001; Pellinen et al., 2002; Avsian-Kretchmer et al., 2004; Passardi et al., 2005; Miller et al., 2008; Wang and Song, 2008). The expression of at least 1–2% of *Arabidopsis* genes is dependent on H_2O_2, and some of them are antioxidant genes and others encode proteins involved in signalling, such as calmodulin, protein kinases and transcription factors (Desikan et al., 2001; Neill et al., 2002).

In biological systems, an increase in ROS production and disturbances of cellular redox potentials were found to be involved either directly or indirectly in the death of individual cells and/or the development of necrotic lesions (Fath et al., 2001; Houot et al., 2001; Pellinen et al., 2002; Wohlgemuth et al., 2002; Dat et al., 2003; Pasqualini et al., 2003; Montillet et al., 2005; Overmyer et al., 2005; Papadakis and Roubelakis-Angelakis, 2005; Yoda et al., 2006; Moschou et al., 2008). NaCl-derived $O_2^{\bullet-}/H_2O_2$ production in the apoplast of pea leaves apparently triggers, by way of an as yet undescribed mechanism, in a necrotic lesion formation (Hernández et al., 2001), which resembles the microburst observed during hypersensitive cell death (Montillet et al., 2005). Similarly, O_3-induced sites

Abbreviations: APX, ascorbate peroxidase; AsC, reduced ascorbate; CAT, catalase; c-PTIO, 2-(4-carboxyl-2-phenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide; DAB, 3,3-diaminobenzidine; DHA, oxidized ascorbate; DPI, diphenylene iodonium; FRAP, ferric reducing/antioxidant power; GR, glutathione reductase; GSH, reduced glutathione; GSSG, oxidized glutathione; GUA, guazatine; JA, jasmonic acid; NBT, nitroblue tetrazolium; PAs, polyamines; POD, peroxidase; Put, putrescine; ROS, reactive oxygen species; SA, salicylic acid; SNP, Na-nitroprusside; SOD, superoxide dismutase; Spd, spermidine; Spm, spermine; STA, staurosporine.

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of ROS accumulation and O_3-necrotic lesions were found in distinct sites (Pellinen et al., 2002; Wohlgemuth et al., 2002; Pasqualini et al., 2003), suggesting that ROS production can function as a regulator of cell death. However, the contribution and interaction between the oxidative stress cross-talk network and salinity that may eventually result in plant death remains elusive (Borsani et al., 2001).

A great deal of research has established that the induction of the cellular antioxidant machinery is important for protection against salt stresses (Mittova et al., 2004; Tuteja, 2007). Transgenic *Arabidopsis* plants that over-express the intracellular vesicle trafficking protein *At*Rab7 showed increased tolerance to salt stresses by reducing ROS (Mazel et al., 2004). In pea plants, transcript levels for mitochondrial Mn-SOD, chloroplastic CuZn-SOD and phospholipid hydroperoxide glutathione peroxidase (PHGPX), and cytosolic GR and APX were strongly induced in the salt-tolerant variety but not in the salt-sensitive one (Hernández et al., 2000). Recently, a role of polyamines (PAs) [putrescine (Put^{2+}), spermidine (Spd^{3+}), and spermine (Spm^{4+})] as direct or indirect efficient ROS-scavengers has been suggested (Papadakis and Roubelakis-Angelakis, 2005; Verma and Mishra, 2005). At the same time, PAs have also been proposed as important substrates for H_2O_2 production, being degraded by copper-containing amine oxidases (Yoda et al., 2006; Moschou et al., 2008). In addition, solid evidence indicates that PAs may participate in loops involving interaction with signal transduction pathways that may control oxidative cell death events (Pignatti et al., 2004; Papadakis and Roubelakis-Angelakis, 2005; Moschou et al., 2008).

Though many reports concerning salinity-induced oxidative stress are available (Hernández et al., 2000, 2001; Borsani et al., 2001; Mazel et al., 2004; Mittova et al., 2004; Huang et al., 2005; Verma and Mishra, 2005; Yadav et al., 2005; Tuteja, 2007) we find a general lack of information concerning the relationship between NaCl-mediated ROS generation and cell death in plants. Against this background, we were interested to investigate the physiological reactions, including visual symptoms, ROS production, oxidative damage, changes in cellular antioxidant machinery, PAs accumulation, and cell death-related responses, occurring in leaves of strawberry plants after NaCl-exposure. On the basis of the results obtained, a possible model of the role of NaCl-driven oxidative stress in the necrotic death-like destruction is proposed.

2. Materials and methods

2.1. Plant material and growth conditions

Six-month-old, strawberry plants (*Fragaria* × *ananassa* Duch., cv. Selva) with one well-developed crown 8–10 mm in diameter were selected for hydroponic culture in a culture room at 22 ± 2 °C temperature with 16 h photoperiod under a light density of 150 μmole m^{-2} s^{-1}. Firstly, plants were cultivated in aerated distilled water for 7 d, and then in aerated 1/2 strength Hoagland's nutrient solution for 7 d. After this period, the media either remained unsupplemented (control plants) or were supplemented with 200 mM NaCl. Fully expanded (but not natural senescent) leaves were used for the sampled after 7 d of NaCl treatment.

2.2. Detection of cell death

To monitor cell death, leaves were incubated in 0.25% (w/v) aqueous solution of Evans blue for 1 h with shaking at 100 rpm at room temperature. Samples were then washed twice for 15 min with distilled water and directly photographed. Cell death was quantified by solubilization of Evans blue-treated leaves with 1% (w/v) SDS in 50% (v/v) MeOH at 50 °C for 10 min, and the optical density was measured at 600 nm.

2.3. DNA isolation and analysis

To determine whether DNA laddering occurred, genomic DNA was isolated from leaf margins as described by White and Kaper (1989). Five microgram of each leaf sample were electrophoresed on a 2% (w/v) agarose gel containing 1xTAE (40 mM Tris–acetate, 1 mM EDTA) and stained with ethidium bromide.

2.4. Determination of H_2O_2 content

The content of H_2O_2 in the leaves was estimated according to Pazdzioch-Czochra and Widenska (2002). The reaction mixture contained 25 mM Na–phosphate buffer (pH 7.5), 0.125 mM homovanillic acid (HVA), peroxidase (1 U ml^{-1}) and sample extract. The mixture was vortexed, incubated for 5 min at 20 °C and the fluorescence intensity was measured at an excitation of 315 nm and an emission of 425 nm in a Shimadzu RF-500 spectrofluorimeter (Shimadzu, Kyoto, Japan).

2.5. H_2O_2 and $O_2^{\bullet -}$ localization in situ

A cytochemical dye, 3,3-diaminobenzidine (DAB), was used for *in situ* detection of H_2O_2 (Wohlgemuth et al., 2002). Leaves from control and NaCl-treated plants were excised and immersed in a 1% DAB solution in 10 mM MES buffer (pH 6.5), and then incubated at room temperature for 8 h. After that, leaves were discolored in boiling 95% (v/v) ethanol for visualization of reddish-brown precipitates characteristic of the reaction of DAB with peroxidases and H_2O_2. To test DAB-specificity, the leaves were also stained in the presence of catalase (200 U ml^{-1}, EC 1.11.1.6, bovine liner, C9322) prior to DAB staining in a control experiment.

Treatments with 600 μM Na-nitroprusside [SNP; nitric oxide (NO) donor], 200 μM 2-(4-carboxyl-2-phenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (c-PTIO; NO scavenger), 1 mM salicylic acid (SA), 100 μM jasmonic acid (JA), 1 mM $LaCl_3$ (Ca^{2+} channel blocker), 10 mM staurosporine (STA; phospholipid/calcium kinase and Ser/Thr protein kinase inhibitor) and 10 mM guazatine (GUA; amine oxidase inhibitor) were supplied in water solutions to excised leaves from plants treated with NaCl and kept for 16 h at 25 °C, except for SNP and SA which were incubated for 4 h. As control reaction, NaCl-treated leaves without modulators were incubated in water for 16 h. After incubation, leaves were used to measure and localize H_2O_2 production, as mentioned above.

For histochemical detection of $O_2^{\bullet -}$ the leaves were immersed in a 0.1% solution of nitroblue tetrazolium (NBT) in 50 mM K–phosphate buffer (pH 6.4), containing 10 mM Na-azide in the absence of light (Hernández et al., 2001). The leaves were subsequently illuminated until appearance of dark spots, characteristic of blue formazan precipitates, followed by boiling in ethanol. Negative control was also performed by adding SOD (100 U ml^{-1}) into the incubation buffer prior to NBT staining.

To study whether ROS accumulation induced by NAD(P)H-oxidase activation, excised leaves from NaCl-treated plants were incubated in the presence of the inhibitor diphenylene iodonium (DPI; 2.5 μM) and then either transferred to the DAB/NBT staining solutions or used for H_2O_2 measurements. In all histochemical studies, the samples were stored in 50% (v/v) ethanol (in the dark for NBT) before photographed.

2.6. Hydroxyl radical scavenging properties

The scavenging properties of leaves against $^{\bullet}OH$, generated by iron-mediated Fenton reactions, were tested using two different assays: deoxy-D-ribose degradation and DNA nicking assays.