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ARTICLE

Ethylene-regulated reactive oxygen species and nitric oxide under salt stress in tomato cell suspension culture

Péter Poór* and Irma Tari

Department of Plant Biology, University of Szeged, Szeged, Hungary

ABSTRACT In the present work the role of ethylene (ET) in the accumulation of reactive oxygen species (ROS) and nitric oxide (NO) was investigated under the effect of sublethal (100 mM) and lethal (250 mM) concentrations of NaCl in tomato cell suspension culture. In these cultures the salt stress increased the production of ET and ROS after 6 hours but NO level was enhanced only at 100 mM NaCl. This corresponded with the lower ratio of dead cells (20%) in these samples suggesting that NO functioned as a protecting compound under moderate salt stress. The synthesis of ET was further enhanced by the addition of ET precursor, 1-aminocyclopropane-1-carboxylic acid (ACC), which increased the ROS production under both moderate and severe salt stress. However, NO levels decreased in the presence of ACC in cells exposed to 100 mM NaCl and did not change after treatment with 250 mM NaCl. The effect of ET on ROS production induced by salt stress could be blocked by silver thiosulphate (STS), an inhibitor of ET action. In accordance with the decreased ROS production STS reduced the death of cells in the presence of 250 mM NaCl. In the presence of ACC the enhanced ROS production concurrently with low NO levels led to the increased cell death after 100 mM NaCl treatment. These results show that the cell viability is determined by the ET generated ROS and NO ratio under salt stress.
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KEY WORDS

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salt stress
tomato suspension culture

Salt stress results in the disturbance of ion homeostasis, water status and redox equilibrium in plant cells (Sun et al. 2010) which can lead to a decrease in biomass production or to death of plants (Joseph and Jini 2010; Shabala 2009).

Under salt stress, a strong membrane depolarization caused by Na^+ uptake results in K^+ efflux via depolarization-activated outward-rectifying K^+ channels. At the same time, salinity elevates cytosolic Ca^{2+} level which leads to a dramatic rise in the level of reactive oxygen species (ROS) by the activation of NADPH oxidase. This causes additional K^+ efflux via ROS-activated non-selective cation channels and at the end the release of K^+ from the cytoplasm causes K^+ deficiency and programmed cell death (PCD) (Shabala 2009; Joseph and Jini 2010).

Regulation of ROS accumulation under salt stress determines the intracellular redox status which is a key factor in cell viability (Sun et al. 2010). ROS, such as H_2O_2 and superoxide radicals ($O_2^{\cdot-}$) are essential component of plant PCD (Lin et al. 2006), because they damage to the cellular building blocks, such as proteins, lipids and DNA (Bi et al. 2009). Both ROS (Mittler et al. 2004) and the other signalling intermediate, nitric oxide (NO) can be synthesized by various enzymatic and non-enzymatic mechanisms and in various cell compartments (Wilson et al. 2008). NO can interact with ROS and their homeostasis determines the fate of the cells (Tari et al. 2010; Gémes et al. 2011).

The gaseous hormone, ethylene (ET) is an important signalling component for many abiotic stresses (Wi et al. 2010). Plants exposed to high salt concentrations in the rooting zones produce ET which plays a role in the early stage of senescence (Morgan and Drew 1997). Internucleosomal cleavage of DNA, the hallmark of PCD, depends on ET production during the aerenchyma formation in maize roots (Gunawardena et al. 2001). H_2O_2 accumulation and simultaneous increase in ET production were observed in camptothecin- (De Jong et al. 2002) and cadmium-induced cell death (Yakimova et al. 2006). Moreover, ET and H_2O_2 can act as self amplifying signal molecules in feed-forward loop (Wi et al. 2010). The biosynthetic pathway of ET involves the conversion of S-adenosylmethione by 1-aminocyclopropane-1-carboxylic acid (ACC) synthase to ACC and then the oxidation of ACC to ethylene by ACC oxidase (Bleecker and Kende 2000). Silver ion can generate ET insensitivity in plants by inhibiting the ET binding to the ET receptor (Kumar et al. 2009). Moreover, it was found that silver thiosulphate (STS) inhibited the camptotechin induced cell death in tomato cell suspension (De Jong et al. 2002).

Although the role of ET-induced ROS production in the PCD induction has already been well documented, the role of ET in NO production and its correlation with tissue damage under salt stress has not yet been described. Our aims were

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*Corresponding author. E-mail: poorpeti@bio.u-szeged.hu

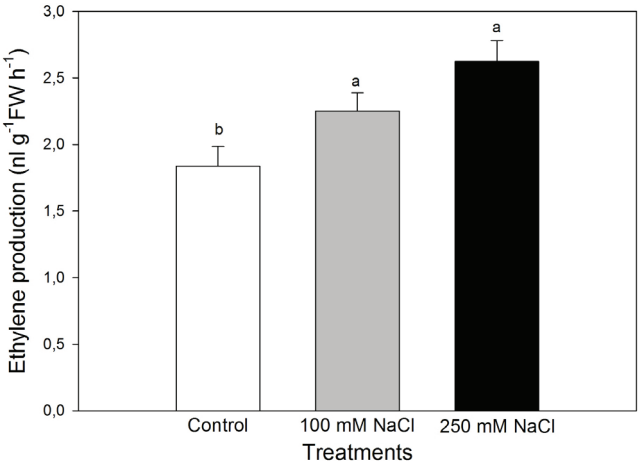

Figure 1. Changes in the ethylene production of tomato suspension cells after 100 and 250 mM NaCl treatments. Means±SE, n=3. Bars with different letters are significantly different at the 0.05 level (Duncan's multiple range test).

to study these signalling mechanisms in case of a lethal (250 mM) and sublethal (100 mM) concentrations of salt stress in tomato cell suspension.

Materials and Methods

Suspension culture

Tomato (*Solanum lycopersicum* L. cvar Rio Fuego) cells in suspension culture were grown in MS medium containing Gamborg B5 vitamins, 30 g l^{-1} sucrose, 5 mM α-naphthyl acetic acid, 1 mM 6-benzyladenine (Yakimova et al. 2008). Suspensions were incubated on rotary shaker (100 rpm) at 25°C in the dark. Cell suspensions were subcultured every 7 days by 1:4 dilutions with fresh medium, then they were treated with 100 or 250 mM NaCl. The effect of salt stress on the production of ROS and NO was determined in the presence of 10 μM ACC or 20 μM STS which were applied in the culture medium for 6 hours.

Measurement of ethylene production

Ethylene production of the suspension cells was measured with a Hewlett-Packard 5890 Series II gas chromatograph equipped with a flame ionization detector and a column packed with activated alumina (Tari et al. 2006). The gas samples were withdrawn from the airspace of the cell suspensions incubated in gas-tight flasks for 24 hours.

Determination of cell death, ROS and NO

Cell death was determined with fluorescein diacetate (FDA), ROS was visualized by using 2',7'-dichlorofluorescein diacetate (H_2DCFDA) and NO production was visualized by using 4,5 diaminofluorescein-diacetate (DAF-2 DA) as described earlier by (Gémes et al. 2011). After staining, the samples were washed in MES-TRIS/KCl buffer (pH 5.8) in the dark at room temperature. Fluorescence intensity was detected with Zeiss Axiowert 200M type fluorescence microscope (Carl Zeiss, Jena, Germany) equipped with an objective 10X. The fluorescence intensity was measured with AXIOVISION REL. 4.5 software. The microscope fields for each sample were chosen randomly.

Statistical analysis

Data presented are average values from at least three independent experiments. Statistical analysis was carried out with Sigma plot 11.0 software (SPSS Science Software). After analysis of variance (ANOVA) Duncan's multiple comparisons were performed. Differences were considered significant if $P \leq 0.05$.

Results

Ethylene production under salt stress

To investigate the role of ET in salt-induced stress or cell death in tomato cell suspension, the hormone was measured by gas chromatography. ET production was increased significantly after treating the tomato cell suspensions culture with 100 and 250 mM NaCl (Fig. 1).

The role of ethylene (ET): Reactive oxygen species (ROS) and nitric oxide (NO) production

The fluorescent probe H_2DCFDA was used to display ROS production of cells. As shown in Figure 2A, the production of ROS was enhanced in tomato suspension cells after the exposure to high salinity.

To investigate if ET affects the oxidative stress in cells under high salinity, ET synthesis was enhanced by the addition of ET precursor ACC. ROS production increased after a simultaneous application of 10 μM ACC and NaCl (Fig. 2A). Addition of STS, an ET receptor blocker, resulted in a reduced ROS production under salt stress (Fig. 2A).

The fluorescent probe DAF2-DA was used to indicate NO production in the cells. NO accumulation increased significantly after the treatment with 100 mM NaCl (Fig. 2B) but decreased in the presence of the ET precursor, ACC (Fig. 2B). The simultaneous application of 20 μM STS with NaCl did not change the NO levels of the suspension cells (Fig. 2B).

The percentage of cell death was determined by fluorescein diacetate (FDA) staining which can detect the viable cells. The cell death was ~20% in case 100 mM NaCl treatment and 80% in case of 250 mM NaCl after 6 hours (Fig. 2C) suggesting that a treatment with the higher concentration of NaCl caused rapid cell death during this time. ACC enhanced the percentage of dead cells only at 100 mM NaCl treatment.

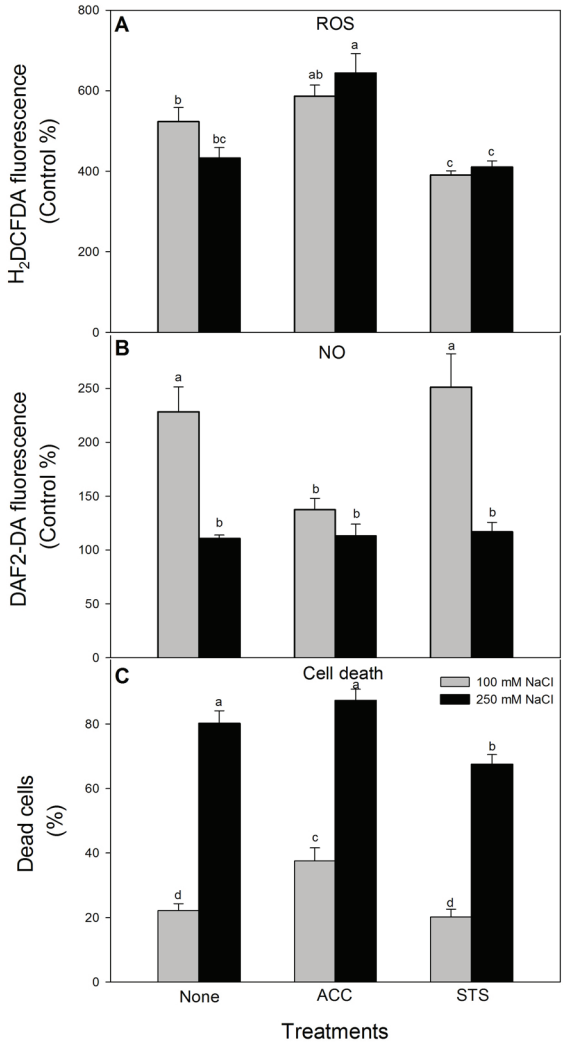

Figure 2. Changes in ROS (A), NO (B) and cell viability (C) in tomato cell suspension after treating the samples with 100 and 250 mM NaCl for six hours or by simultaneous application of 10 μM ethylene precursor, 1-aminocyclopropane-1-carboxylic acid (ACC) or 20 μM ethylene receptor blocker, silver thiosulphate (STS). Data are expressed as a percent of untreated controls after counting 100-100 cells from six random samples. Percent of dead cells was about 7% in control samples. Means±SE, n=3. Bars with different letters are significantly different at the 0.05 level (Duncan's multiple range test; ns: not significant).

Discussion

Treatment with sublethal (100 mM) and lethal (250 mM) concentrations of NaCl were compared in this present work in order to reveal the role of salt stress-induced ET production on the accumulation of ROS and NO. It was of interest how the balance between these active molecular species can determine tissue viability and tissue damage.

ET has an important role in triggering PCD in plant cells (Love at al. 2008) and the hormone accumulated in tomato suspension cells exposed to medium level or severe salt stress. Our results also suggest that this ET production may enhance the PCD during salt stress. De Jong et al. (2002) found that ET increased ROS production which enhanced cell death and caused the fragmentation of DNA. To investigate if ET plays a role in salt-induced cell death, different ET modulators were added to the tomato cell suspension to detect their effect on the cell viability. The application of ET precursor ACC enhanced the production of ROS both at 100 mM or 250 mM NaCl but it increased the percent of dead cells only at lower salt concentration. However, the application of the ET receptor blocker STS decreased the NaCl-induced cell death significantly only at 250 mM NaCl. This shows that ET signalling is an important component of salt-induced cell death similarly to a camptothecin- (De Jong et al. 2002) or a cadmium-induced PCD (Yakimova et al. 2006; Yakimova et al. 2008), but the ET effect displays a salt concentration dependency.

To clarify if ET also plays a role in the NO production of cells, the modulators were used together with the NaCl treatment. The addition of ET precursor, ACC which increased the ROS production of the cells in case of both treatments, decreased the NO level only in cells exposed to 100 mM NaCl. In these samples the enhanced ROS and lower NO production after the ACC application enhanced the death of cells. Addition of STS, an ET receptor blocker, resulted in reduction of ROS production induced by 100 mM NaCl stress and did not change the NO levels, and in these suspension cultures the percentage of dead cells remained as high as in the salt treated controls. If the ET signalling was blocked at this moderate salt stress high NO contents did not affect the vitality of cells. However at 250 mM NaCl blocking of ET signalling by STS significantly decreased the percentage of dead cells.

These results show that NaCl induces cell death by generating oxidative stress which can be enhanced by ET, and the cell viability is determined by the ET generated ROS and NO balance.

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