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ORIGINAL ARTICLE

Ethylene signaling in salt stress- and salicylic acid-induced programmed cell death in tomato suspension cells

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Abstract Salt stress- and salicylic acid (SA)-induced cell death can be activated by various signaling pathways including ethylene (ET) signaling in intact tomato plants. In tomato suspension cultures, a treatment with 250 mM NaCl increased the production of reactive oxygen species (ROS), nitric oxide (NO), and ET. The 10^{-3} M SA-induced cell death was also accompanied by ROS and NO production, but ET emanation, the most characteristic difference between the two cell death programs, did not change. ET synthesis was enhanced by addition of ET precursor 1-aminocyclopropane-1-carboxylic acid, which, after 2 h, increased the ROS production in the case of both stressors and accelerated cell death under salt stress. However, it did not change the viability and NO levels in SA-treated samples. The effect of ET induced by salt stress could be blocked with silver thiosulfate (STS), an inhibitor of ET action. STS reduced the death of cells which is in accordance with the decrease in ROS production of cells exposed to high salinity. Unexpectedly, application of STS together with SA resulted in increasing ROS and reduced NO accumulation which led to a faster cell death. NaCl- and SA-induced cell death was blocked by Ca^{2+} chelator EGTA and calmodulin inhibitor W-7, or with the inhibitors of ROS. The inhibitor of MAPKs, PD98059, and the cysteine protease inhibitor E-64 reduced cell death in both cases. These results show that NaCl induces cell death mainly by ET-induced ROS production, but ROS generated by SA was not controlled by ET in tomato cell suspension.

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Abbreviations

ACC	1-Aminocyclopropane-1-carboxylic acid
AVG	Aminoethoxyvinyl glycine
CAT	Catalase
cPTIO	Carboxyphenyl-tetramethylimidazoline-oxide
DAF-2 DA	4,5 Diaminofluorescein-diacetate
DPI	Diphenyleneiodonium chloride
E-64	*N*-(*trans*-epoxysuccinyl)-L-leucine 4-guanidinobutylamide
EGTA	Ethylene glycol tetraacetic acid
EL	Relative electrolyte leakage
ET	Ethylene
FDA	Fluorescein diacetate
H_2DCFDA	2′,7′-Dichlorofluorescein diacetate
HR	Hypersensitive response
KOR	Depolarization-active outward-rectifying K^+ channels
MAPK	Mitogen-activated protein kinase
NO	Nitric oxide
Nr	*Never ripe* tomato (ethylene receptor) mutant
NSCC	Non-selective cation channels
PBS	Phosphate-buffered saline
PCD	Programmed cell death
PD98059	Amino-methoxyphenyl-benzopyran
PM	Plasma membrane
ROS	Reactive oxygen species
SA	Salicylic acid
SNP	Sodium nitroprusside
SOD	Superoxide dismutase
STS	Silver thiosulphate
TUNEL	Terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling
W-7	*N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride

Introduction

Programmed cell death (PCD) is a controlled cellular suicide which plays an important role in the plant development. PCD is associated with specific morphological and biochemical features such as nuclear DNA degradation or the activation of specific proteases (Danon et al. 2000; Lam et al. 2001; Rogers 2005; Woltering 2004). PCD is induced by various abiotic stressors among others by high salinity (Shabala 2009; Joseph and Jini 2010) and the HR, a special type of PCD in plants during biotic stress is mediated by salicylic acid (SA) (Alvarez 2000; Lam et al 2001).

Ethylene (ET), a gaseous plant hormone, is not only involved in plant growth and development but also in plant responses to different abiotic and biotic stresses and thus can be crucial in plant PCD (Dangl et al. 2000). Internucleosomal cleavage of DNA, TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling)-positive nuclei, the hallmarks of PCD, were observed during aerenchyma formation in hypoxic maize roots which were dependent on ET (Gunawardena et al. 2001). The biosynthetic pathway of ET involves the conversion of *S*-adenosylmethione to 1-aminocyclopropane-1-carboxylic acid (ACC) by ACC synthase and then to ET by ACC oxidase (Bleecker and Kende 2000). Silver ions are capable of blocking the ET receptor (Kumar et al. 2009), and silver thiosulfate (STS) could inhibit the camptothecin- (De Jong et al. 2002) and the salt stress-induced cell death in tomato cell suspension (Poór and Tari 2011). ET has been shown to modulate plant PCD induced by O_3 exposure (Overmyer et al. 2003) and organ senescence (Quirino et al. 2000; Byczkowska et al. 2012), and it was found that SA is required to produce ET in response to O_3 (Rao et al. 2002). In our earlier work, it was found that high ethylene production was associated with SA-induced PCD in the root tips of tomato (Gémes 2011). Contrarily, SA can inhibit the ET synthesis by blocking the conversion of ACC to ET (Leslie and Romani 1986).

Camptothecin- and cadmium-induced cell death was accompanied by an increased production of ET and simultaneously by H_2O_2 accumulation in tomato cell suspension (De Jong et al. 2002; Yakimova et al. 2006). It was found that H_2O_2 stimulated ET emission and ACC enhanced H_2O_2 production in salt-treated *Arabidopsis* calli (Wang et al. 2010a), suggesting that ET and H_2O_2 can act as self-amplifying signal molecules in feed-forward loop (Wi et al. 2010). ROS, such as H_2O_2 and superoxide radicals (O_2^-), are essential mediators of plant PCD, because they damage the cellular components, such as proteins, lipids, and DNA (Bi et al. 2009). ROS can be synthesized by different pathways, e.g., by PM-localized nicotinamide adenine dinucleotide phosphate (NADPH) oxidases and modulate signaling networks that control growth, development, and stress response (Mittler et al. 2004). The H_2O_2 signal is mediated through alterations in Ca^{2+} fluxes, redox changes, activation of mitogen-activated protein kinase (MAPK) cascade, and interactions with NO (Gechev and Hille 2005).

The gaseous free radical NO can interplay with ROS in a variety of ways, and it is a crucial partner in determining cell fate or in signaling response to a number of physiological and stress-related conditions such as ET-, salt-, or SA-induced signaling (Ederli et al 2006; Bastianelli et al. 2010; Gémes et al. 2011). Ederli et al. (2006) demonstrated that ET accumulation was dependent on NO generation, whereas ET did not induce NO emission in tobacco leaf discs. Wang et al. (2009) found that, under salt stress, the NO accumulation and ET emission appeared at an early time. While sodium nitroprusside (SNP), an NO generator, greatly stimulated ET emission in *Arabidopsis* callus, ACC did not enhance the production of NO. In accordance with this, Ahlfors et al. (2009) reported that SNP activates ET biosynthesis after 1 h in *Arabidopsis thaliana* plants. NO plays an important role in cytoprotection by regulating the level and toxicity of ROS, and it is involved in tolerance to salt stress (Siddiqui et al. 2011).

The early signaling component in stress response pathway is the activation of specific MAPKs. MAPK cascades are evolutionarily conserved signaling modules which regulate a wide variety of cellular processes. Pathogens and pathogen-derived elicitors, ET, salt stress, SA, as well as ROS and H_2O_2 can activate MAPK cascades which can lead to defense gene activation or cell death (Zhang and Klessing 2001; del Pozo et al. 2004; Gechev and Hille 2005).

The cytosolic caspase-mediated cell death pathway is highly conserved in animal cells, but such a death cascade has not been found in plant cells (Vartapetian et al. 2011). However, there are studies which present similarities between plant PCD and animal caspase-mediated apoptotic cell death program (De Jong et al. 2000). Plant cysteine proteases can have putative homology to caspases (Hoeberichts et al. 2003) and are ubiquitously present in plant PCD. Components of the cysteine protease subfamily C1A (papain-like cysteine proteases) trigger PCD during abiotic stress and senescence (Trobacher et al. 2006). Activation of caspase-like proteases may lead to PCD in case of salt stress, too (Shabala 2009). It was found by Jones et al. (2005) that ET sensitivity regulated cysteine protease gene expression in petunia corollas; nine of them showed increased transcript abundance during petal senescence. The expression of these cysteine proteases was delayed in ET-insensitive flowers.

In earlier works, it was shown that the sublethal concentrations of SA (10^{-7}–10^{-4} M) applied in hydroponic culture through the root system generated pre-adaptation responses which led to salinity tolerance. These concentrations of SA could improve acclimation to high salinity by enhancing the

net photosynthetic rate in a salt-tolerant wheat genotype (Arfan et al. 2007) or in tomato (Poór et al. 2011a) and by stimulating abscisic acid accumulation and polyamine content (Szepesi et al. 2009). However, the excess of SA (10^{-3} M) or NaCl (250 mM) caused the death of plants or cells (Poór and Tari 2011) and the decrease of the intracellular K^+ concentration, and K^+/Na^+ ratio was found a common phenomenon in the cell death program triggered by high salinity and lethal concentration of SA (Poór et al. 2011b).

We have recently revealed that ET signal transduction determines the balance between ROS and NO production induced by SA in the apex of adventitious roots in wild-type and in the ET-insensitive *Never ripe* (*Nr*) mutants of tomato. In the absence of functional ET signaling in *Nr* mutants, the NO production was elevated as a function of SA concentration compared with wild-type controls (Tari et al. 2011).

Although a number of papers appeared about the role of ET, H_2O_2 (Wang et al. 2010a), and NO (Wang et al. 2009) in the salt stress adaptation of *Arabidopsis*, no single study has investigated the role of ET and ET signaling and its relationship with ROS and NO balance in salt stress- and SA-induced PCD in time course experiments. Garcia-Heredia et al. (2008) reported that 10^{-3} M SA induced the mitochondrial way of cell death program, but they did not show the role of intracellular NO levels in this process.

In this work, the common features and differences in the signaling of NaCl- and SA-induced cell death in tomato cell suspension culture were studied. The role of ET in ROS and NO production and its correlation with cell damage were investigated during this process. It was also of interest whether the modulation of ET production by ACC or inhibition of ET signaling with STS could shift the balance of ROS and NO and could affect the cell death program in tomato suspension culture.

Materials and methods

Plant material and establishment of suspension cultures

Tomato (*Solanum lycopersicum* L. cvar Rio Fuego) cells in suspension culture were grown in a 100-ml Erlenmeyer flask containing 30 ml of MS salt (Murashige and Skoog 1962), Gamborg B5 vitamins (Gamborg et al. 1968), 30 gl^{-1} sucrose, 5 μM α-naphthaleneacetic acid, and 1 μM 6-benzyladenine (Yakimova et al. 2006). Suspensions were incubated on a rotary shaker (100 rpm) at 25 °C in darkness and were subcultured every 7 days by 1:4 dilutions with fresh medium. For treatments, cells were used 4–5 days after subculture (Poór and Tari 2011).

Chemical treatments

Suspension cultures were treated with 250 mM NaCl or 10^{-3} M SA (pH 5.6). These concentrations just in excess of physiological range were chosen in order to provoke fast and significant induction of PCD under stressful conditions.

Most of the chemicals were taken from concentrated stock solution allowing microliter volumes to be added to the cell cultures 10 min before the NaCl or SA treatments. Ten micromoles ethylene glycol tetraacetic acid (EGTA), 200 U superoxide dismutase (SOD), 100 U catalase (CAT), 10 mM diphenyleneiodonium chloride (DPI), 10 μmol ACC, 10 μmol aminoethoxyvinyl glycine (AVG), and 1 μmol *N*-(*trans*-epoxysuccinyl)-L-leucine 4-guanidinobutylamide (E-64) were dissolved in water. STS was prepared by mixing 0.1 M sodium thiosulphate with a molar ratio of 1:4 between silver and thiosulfate, respectively, and was used in 20 μmol concentration. Ten micromoles *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride (W-7), 200 μmol carboxyphenyl-tetramethylimidazoline-oxide (cPTIO), and 0.1 μmol amino-methoxyphenyl-benzopyran (PD98059) were prepared with dimethyl sulfoxide (DMSO). DMSO was tested alone, and no effect on cell viability was detected. All chemicals were from Sigma (Sigma-Aldrich, St. Louis MO, USA).

Cell death determination

Cell death was determined with 0.002 % fluorescein diacetate (FDA). After staining for 10 min with FDA, cells were rinsed once with 10 mM MES-TRIS/KCl buffer (pH 5.8). The cells were detected by Zeiss Axiovert 200 M type fluorescent microscope (Carl Zeiss Inc., Jena, Germany) with an objective ×10 and counted by Axiovision Rel. 4.8 software (Carl Zeiss Inc., Munich, Germany). The microscope fields of each different sample were chosen randomly. The cell death was calculated as a percentage of dead cells to the total number of cells (Yakimova et al. 2006).

Electrolyte leakage was determined following the method of Sun et al. (2010), with some modifications (Poór et al. 2011b). Briefly, 0.5 g of cells was transferred to 15 ml double-distilled water. After 2 h of incubation at 25 °C, the conductivity of the bathing solution was determined (C1) with conductivity meter (OK-102/1 Radelkis, Budapest, Hungary). Cell samples were then heated at 95 °C for 40 min, and the total conductivity (C2) was measured. Relative electrolyte leakage (EL) was expressed as a percentage of total conductivity, EL (%)=(C1/C2)×100.

TUNEL assay

For in situ detection of DNA fragmentation in the cells treated with 250 mM NaCl or 10^{-3} M SA, samples were

fixed in phosphate-buffered saline (PBS, pH 7.4) containing 4 % paraformaldehyde for 30 min at room temperature in glass-bottom culture dishes (MatTek Co., Ashland MA, USA). The fixed and washed samples were incubated in permeabilization solution containing 0.1 % TritonX-100 and 0.1 % Na-citrate. The cells were washed twice in PBS and labelled on the 3'OH groups of nuclear DNA by TUNEL assay using digoxigenin-dUTP according to the manufacturer's instructions (TUNEL kit, Roche Applied Science, Mannheim, Germany) (Bi et al. 2009). TUNEL labelling was done by applying 50 μL of TUNEL reaction solution and incubating the samples at 37 °C for 1 h in the dark. The labelled samples were washed in fresh PBS, and the nuclei were counterstained with 0.002 % Hoechst 33258 before detecting it with Zeiss Axiovert 200 M-type fluorescent microscope (Carl Zeiss Inc., Jena, Germany).

Detection of DNA fragmentation

Detection of DNA fragmentation was determined following the method of Kubis et al. (2003), with some modifications. Cells were collected on a filter paper and washed twice with 50 ml double-distilled water, and 200 mg cells were frozen and grinded in liquid nitrogen. The resulting powder was mixed with 10 ml extraction buffer (0.1 M NaCl, 2 % SDS, 50 mM TRIS–HCl pH 9, 10 mM EDTA) for 10 min at room temperature. Then, 300 μL of phenol–chloroform 1:1 (*v/v*) solution was added to the reaction mixture, which was then centrifuged for 10 min at 4 °C, 3,000×*g*. The phenol–chloroform step was repeated with the supernatant. The supernatant was then added to 0.5 ml of chloroform–isoamyl alcohol (24:1) and centrifuged again (10 min at 4 °C, 3,000×*g*). The supernatant was incubated for 3 h in the mixture of 550 μl isopropanol and 20 μl Na-acetate. Then, the samples were centrifuged (10 min at 4 °C, 11,300×*g*), and the pellet was washed and centrifuged twice for 10 min (4 °C, 11,300×*g*) in 70 % ethanol. Finally, the pellet was dried and dissolved in 20 μL TE buffer (10 mM TRIS pH 8.0, 1 mM EDTA); 0.1 μg ml^{-1} DNase-free RNase was added, and the samples were incubated for 10 min at 37 °C before the agarose gel (3 %) electrophoresis was performed (80 mV, 2 h).

Measurement of ET production

One gram of collected and washed cells were incubated in 0.5 ml suspension liquid with 250 mM NaCl or 10^{-3} M SA in gas-tight flasks fitted with a rubber serum stopper. The flasks were shaken in the dark for 6 h. The 2.5 ml of the gas was removed from the tubes with a gas-tight syringe and injected to GC. A set of ET standards was used to calculate the amount of ET generated by the cells. ET 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. Flow rates were 35 mL min^{-1} for He, 30 mL min^{-1} for H_2, and 300 mL min^{-1} for air. The oven, injector, and detector temperatures were 100, 120, and 200 °C, respectively (Tari et al. 2006).

Detection of ROS and NO

ROS was visualized by using 10 μM 2′,7′-dichlorofluorescein diacetate (H_2DCFDA) for 20 min in 10 mM MES-TRIS/KCl buffer (pH 5.8) in the dark at 37 °C and rinsed once with 10 mM MES-TRIS/KCl buffer (pH 5.8) (Gémes et al. 2011).

NO production was visualized by using 10 μM 4,5 diaminofluorescein-diacetate (DAF-2 DA) for 20 min in 10 mM MES-TRIS/KCl buffer (pH 5.8) in the dark at room temperature and rinsed once with 10 mM MES-TRIS/KCl buffer (pH 5.8) (Gémes et al. 2011).

Fluorescence intensity was detected with Zeiss Axiowert 200 M-type fluorescent microscope (Carl Zeiss Inc., Jena, Germany) equipped with an objective ×10. Digital photographs were taken from the samples with a high-resolution digital camera (Axiocam HR, HQ CCD camera; Carl Zeiss Inc., Jena, Germany). The fluorescence intensity was measured with AXIOVISION REL. 4.5 software (Carl Zeiss Inc., Munich, Germany) using a filter set 10 (excitation 450–495 nm, emission 515–565 nm). The microscope fields of each different sample were chosen randomly.

Statistical analysis

Data presented as average values resulted from at least three independent experiments. Statistical analysis was carried out with Sigma plot 11.0 software (Systat Software Inc., Erkrath, Germany). Statistical analyses were performed using Student's *t* test, and differences were considered significant if $P \leq 0.05$.

Results

NaCl- and SA-induced cell death

To investigate the role of high salinity and SA in the induction of cell death in tomato cells, 250 mM NaCl and 10^{-3} M SA were added to the suspension cultures. This increased cell death within 6 h significantly, and in short-term experiments SA treatment was not as harmful as salt stress. The number of living cells was determined by FDA staining, and the percentage of dead cells was calculated after counting the total cell number. Treatment with 250 mM NaCl caused rapid cell death after 2 h, while the PCD initiated by SA developed later, 10^{-3} M SA caused significant increase in PCD after 5 h (Fig. 1a). The ratio of dead cells was 80 % in

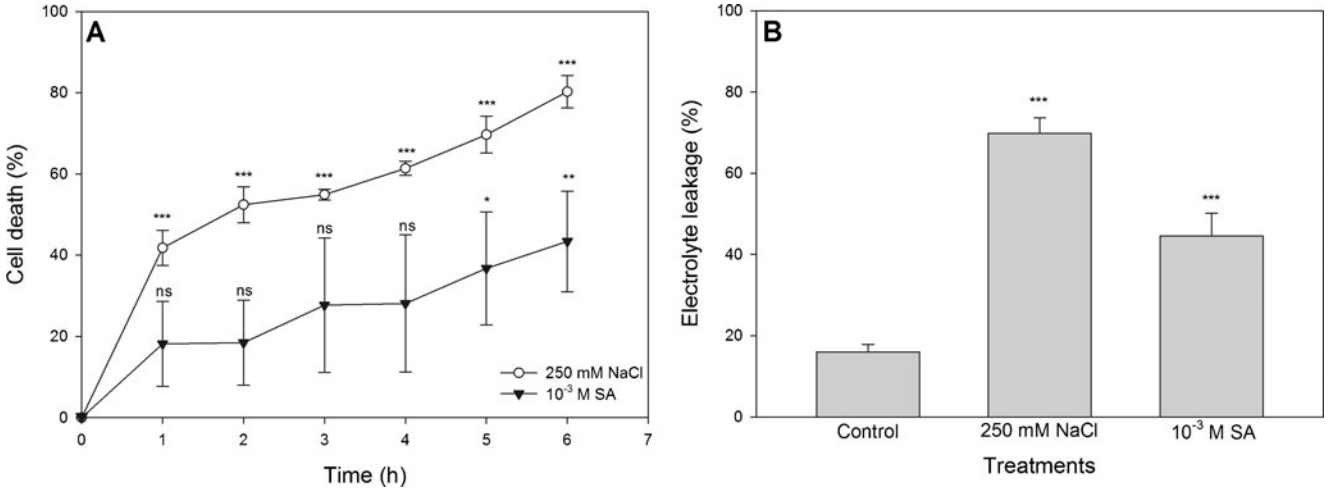

Fig. 1 Cell death was determined by FDA staining (**a**) and by electrolyte leakage (**b**) in tomato cell suspension induced by 250 mM NaCl or 10^{-3} M salicylic acid (SA) for 6 h. Results are the average ± SE from at least three to four independent experiments. *, **, *** indicate significance levels compared with the untreated control at $P<0.05$, 0.01, 0.001, respectively, in each time point (Student's *t* test; *ns*: not significant)

case of treatment with 250 mM NaCl and 40 % in case of treatment with 10^{-3} M SA after 6 h. These data were confirmed by the measurement of electrolyte leakage (Fig. 1b). Six-hour-long treatments were chosen for the following experiments. Recovery was performed to detect if 250 mM NaCl and 10^{-3} M SA treatments induced irreversible damage in cell cultures. After a 6-h-long treatment, cells were collected on a filter paper and washed twice with 30 ml suspension medium and transferred into a new 100-ml Erlenmeyer flask containing 30 ml of fresh suspension medium for 24 h. After recovery, the percentage of dead cells was determined by FDA staining. The results revealed that the recovery was unsuccessful because both treatments induced PCD, and all of the cells died in fresh medium.

After 6 h TUNEL assay, a marker of DNA fragmentation was used to demonstrate the DNA damage. TUNEL-positive nuclei were visible in both 250 mM NaCl and 10^{-3} M SA-treated cells (Fig. 2a). This observation on TUNEL staining was confirmed in case of 10^{-3} M SA-induced PCD, but it occurred later than in the case of 250 mM NaCl in tomato suspension culture. The TUNEL-positive nuclei were about 60 % in case of 250 mM NaCl treatment and about 22 % after the 10^{-3} M SA exposure within 6 h. DNA isolation and separation by agarose gel electrophoresis was performed to study the effects of NaCl and SA on DNA degradation. DNA fragmentation was detected in 250 mM NaCl-treated cells, but not in 10^{-3} M SA-treated cells after 6 h of the treatment (Fig. 2b).

ET production

ET production increased significantly after treating tomato suspension culture with 250 mM NaCl. However, treatment with 10^{-3} M SA did not cause an elevated ET production in our system (Fig. 3). The ET production increased with the addition of ET precursor, ACC both in controls, and in cells exposed to 250 mM NaCl and 10^{-3} M SA (Fig. 3). Addition of STS, an ET receptor blocker, did not decrease the ET production alone or with simultaneous application of SA, but it decreased the ET induced by high salinity (Fig. 3).

ET signaling: ROS and NO production

The fluorescent probe H_2DCFDA was used to display ROS production of cells. As shown in Fig. 4a and b, the production of ROS in cells exposed to NaCl and SA were enhanced in tomato suspensions. ROS production reached its peak following a 1-h-long NaCl and SA treatment and then decreased throughout the experiment. The degree of oxidative burst correlates well with the percentage of dead cells.

To investigate if ET also plays a role in the oxidative burst in cells treated with NaCl or SA, ET synthesis was enhanced by the addition of ET precursor, ACC. In the first hour, ACC did not change the production of ROS in the 250 mM NaCl- treated samples, but, after the second hour, it triggered and elevated ROS accumulation (Fig. 4a). In the presence of 10^{-3} M SA and ACC, the level of ROS was higher than in the control after the third hour (Fig. 4b). Addition of STS, an ET receptor blocker, resulted in a reduction in ROS production under salt stress in the first hours, but ROS did not change significantly after the fourth hour from the salt-treated control (Fig. 4a). Interestingly, the ROS production increased significantly and permanently by simultaneous application of SA and STS (Fig. 4b). Addition of ACC alone increased the ROS production of untreated

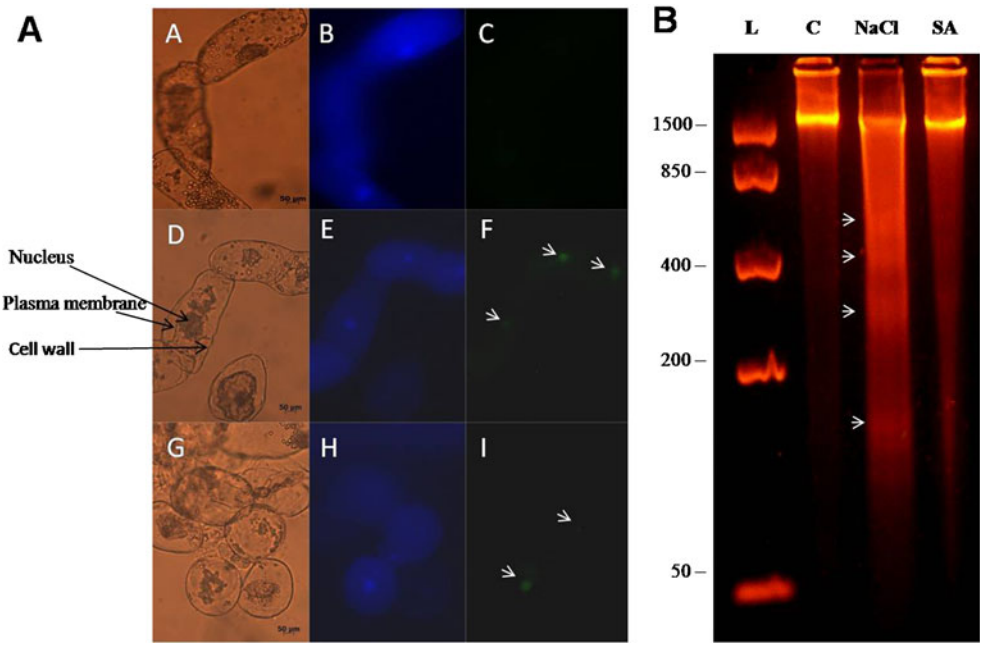

Fig. 2 DNA fragmentation was determined by TUNEL staining (**a**), and DNA degradation was detected by agarose gel electrophoresis (**b**) in tomato cell suspension treated with 250 mM NaCl or 10^{-3} M salicylic acid (SA) for 6 h. Part *A*: control (**a**, **b**, **c**), 250 mM NaCl (D, E, F), 10^{-3} M SA (G, H, I), bright field microscopy (**a**, **d**, **g**); Hoechst 33258 staining (**b**, **e**, **h**); TUNEL-positive nuclei (**c**, **f**, **i**). Part *B*: *L* (ladder); *C* (control); *NaCl* (250 mM NaCl); *SA* (10^{-3} M salicylic acid)

cells (143±13 %), but STS did not change it significantly (103±10 %) (data not shown).

The fluorescent probe DAF2-DA was used to indicate NO production in the cells, which increased significantly in both cases. The fluorescence intensity was time-dependent and reached its maximum after 1 h of 10^{-3} M SA treatment and after 2 h in case of 250 mM NaCl exposure (Fig. 5a, b).

The ET precursor ACC increased the NO production in the first hour in case of salt treatment, but it was not significantly different from control at the end of the 6-h incubation period. It was found that 10 μM ACC did not affect NO production significantly if it was applied in the presence of 10^{-3} M SA (Fig. 5a, b). The simultaneous application of STS eliminated the small, 40 % increase of NO production in salt-stressed cells and significantly decreased NO production induced by SA throughout the experiments (Fig. 5b). Addition of ACC alone did not change significantly the NO production of untreated cells (108±5 %), but STS increased to 136±15 % (data not shown).

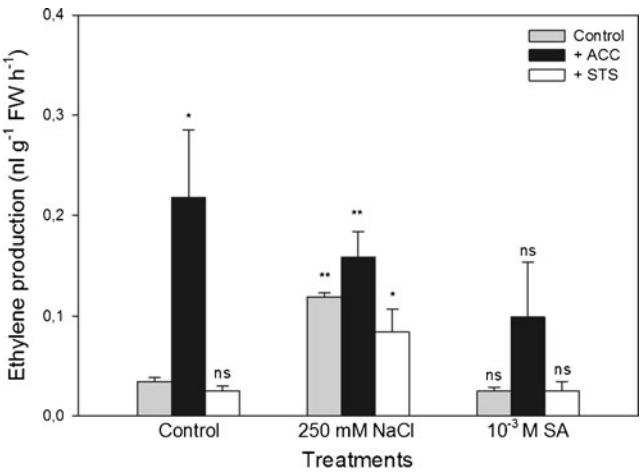

Fig. 3 Changes in the ethylene production of tomato suspension cells after treating the samples with 250 mM NaCl or 10^{-3} M salicylic acid (SA) alone or by simultaneous application of 10 μM ethylene precursor, 1-aminocyclopropane-1-carboxylic acid (ACC), or 20 μM ethylene receptor blocker, silver thiosulphate (STS). Results are the average ±SE from at least three to four independent experiments. *, **, *** indicate significance levels compared with the untreated control at $P<0.05$, 0.01, 0.001, respectively, in each time point (Student's *t* test; *ns*: not significant)

The components of PCD signaling

The 250 mM NaCl and 10^{-3} M SA-induced cell death decreased significantly after treating the suspensions with Ca^{2+} chelator EGTA and calmodulin inhibitor W-7 (Table 1). The addition of O_2^- scavenging enzyme SOD, the H_2O_2 scavenging enzyme CAT, and the NADPH oxidase blocking DPI markedly decreased NaCl- and SA-induced cell death (Table 1). The NO scavenger, cPTIO, did not have significant effects on the NaCl- and SA-induced cell death, suggesting that these small changes in NO accumulation did not affect PCD induction in early phase of stress exposure (Table 1). Application of the ET precursor ACC together with NaCl increased the cell death in the tomato cell suspension but had no effect on SA-induced cell death program. AVG, an ET synthesis inhibitor, had a slight increasing effect on cell viability in salt-stressed cells. In order to reveal the participation of possible signaling pathways in NaCl- and SA-induced PCDs, we determined the effects of specific inhibitors in salt- and SA-treated suspensions on the percentage of dead cells. Adding the ET receptor blocker STS decreased the salt stress-induced PCD but

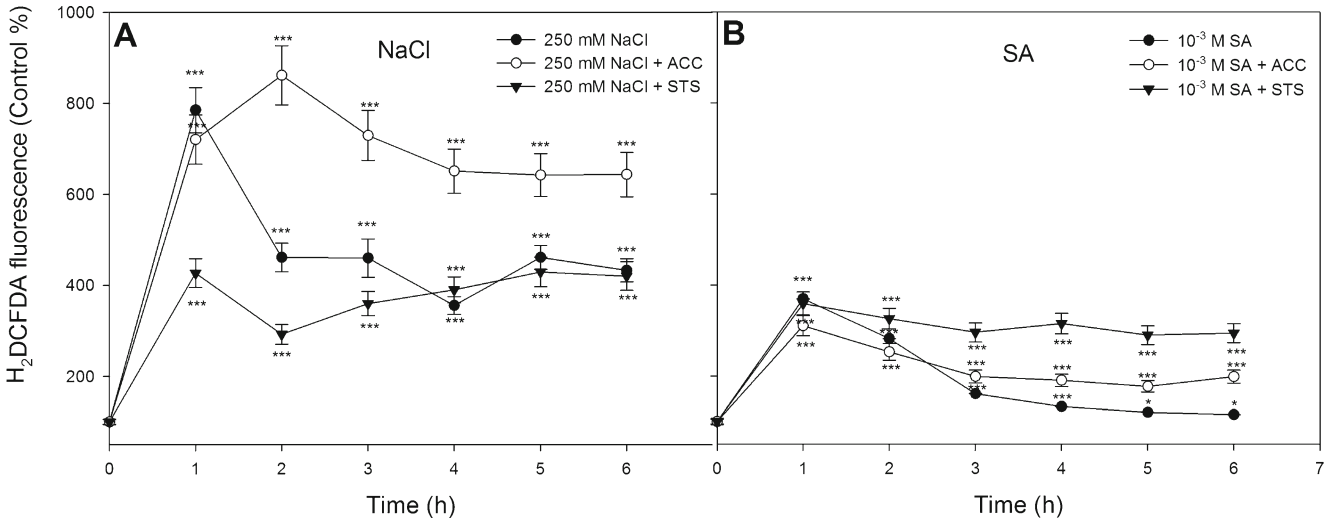

Fig. 4 Kinetics of reactive oxygen species production in tomato cell suspension after treating the samples with 250 mM NaCl (**a**) or 10^{-3} M salicylic acid (SA) (**b**) for 6 h alone or by simultaneous application of 10 μM ethylene precursor, 1-aminocyclopropane-1-carboxylic acid (ACC), or 20 μM ethylene receptor blocker, silver thiosulphate (STS). Results are the average ±SE from at least three to four independent experiments. *, **, *** indicate significance levels compared with the untreated control at $P<0.05$, 0.01, 0.001 in each time point (Student's *t* test; *ns*: not significant)

increased the SA-induced cell death, which correlated well with STS-induced ROS generation. MAPK blocker PD98059 and the cysteine protease inhibitor E-64 decreased both the NaCl- and SA-induced cell death (Table 1).

Discussion

In the present work, the role of ET and ET-induced signaling intermediates were compared in NaCl- and SA-induced PCD. The 250 mM NaCl and 10^{-3} M SA caused the death of about 80 % and 40 % of the cells, respectively, within 6 h. The mortality of the cells increased significantly after 1 h following the 250 mM NaCl treatment and after 5 h following the 10^{-3} M SA treatment. In accordance with the results of Huh et al. (2002) and Garcia-Heredia et al. (2008), who revealed that SA-induced cell death is a relatively slow process, the decrease of cell viability was faster in case of 250 mM NaCl because the excess of Na ions generated not only oxidative stress but strong ionic and osmotic stress as well.

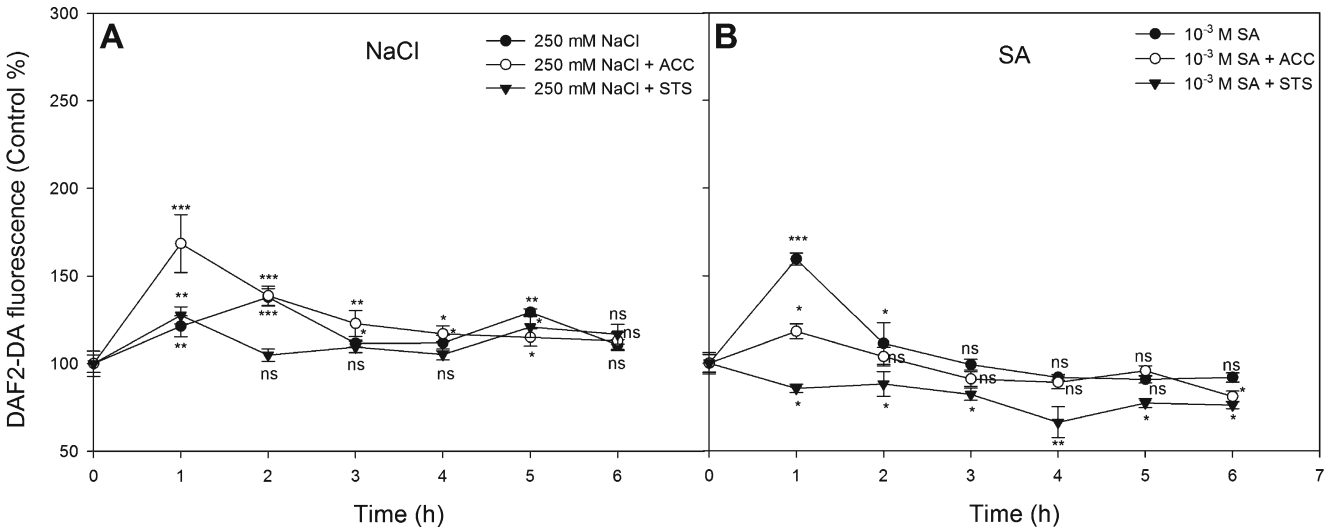

Fig. 5 Kinetics of nitric oxide production in tomato cell suspension after treating the samples with 250 mM NaCl (**a**) or 10^{-3} M salicylic acid (SA) (**b**) for 6 h alone or by simultaneous application of 10 μM ethylene precursor, 1-aminocyclopropane-1-carboxylic acid (ACC), or 20 μM ethylene receptor blocker, silver thiosulphate (STS). Results are the average ±SE from at least three to four independent experiments. *, **, *** indicate significance levels compared with the untreated control at $P<0.05$, 0.01, 0.001, respectively, in each time point (Student's *t* test; *ns*: not significant)

Table 1 Effect of inhibitors of the putative signaling pathways of PCD on the percentage of dead cells 6 h after the application of 250 mM NaCl or 10^{-3} M salicylic acid (SA) in the tomato cell suspension culture

Chemical treatments														
Treatments		+	+	+	+	+	+	+	+	+	+	+	+	+
		10 μM	10 μM	200 U	100 U	10 mM	200 μM	10 μM	10 μM	20 μM	0.1 μM	1 μM		
		EGTA	W-7	SOD	CAT	DPI	cPTIO	ACC	AVG	STS	PD98059	E-64		
Dead cells (%)	250 mM NaCl	80.2±3.9	71.0±1.0*	67.7±4.2*	58.6±3.0**	52.1±1.0**	63.0±1.7**	77.4±2.0 ns	89.3±2.5*	64.7±4.8*	67.5±3.0*	72.8±1.0*	68.1±1.0*	
	10^{-3} M SA	43.3±2.3	33.8±1.0**	37.0±2.1*	21.5±1.4***	30.1±1.0**	15.0±1.0***	43.7±1.0 ns	45.6±3.6 ns	45.1±1.0 ns	55.6±1.0***	30.3±2.7**	29.7±1.3**	

Chemical names and abbreviations: *EGTA* ethylene glycol tetraacetic acid, *W-7 N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride, *SOD* superoxide dismutase, *CAT* catalase, *DPI* diphenyleneiodonium chloride, *cPTIO* carboxyphenyl-tetramethylimidazoline-oxide, *ACC* 1-aminocyclopropane-1-carboxylic acid, *AVG* aminoethoxyvinyl glycine, *STS* silver thiosulphate, *PD98059* amino-methoxyphenyl-benzopyran, *E-64 N*-(*trans*-epoxysuccinyl)-L-leucine 4-guanidinobutylamide

*, **, ***P values indicating significance levels compared with the untreated control at $P<0.05$, 0.01, 0.001, respectively, in each time point (Student's t test; *ns*: not significant)

Loss of the cell membrane integrity and stability under stress conditions is an important feature of cell death induction. It is well demonstrated that NaCl and SA can induce electrolyte leakage and can increase the membrane permeability (Shi et al. 2006; Sun et al. 2010). An enhanced electrolyte leakage was detected after both NaCl and SA treatments, which correlates well with the results of FDA staining.

NaCl and SA induced chromatin condensation and cell shrinkage, the morphological characteristics of plant PCD. According to new definitions of van Doorn (2011), a decrease in cytoplasmic volume, an increase in cytoplasmic free Ca^{2+} concentration, induction of MAPK signaling, DNA degradation, condensation of the nucleus to smaller diameter, or activation of serine/cysteine proteases are associated with autolytic PCD. Moreover, Wang et al. (2010b) presented transmission electron micrographs of *Thellungiella halophila* cell suspension and detected the ultrastructual features of autophagy in cells 6 h after 300 mM NaCl exposure. The autolytic PCD in some cell types required hydrogen peroxide (Duan et al. 2010). Thus, the morphological features of NaCl-induced PCD can be classified as autolytic.

The SA-induced PCD is not accompanied by rapid clearance of the cytoplasm, but the influx of Ca^{2+} into the cytoplasm, the activation of MAPKs, the accumulation of reactive oxygen species and nitric oxide, and the collapse of the mitochondrial transmembrane potential (Garcia-Heredia et al. 2008) have been shown in this process.

DNA fragmentation was observed after a 6-h treatment with 250 mM NaCl. It is in accordance with the result of Affenzeller et al. (2009), who found that DNA laddering can be observed in an early phase of salt stress. DNA laddering was not found in SA-treated cells after 6 h, but there were TUNEL-positive nuclei in these samples. To evaluate the effect of increasing NaCl concentration in *T. halophila* cell suspension culture on cell viability, Wang et al. (2010b) found that the percentage of dead cells was significantly different from TUNEL-labelled nuclei. This suggests that TUNEL-labelling indicates only some kind of DNA fragmentation, but it is not suitable for quantitative estimation of cell death.

Garcia-Heredia et al. (2008) observed cytochrome c release from mitochondria to cytosol and a decrease in mitochondrial membrane potential only 12 h after the treatment by 10^{-3} M SA. These PCD hallmarks strengthen that the PCD induction by 10^{-3} M SA is slower than by 250 mM NaCl in the tomato suspension culture. In contrast, the results of the unsuccessful recovery confirmed that 250 mM NaCl and 10^{-3} M SA treatments induced the cell death process within 6 h in the tomato cells.

ROS and NO are involved in the rapid adjustments of plant response to various stress factors, but the relationship

between ROS and NO in the activation of cell death is not clearly defined. ROS production increased significantly in the first hour after treating with 250 mM NaCl, and after the second hour, it decreased but remained permanently high. In case of the treatment with 10^{-3} M SA, ROS accumulation showed a transient increase and was not as high as in the samples exposed to high salinity. This ROS production proved to be the most important component of cell death signal. Suppression of ROS accumulation by O_2^- scavenging enzyme SOD, the H_2O_2 scavenging enzyme CAT, and the NADPH oxidase blocker DPI markedly decreased NaCl- and SA-induced cell death. These results confirmed the main role of the oxidative burst and ROS-induced damage in salt stress- or SA-induced cell death (Garcia-Heredia et al. 2008; Shabala 2009; Sun et al. 2010). In contrast, NO production increased significantly in the first hours after both treatments and was significantly higher in SA-treated cells, but it decreased later as a function of time. NO can act synergistically with ROS to potentiate cell death and can act independently of ROS to induce defense mechanism (Delledonne et al. 1998; Clarke et al. 2000). However, the percentage of dead cells was not affected by the NO scavenger, cPTIO. As a conclusion, it can be stated that NO does not play a role in the induction of PCD induced by NaCl or SA in early phase of the process in our system. However, especially in the case of NaCl treatment, the permanent accumulation of ROS is crucial in the rapid induction of cell death.

Because ET itself can trigger PCD in plant cells (Love et al. 2008), it was of interest how the ET generator ACC and STS, the inhibitor of ET action affects ROS and NO production, and the cell death process in tomato cell suspension exposed to salt stress and SA. ET production increased significantly after a treatment with 250 mM NaCl, but it did not change after SA treatment. These results suggest that ET production may enhance the PCD during salt stress. The finding that ET increased ROS production, which enhanced cell death and caused the fragmentation of DNA, corresponds well with the results of De Jong et al. (2002). The lack of ET induction is one of the most characteristic differences in the salt- and SA-induced cell death program at the concentrations used. To investigate if ET really plays role in stress-induced cell death, different ET modulators were added to the tomato cell suspension to detect the cell viability. The ET biosynthesis inhibitor AVG resulted in a slight increase in cell viability under salt stress, but it did not have significant effect on SA-treated cells. The ET receptor blocker STS decreased the NaCl-induced cell death significantly suggesting that ET signal transduction has a direct control over PCD induction, or ET signaling can modulate ROS production contributing to the initiation of PCD. ET-enhanced cell death in the tomato suspension cells treated with NaCl was confirmed by the application of ET precursor ACC, which increased oxidative stress and caused a slight increase in the percentage of dead cells. These data suggest that the salt stress-induced ET signaling directly controls ROS accumulation and shows that ET signaling is an important component of salt-induced cell death similarly to the camptothecin- (De Jong et al. 2002) or cadmium-induced PCDs (Yakimova et al. 2006; Yakimova et al. 2008).

The ineffectiveness of ACC and AVG suggests that ET is not an initiator in SA-induced cell death.

The addition of ET precursor ACC after 1 h increased the NO production of the cells in case of salt stress, but did not change the NO levels after SA treatment. It was unexpected that the application of STS together with SA resulted in increasing ROS and decreasing NO production which led to faster cell death. This suggests that, in these samples, increasing ROS production could lead to NO scavenging. STS has been employed in tissue studies for inhibiting ET action because its water solubility (Kumar et al. 2009), but there is no information about its effect on the redox homeostasis, which may also be involved in PCD induction especially in case of SA treatment.

Ca^{2+} is a second messenger and an important element in the cell death signaling during salt stress- (Shabala 2009) and SA-induced signaling events (Zottini et al. 2007), thus the effect of Ca^{2+} chelator EGTA and calmodulin inhibitor

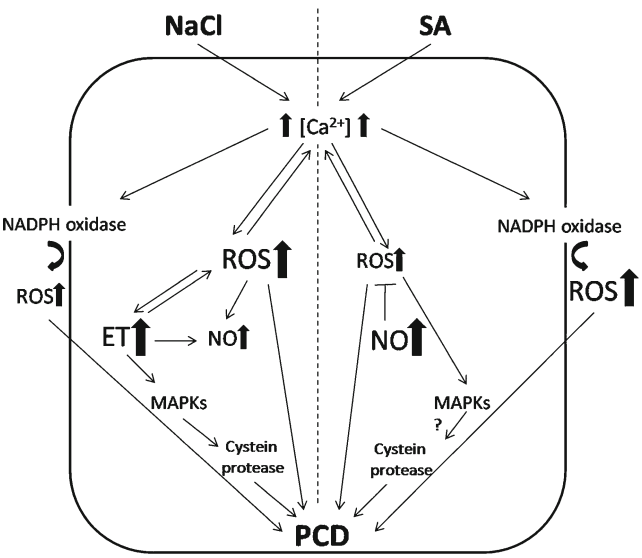

Fig. 6 A schematic model of salt stress- and salicylic acid (SA)-induced programmed cell death (PCD) in tomato suspension cells. Salt- and SA-treatment elevated free calcium level in the cytoplasm and induced a strong oxidative stress. Both treatments activated the NADPH oxidase complex and the produced reactive oxygen species (ROS) leading to PCD. NO can act synergistically with ROS to potentiate cell death and can act as a ROS scavenger or independently of ROS it can induce defence mechanisms. The activation of MAPKs and cysteine proteases can mediate the cell death signaling, and ethylene (ET) can accelerate the process only in cells exposed to high salinity

W-7 were tested in the tomato suspension culture during the cell death process. These chemicals prevented NaCl- and SA-induced cell death indicating that Ca^{2+}-dependent processes are essential in triggering cell death in tomato cell suspension. Similar results were found concerning the effect of EGTA and W-7 in cadmium-induced cell death (Yakimova et al. 2008).

To investigate the role of MAPKs in NaCl- or SA-induced cell death, PD98059, a specific inhibitor of mammalian MAPK activation, was added to the tomato suspension culture. Clarke et al. (2000) showed that PD98059 did not inhibit the effects of NO but blocked H_2O_2-induced cell death, suggesting that activation of MAPKs may mediate the H_2O_2-induced PCD. The MAPK inhibitor PD98059 decreased the NaCl- and SA-induced cell death, which indicates the role of the PD98059-sensitive MAP kinase cascade in PCD in case of both treatments which can also activate the changes in cytosolic Ca^{2+} level and generation of ROS through a feed-forward loop.

The role of ET on the expression and activity of cysteine proteases are complex. Some of them had been induced earlier in senescing petals of petunia hybrid wild-type plants than in ET-insensitive transgenic plants expressing 35 S: *etr1-1* gene (Jones et al. 2005). Thus, ET may modulate the time course of PCD by controlling the expression and activity of some isoenzymes of cysteine proteases. The cysteine protease inhibitor E-64 decreased the NaCl- and SA-induced cell death, suggesting that cysteine proteases are important components of the NaCl- and SA-induced cell death program.

Moreover, under salt stress, a strong membrane depolarization causes Na^+ uptake and results in K^+ efflux. Release of K^+ from the cytoplasm may activate cysteine proteases which are the effectors of PCD (Shabala 2009; Joseph and Jini 2010). In our studies, it was revealed that the disturbance of ionic homeostasis, especially the K^+ deficiency, contributes to the induction of PCD in case of SA, too (Poór et al. 2011b).

These results show that salt stress-induced ET signaling is necessary for a fast and significant ROS production in tomato cell suspension which is accompanied only with moderate increase in NO accumulation (Fig. 6). STS, an ethylene receptor blocker, could reduce ROS production but had no significant effect on NO accumulation under salt stress. Inhibition of ET signal transduction in presence of SA prevented NO accumulation in tomato cells and increased the production of ROS which suggests that the cells were not able for effective ROS scavenging in the absence of NO. This contrasts with the results found in the apical tissues of control and ET receptor mutant, *Nr* tomato plants where higher NO levels were detected in the mutant plants than in the wild-type in the presence of SA (Tari et al. 2011). ROS production in the presence of SA was not controlled by ET, but it was initiated by other factors. In summary, the cause of salt stress and SA-induced the cell death is a strong oxidative stress, which, by the activation of MAPKs and cysteine proteases, can mediate the cell death signaling, and ET can accelerate the process only in cells exposed to high salinity (Fig. 6). It can be concluded that blocking the ET-induced signaling by STS in tomato cell suspension resulted in contradictory results concerning ROS/NO balance than the intact root tissues of control and ET receptor mutant tomato.

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Conflict of interest The authors declare that they have no conflict of interest.

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