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Salt stress-induced production of reactive oxygen- and nitrogen species and cell death in the ethylene receptor mutant *Never ripe* and wild type tomato roots

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Abstract

The salt stress triggered by sublethal, 100 mM and lethal, 250 mM NaCl induced ethylene production as well as rapid accumulation of superoxide radical and H_2O_2 in the root tips of tomato (*Solanum lycopersicum* cv. Ailsa Craig) wild type and ethylene receptor mutant, *Never ripe* (*Nr/Nr)* plants. In the wild type plants superoxide accumulation confined to lethal salt concentration while H_2O_2 accumulated more efficiently under sublethal salt stress. However, in *Nr* roots the superoxide production was higher and unexpectedly, H_2O_2 level was lower than in the wild type under sublethal salt stress. Nitric oxide production increased significantly under sublethal and lethal salt stress in both genotypes especially in mutant plants, while peroxynitrite accumulated significantly under lethal salt stress. Thus, the nitro-oxidative stress may be stronger in *Nr* roots, which leads to the programmed death of tissues, characterized by the DNA and protein degradation and loss of cell viability under moderate salt stress. In *Nr* mutants the cell death was induced in the absence of ethylene perception. Although wild type roots could maintain their potassium content under moderate salt stress, K^+ level significantly declined leading to small K^+/Na^+ ratio in *Nr* roots. Thus *Nr* mutants were more sensitive to salt stress than wild type and the viability of root cells decreased significantly under moderate salt stress. These changes can be attributed to a stronger ionic stress due to the K^+ loss from the root tissues.

Key Words

Ethylene; *Never ripe* mutant; Programmed cell death; Reactive nitrogen forms; Reactive oxygen species; Salt stress; Tomato roots

Abbreviations

ACC	1-aminocyclopropane-1-carboxylic acid
ACO	ACC oxidase
ACS	ACC synthase
APF	Aminophenyl fluorescein
AR	10-Acetyl-3,7-dihydroxyphenoxazine
DAF-FM DA	4-Amino-5-methylamino- 2′,7′-difluorofluorescein
DHE	Dihydroethidium
DTT	Dithiothreitol
E-64	*trans*-Epoxysuccinyl-L-leucylamido(4-guanidino)butane
EL	Electrolyte leakage
ERF	Ethylene response factor
FDA	Fluorescein diacetate
H_2DCFDA	2',7'-dichlorofluorescein diacetate
MES	2-(N-morpholino)ethanesulfonic acid

Nr	*Never-ripe* tomato (ethylene receptor) mutant
NO	Nitric oxide
PCD	Programmed cell death
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SAM	S-adenosylmethione
SNP	Sodium nitroprusside
STS	Silver thiosulphate
TCA	Trichloroacetic acid

1. Introduction

The plant hormone, ethylene plays an important regulatory role in the growth and development and in the response of plants to different biotic- or abiotic stresses, such as high salinity (Kieber, 1997). The biosynthesis of ethylene is well defined in higher plants and starts with the conversion of L-methionine to S-adenosyl methionine (SAM). The subsequent conversion of SAM to the immediate precursor, 1-aminocyclopropane-1-carboxylic acid (ACC) and the oxidation of ACC to ethylene are catalyzed by ACC synthase (EC 4.4.1.14, ACS) and ACC oxidase (AC 1.4.3, ACO), respectively. Both ACSs and ACOs are encoded by multigene families and the isoenzymes are regulated by several developmental and environmental factors (Yang and Hoffman, 1984). Ethylene receptors are transmembrane proteins bound to endoplasmic reticulum (ER) membranes and have structural similarity to bacterial two-component histidine kinases. In tomato there are eight ethylene receptors, LeETR1, -2, to -7, and *Never ripe* (NR) and five of them were shown to bind ethylene with high affinity (Kamiyoshihara et al., 2012). *Never-ripe* (*Nr*) locus encodes a protein with high homology to the *Arabidopsis* ethylene receptor ETR3 (Zhong et al., 2008). Ethylene binds to the receptors via a copper cofactor, which is delivered by the copper transporter RAN1 protein to the ER membrane. Silver ions can substitute Cu^{+} ions in the receptor thus Ag^{+} is applied as effective blocker of the ethylene signal transduction (Kieber, 1997). The subsequent well-characterized components in ethylene signalling pathway are the negative regulator Raf-like serine/threonine kinase CTR1 (CONSTITUTIVE TRIPLE RESPONSE1), the positive regulator integral membrane protein EIN2 (ETHYLENE INSENSITIVE2), the transcription factors EIN3/EIL1 (ETHYLENE INSENSITIVE3) and ethylene response factors (ERFs) (Guo and Ecker, 2004). It has been confirmed by bimolecular fluorescence complementation that Never Ripe (NR) protein was targeted to the ER and interacted with multiple CTR proteins (LeCTR1, 3 and 4) when transmitted the signal to the downstream CTRs (Zhong et al., 2008).

The ethylene receptor- and signalling mutants have allowed for a more precise analysis of the physiological functions of ethylene. The *Nr* mutants exhibit insensitivity to ethylene not only in fruit ripening but in the triple response, leaf petiole epinasty, senescence of petals and flower abscission. However, the *Nr/Nr* plants were able to produce ethylene after pathogen attack indicating that the mutants are not impaired in ethylene biosynthesis

(Lanahan et al., 1994). Although *Nr/Nr* plants displayed severely reduced ethylene perception, they maintained a very low ethylene sensitivity in ripening tomato fruit (Barry et al., 2005).

The literature has numerous reports to support the involvement of ethylene production and signalling in salinity tolerance. Quinet et al. (2010) found that salt-tolerant rice cultivars showed higher ethylene emission than the salt-sensitive ones. Similar results were found in soybean (Ma et al., 2012) and wheat cultivars (Khan et al., 2012). Besides ethylene, the ACC content also increased after salt exposure in the roots of tomato (Albacete et al., 2008). Salt stress inhibited the germination of *Suaeda salsa* seeds which was alleviated by exogenous ACC (Li et al., 2005). The germination was also delayed under salt stress in ethylene insensitive *Arabidopsis* mutants (*ein2-5*) compared to wild type Col-0 plants. Moreover, mutation of EIN2 led to extreme salt sensitivity, while over-expression of the C-terminus of EIN2 mitigated the salt sensitivity of *ein2-5* mutant, indicating that EIN2 is required for salt tolerance (Lei et al., 2011). ERFs were generally found to be important components in establishing salt tolerance in many plant species such as tomato (Pan et al., 2012) and *Arabidopsis* (Zhang et al., 2012). However, the over-expression of rice *OsERF922* (encoding an APETALA2/ethylene response factor, AP2/ERF) decreased the tolerance to salt stress by increasing Na^+/K^+ ratio in the shoots (Liu et al., 2012). Moreover, the ethylene-insensitive *etr1-3* mutants were more sensitive to salt stress than wild type plants (Wang et al., 2009).

On the other hand, ethylene at high concentration can induce programmed cell death (PCD) in plants (Trobacher, 2009) by the initiation of chromatin condensation and DNA laddering, the hallmarks of PCD (Byczkowska et al., 2013). The PCD inducing role of ethylene was also supported by the experiments of Wi et al. (2010), who demonstrated that the inhibition of salt stress-induced biphasic ethylene production enhanced salt tolerance in tobacco by reducing the accumulation of ROS.

PCD has been associated in various organs with decreased protein level and increased proteolytic activity (Jones, 2004). Although several senescence-associated cysteine protease genes were up-regulated following ethylene treatment in senescing tissues (Cervantes et al., 1994), the role of ethylene in the initiation and execution of proteolysis during cell death proved to be controversial. The lack of ethylene perception only delayed but did not prevent the expression of four senescence-associated cysteine protease genes in the corollas of ethylene insensitive, transgenic petunia line constitutively expressing the mutant ethylene receptor 35S:*etr1-1*. This suggests that the up-regulation of these cysteine protease genes was not dependent on ethylene signalling (Jones et al., 2005).

There is tight connection between the production of ethylene and the accumulation of reactive oxygen species (ROS) and nitric oxide (NO) under salt stress (Wang et al., 2010). While external addition of ethylene increased the accumulation of H_2O_2 (Overmyer et al., 2003), exogenous application of H_2O_2 stimulated ethylene emission in salt-treated wild type but not in *etr1-3* mutant *Arabidopsis* callus tissue (Wang et al., 2009) suggesting that ethylene and H_2O_2 can act as self-amplifying signal molecules in feed-forward loop (Wi et al., 2010). Poór et al. (2013) found that the lethal concentration of NaCl induced ROS and NO production in tomato cell suspension culture which was time-dependent and these signalling pathways acted synergistically to potentiate cell death. Treatment with ACC together with 250 mM NaCl enhanced ROS production in parallel with increasing cell death ratio in tomato cell suspension culture. The ethylene receptor blocker silver thiosulphate (STS) decreased the NaCl induced ROS production and cell death in this system suggesting that ethylene signal transduction had a direct control over PCD induction. ROS, such as H_2O_2 and superoxide radicals ($O_2^{\cdot-}$) that are dramatically induced by high salinity are essential mediators of plant PCD, since they can damage cellular components, such as proteins, lipids and DNA (De Pinto et al., 2012).

ROS and reactive nitrogen species (RNS), especially H_2O_2 and NO can modulate signalling networks that control growth, development and stress response both independently and synergistically (Wang et al., 2013; Petrov et al. 2015). NO and superoxide can generate other toxic molecular species, such as peroxynitrite ($ONOO^-$), which can initiate cell death only at high concentration (Delledonne et al., 2001).

In many species and tissues NO can enhance salt tolerance of plants (Poór et al., 2015). Wang et al. (2009) found that treatment with NaCl together with sodium nitroprusside (SNP), an NO generator decreased the NaCl induced electrolyte leakage (EL) in the wild type but not in calli of the *etr1-3 Arabidopsis* mutant. While SNP greatly stimulated emission of ethylene from *Arabidopsis* callus, exogenous ACC did not enhance the production of NO in this system. The NO donor SNP alleviated the NaCl-induced injury by maintaining lower Na^+/K^+ ratio and an increased plasma membrane ATP-ase activity in *Arabidopsis* callus tissues. It can be concluded that NO plays an important role in cytoprotection and in the induction of salt tolerance, but it can also promote salt-induced PCD (Wang et al. 2013).

Although a number of papers appeared about the effects of ethylene in the response of plants to high salinity, the salt stress acclimation and salt stress-induced PCD has not been compared in the ethylene receptor *Never ripe* mutant and in the wild type tomato plants.

Since various types of ROS and RNS may activate distinct signalling pathways (Petrov et al., 2015), the aim of this work is to reveal the role of ethylene signalling in the accumulation of various reactive oxygen ($O_2^{\cdot-}$ and H_2O_2) and nitrogen (NO and $ONOO^-$) forms in the root apices of wild type and ethylene receptor mutants and to reveal correlations between their accumulation and cell viability. We are also interested in how various forms of reactive oxygen and nitrogen affect the acclimation to salt stress or the initiation of PCD in *Nr* mutants exposed to salinity stress which had been found to be sublethal and lethal stress in wild type tomato roots (Poór et al. 2014).

2. Methods

2.1. Plant material and growth conditions

Wild type and ethylene receptor mutant *never ripe (Nr)* tomato plants (*Solanum lycopersicum* L. cv. Ailsa Craig) were grown in a controlled environment under 300 μmol m^{-2} s^{-1} light intensity (F36W/GRO lamps, Sylvania, Germany), 12 h light/12 h dark period, 24/22 ^oC day/night temperature and 55–60 % relative humidity in 25 cm diameter pots in soil culture. Pots were filled with 2.5 kg of commercial soil (Bioland Tőzegfeldolgozó Kft., Biatorbágy, Hungary) containing N (200-500 mg L^{-1}), P_2O_5 (200-500 mg L^{-1}); K_2O (300-600 mg L^{-1}), white peat (50 m/v %), black peat (50 m/v %), and $CaCO_3$ (2 kg m^{-3}), (pH 5.5-7.5).

Nr/Nr mutants were homozygous for *Nr* in Ailsa Craig background. Using the possibility that *Nr* mutants are capable to initiate adventitious roots, the plants were propagated vegetatively (Lanahan et al. 1994). For the experiments shoots at three-leaf-stage were cut and rooted for 4 weeks in a hydroponic culture containing 2 mM $Ca(NO_3)_2$, 1 mM $MgSO_4$, 0.5 mM KH_2PO_4, 0.5 mM Na_2HPO_4, 0.5 mM KCl, micronutrients (0.001 mM $MnSO_4$, 0.005 mM $ZnSO_4$, 0.0001 mM $CuSO_4$, 0.0001 mM $(NH_4)_6Mo_7O_{24}$, 0.01 mM H_3BO_4) and 0.02 mM Fe(III)-EDTA at pH 5.8, under the same controlled condition (Tari et al., 2011). The nutrient solution was changed twice a week. The uniform, adventitious roots were used in the experiments.

Plants were treated with 100 mM or 250 mM NaCl, with sublethal and lethal concentrations, respectively, for 24 h through the root system in the hydroponic culture solution. The experiments were performed at 9 o'clock a.m and repeated 3-4 times in independent experiments.

2.2. Measurement of ethylene production

Five hundred mg of washed root tip segments were incubated in gas-tight flasks fitted with a rubber stopper after salt treatments. The tissue hydration was maintained with 0.5 mL distilled water during the incubation. The flasks were shaken in the dark for 1 hour at the indicated time intervals. 2.5 mL of the gas was removed from the tubes with a gas-tight syringe and injected to gas chromatograph. Production of ethylene 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 300, 120 and 100 ^{o}C, respectively (Poór et al., 2014). A set of ethylene standards was used to calculate the amount of ethylene generated by the root segments.

2.3. Fluorescence microscopy

Superoxide radical was visualized by 10 μM dihydroethidium (DHE) (Sigma-Aldrich, St. Louis, MO) and H_2O_2 by 50 μM 10-acetyl-3,7-dihydroxyphenoxazine (AR; ADHP or Ampliflu™ Red) (Sigma-Aldrich, St. Louis, MO) fluorescent dyes in the roots of tomato plants (Pető et al., 2013). NO was detected with a specific fluorescent dye, 10 μM 4-amino-5-methylamino- 2′,7′-difluorofluorescein (DAF-FM DA) (Sigma-Aldrich, St. Louis, MO) and peroxynitrite generation was monitored using 10 μM aminophenyl fluorescein (APF) according to Lehotai et al. (2011). The samples were incubated in the dark in the presence of specific dyes at room temperature for 30 min and then were washed twice with 10 mM *Tris*(hydroxymethyl)aminomethane (TRIS–HCl, pH 7.4) buffer for 10 min.

Cell viability was determined by fluorescein diacetate (FDA) (Sigma-Aldrich, St. Louis, MO). Root tip segments were stained at room temperature for 10 min in the dark with 10 μM FDA dissolved in 3 mL 10 mM 2-(N-morpholino)ethanesulfonic acid (MES)/ potassium chloride (KCl) buffer (pH 6.15). After the incubation samples were washed two times for 10 min with MES/KCl buffer (pH 6.15) (Lehotai et al., 2011).

Fluorescence intensity was detected with Zeiss Axiowert fluorescence microscope (200M type, Carl Zeiss Inc., Jena, Germany) equipped with an objective 5X. Digital photographs were taken with a high-resolution digital camera (Axiocam HR, HQ CCD camera; Carl Zeiss Inc., Jena, Germany) using a filter set 10 (excitation 450-495 nm, emission

515-565 nm) or filter set 20HE (excitation: 535–585 nm, emission: 600–655 nm) and pixel intensity was quantified with AXIOVISION REL. 4.8 software (Carl Zeiss Inc., Munich, Germany). For the determination of pixel intensity constant circles of 150 μm diameter were used in all experiments and the pixel intensity was measured at 2 mm distance from the root apex.

2.4. Cell death determination

Cell death was determined with the measurement of electrolyte leakage (EL) by the method of Poór et al. (2014). One g of root segments was transferred to 15 mL double distilled water. After 2 h of incubation at 25 ^{o}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 ^{o}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)x100.

DNA fragmentation was determined following the method of Kubis et al. (2003), with some modifications. Roots were washed twice with 50 mL double distilled water and 100 mg of root tip segments 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 centrifuged for 10 min at 4 ^{o}C, 3000 g. The phenol-chloroform step was repeated with the supernatant. Then 0.5 mL of chloroform-isoamyl alcohol (24/1) was added to the supernatant and centrifuged again (10 min at 4 ^{o}C, 3000 g). The supernatant was incubated for 3 hours in the mixture of 550 μL isopropanol and 20 μL Na-acetate. Then the samples were centrifuged (10 min at 4 ^{o}C, 11300 g) and the pellet was washed and centrifuged twice for 10 min (4 ^{o}C, 11300 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 minutes at 37 ^{o}C before the agarose gel (3 %) electrophoresis was performed (80 mV, 2 h).

2.5. Determination of macroelement contents

Roots were collected on filter paper, washed twice with 50 mL double distilled water, one g were put into glass tubes (Packard, Groningen, Netherlands) and dried (80 ^{o}C, 24 h). After

measuring the dry weight (DW), 6 mL HNO_3 (Reanal, Budapest, Hungary) and 2 mL 30 % H_2O_2 (Reanal, Budapest, Hungary) were added to 100 mg plant material for 20 h. The samples were digested in microwave destructor (MarsXpress CEM, Matthews NC, USA) at 200 ^oC for 25 min and after cooling they were diluted with 12 mL double distilled water. Potassium and sodium contents of the root cells were determined by AAS (Hitachi Z-8200, Tokyo, Japan) (Poór et al., 2014).

2.6. Determination of proteolytic activity

Soluble protein was extracted from 500 mg root tissues with 0.5 mL extraction buffer (50 mM sodium acetate, 1 mM dithiothreitol (DTT, pH 6.1) at 4 ^oC. The slurry was vortexed and centrifuged (10 min at 4 ^oC, 11300 g) and supernatants were used for the measurements.

Protein contents of the supernatants were determined spectrophotometrically at 595 nm (KONTRON, Milano, Italy) according to the method of Bradford (1976) using bovine serum albumin as a standard.

Azocasein (Sigma-Aldrich, St. Louis, MO) was applied as a non-specific substrate to measure the total proteolytic activity. 50 μL of tissue extract, 0.3 mL 1 % azocasein (w/v) and 650 μL potassium phosphate buffer (pH 5.5) were incubated at 37 ^oC for 2 h. The reaction was stopped by the addition of 300 μL 10 % (w/v) trichloroacetic acid (TCA) at 4 ^oC for 20 min. After 20 min on ice, the samples were centrifuged (10 min at 4 ^oC, 11300 g) and the yellow color of the supernatant was measured at 440 nm. One unit of total proteolytic activity (*U*) was defined as the amount of enzyme yielding 0.01 unit of absorbance per min under the assay conditions. To determine the cysteine protease activity, the extracts were incubated with specific cysteine protease inhibitor, *trans*-epoxysuccinyl-L-leucylamido(4-guanidino)butane (E-64, Sigma-Aldrich, St. Louis, MO). Stock solution of E-64 was dissolved in water and it was mixed with tomato root extract containing the enzymes. Final concentration of the inhibitor was 10 mM. After 1 h at 20 ^oC azocasein was added to the mixture. Cysteine protease activity was calculated in relation to uninhibited activity (Rossano et al., 2011).

2.7. Statistical analysis

Data are presented as average values resulted from at least three independent experiments. Statistical analyses were performed with Sigma Plot 11.0 software (Systat Software Inc.,

Erkrath, Germany) using Duncan's multiple range test after analysis of variance (ANOVA) and differences were considered significant if $P<0.05$.

3. Results

3.1. NaCl-induced ethylene production

To investigate the role of ethylene perception in salt stress-induced ROS and NO accumulation, the ethylene production of root segments was measured after the treatment with sublethal (100 mM) and lethal (250 mM) concentration of NaCl. Ethylene production was enhanced significantly by both salt concentrations after one-hour-long treatments in the roots of ethylene receptor mutant *Nr* (Fig. 1A).

Addition of the sublethal concentration of 100 mM NaCl to the nutrient solution increased the ethylene production both in the wild type and the *Nr* tomato genotypes within 6 hours and this tendency lasted to 24 h (Fig. 1B). To the end of the experiments the ethylene production decreased significantly both in the wild type and the *Nr* roots exposed to 250 mM NaCl (Fig. 1C).

3.2. NaCl-induced, ethylene-dependent ROS production

Fluorescent probes were used to determine ROS and RNS production in root cells. Both salt treatments induced rapid superoxide anion radical accumulation within one hour in the root tips of wild type and *Nr* tomatoes, which was significantly higher in *Nr* roots (Fig. 2A). In the presence of 100 mM NaCl, the superoxide production decreased after 6 hours in the root apices of wild type tomato but remained significantly higher in the roots of *Nr* mutants compared to the control (Fig. 2B). 250 mM NaCl induced significant and permanently high superoxide production both in wild type and *Nr* roots, but it was much higher after the first and sixth hours in the roots of *Nr* than in the wild type (Fig. 2).

Hydrogen peroxide production was elevated by both salt treatments in the first hour in the root apices of wild type tomato (Fig. 3A) but increased only later in the roots of *Nr* plants exposed to 250 but not to 100 mM NaCl. (Fig. 3).

3.3. NaCl-induced, ethylene dependent RNS production

Both genotypes increased NO production under salt stress in the first hour but it was higher in root apices of *Nr* mutant (Fig. 4A). While NO accumulation proved to be transient at lethal, 250 mM NaCl concentration, NO level remained above the untreated wild type control in the presence of sublethal salt stress (Fig. 4C).

Peroxynitrite ($ONOO^-$) production was elevated significantly only by the lethal, 250 mM NaCl treatments in the *Nr* mutant from the 1st to the 6th hours and the $ONOO^-$ accumulation was slightly but not significantly higher in *Nr* roots compared to the wild type (Fig. 5).

3.4. NaCl-induced, ethylene dependent physiological responses

The harmful effects of NaCl treatments were determined by FDA staining in the root apices of wild type and *Nr* tomato plants. The cell viability decreased significantly by 250 mM NaCl treatments from the 6th hour after salt exposure both in wild type and *Nr* roots (Fig. 6B). Interestingly, cell viability also decreased significantly in *Nr* roots after the exposure of 100 mM NaCl within 6 hour but it was not changed in wild type roots. This suggests that cells in the root apices of *Nr* mutants were more sensitive to salt stress than that of the wild type. The damage caused by 100 mM NaCl treatment was more pronounced after 24 h in *Nr* root apices (Fig 6C).

The changes in cell viability determined with FDA were confirmed by the measurement of electrolyte leakage (EL) from the root tissues (Fig. 7A). The EL was about 80 % after strong salt stress both in wild type and *Nr* plants and 55 % in case of 100 mM NaCl treatment in the roots of *Nr* plants 24 h after the salt treatment, suggesting that the *Nr* mutants were more sensitive to salinity stress than the wild type.

Since DNA fragmentation may be a hallmark of apoptotic-like PCD in plants, DNA isolation and separation by agarose gel electrophoresis were performed to study the effects of NaCl treatments on DNA degradation. DNA fragmentation was detected in 250 mM NaCl treated wild type and *Nr* roots, as well as in *Nr* roots after 100 mM NaCl treatment (Fig. 7B).

Maintenance of ion homeostasis, especially the optimal K^+/Na^+ ratio is fundamental for cell survival under salt stress because irreversible changes in the ion balance can activate PCD. After 24-hour-long treatments the K^+ content decreased significantly in the root cells of both wild type and *Nr* mutants exposed to 250 mM NaCl and in the case of *Nr* plants treated with 100 mM NaCl (Table 1). Na^+ accumulated significantly in the roots of wild type and

ethylene receptor mutant tomato after the treatment with sublethal and lethal concentration of NaCl (Table 1). However, due to the low K^+ content, the ratio of K^+/Na^+ decreased only in the roots of *Nr* plants exposed to both salt concentrations and in wild type roots treated with 250 mM NaCl (Table 1).

250 mM NaCl decreased the protein content of both wild type and Nr roots (Fig. 8A) and in parallel increased the proteolytic activity in these cells (Fig. 8B). The roots of ethylene receptor mutant *Nr* plants showed small increases in protease activity and thus reduction in protein content after treatment with 100 mM NaCl but proteolytic activity increased significantly in both genotypes under lethal salt stress (Fig. 8). The use of specific cysteine protease inhibitor, E-64 decreased the total proteolytic activity significantly in the root samples which confirmed that the salt stress induced the activity of cysteine proteases in both genotypes (Fig. 8B) and most of the protease activity increase originated from cysteine proteases.

4. Discussion

Exposure of tomato roots to different concentrations of NaCl led to an increased ethylene production both in the wild type and ethylene receptor mutant, *Nr* plants. The ethylene production was not only dependent on the salt concentration but also on the duration of salt stress. Higher ethylene production could be detected in both genotypes at lower salt concentration, but its maximum appeared earlier, after one hour in *Nr* roots. In the wild type the maximal ethylene production was detected six hours after salt treatment. This result suggests that the ethylene biosynthesis in the *Nr* mutants is more sensitive to high salinity than in the wild type plants and the partial lack of ethylene perception stimulates ethylene production under salt stress conditions. Similar results were found by other authors, Lanahan et al. (1994) also confirmed that ethylene biosynthesis was not impaired in *Nr* plants. These results are also in accordance with our previous findings which revealed that the sublethal salt stress increased while the lethal concentration of NaCl reduced the ethylene production to a very low level in the root tips of Rio Fuego tomato cultivar (Poór et al., 2014).

Since the most obvious biochemical response during high salinity is the enhanced ROS production, the results with *Nr* mutant provide some extra insight into the role of ethylene sensitivity in salt stress-induced oxidative stress.

Moreover, there is a tight connection between ethylene and other signalling molecules, such as ROS and RNS (Wang et al., 2010b; Poór et al., 2013). Salt stress induced rapid ROS accumulation (superoxide anion radical and H_2O_2) in both genotypes within one hour. In wild type plants superoxide accumulation displayed a strict concentration dependency and was confined to the lethal salt concentration while H_2O_2 accumulated more efficiently under sublethal salt stress with one- and six-hours maxima at sublethal and lethal stress, respectively. In *Nr* roots the superoxide levels were significantly higher at both NaCl concentrations and unexpectedly, H_2O_2 level was lower than in the wild type after one hour under sublethal salt stress. In other cases H_2O_2 content did not increase above the control level in the mutant tissues. These results show that the lack or partial block of ethylene perception in *Nr* mutant resulted in a shift in various reactive oxygen species, from H_2O_2 to superoxide. Since strong positive correlation was found between superoxide anion radical, H_2O_2 content and protein carbonylation in beech seeds during ageing process (Kalemba and Pukacka, 2014) and H_2O_2 proved to be the most important signalling molecule, which activates the defence mechanisms during oxidative stress (Petrov et al., 2015), the observed changes in *Nr* root tips led to higher stress sensitivity.

H_2O_2 content increased slightly in the roots of *Nr* mutant introgressed into Micro-Tom (MT) background under moderate salt stress. Although *Nr* roots exhibited enhanced catalase (EC 1.11.1.6), ascorbate peroxidase (EC 1.11.1.11) and glutathione reductase (EC 1.6.4.2) activities under control conditions when compared to the wild type, these enzyme activities as well as superoxide dismutase (EC 1.15.1.1, SOD) activity were very similar in the MT wild type and *Nr* mutants under salt stress. Moreover, similar reductions in SOD activity pattern investigated by non-denaturating PAGE were observed in the roots of MT and *Nr* plants treated with 100 mM NaCl, which raises the possibility that a H_2O_2 generating system other than SOD can be found in the mutants (Monteiro et al., 2011). These mutants proved to be more tolerant to salinity stress suggesting that the effect of *Nr* mutation on salt stress response can be diverse in different genetic background.

When the scavenging mechanisms keeping ROS and RNS production under control are exhausted, the excess of NO may contribute to the initiation of PCD. NO production increased significantly under salt stress after one hour both in the wild type and *Nr* roots but this was more pronounced in mutant plants. The difference between sublethal and lethal salt stress in wild type plants is that plants which survive salt stress show a long-lasting increase in NO accumulation during the first 24 hours of the stress while under lethal salt stress NO

accumulation is limited to the 1st hour. *Nr* plants displayed a significantly higher NO accumulation during the first hour under salt stress.

It has been shown recently that under Cd stress the generation of NO was accompanied by the activation of plasma membrane NADPH-oxidase (EC 1.6.3.1) and a subsequent superoxide anion accumulation (Arasimowicz-Jelonek et al., 2012). The lack of simultaneous H_2O_2 accumulation during the experiment suggests that $O_2^{\bullet-}$ rather than H_2O_2 cooperate with NO to induce PCD (Chmielowska-Bąk et al., 2014). We found similar changes in the root apices of tomato exposed to high salinity.

Since NO can react readily with superoxide to form peroxynitrite, the accumulation of peroxynitrite is dependent on the generation of the precursors. Peroxynitrite accumulated significantly under lethal salt stress both in the wild type and the *Nr* plants. Although $ONOO^-$ is not considered to be very toxic to plant cells (Delledonne et al., 2001), its accumulation was much more intensive under cell death-inducing salt concentration, however, the peroxynitrite accumulation in the ethylene response mutant *Nr* did not show significant differences from the wild type plants. Summarizing the results salt stress can induce oxidative- and nitrosative stress in plants by regulating ROS and RNS accumulation, which at low concentration can promote stress tolerance but at high concentrations can initiate cell death (Poór et al., 2015). The oxidative and nitrosative stress may be significantly stronger in the root apices of the ethylene insensitive *Nr* plants than in the wild type which can lead to the programmed death of tissues in the root apex at lower salt concentration. It can also be concluded that in ethylene receptor *Nr* mutants the oxidative or nitrosative stress responses have been developed in the absence of ethylene perception under salt stress.

On the other hand, ethylene may regulate PCD signalling in the wild type plants (Trobacher, 2009). Ethylene induced fragmentation of chromatin and DNA laddering were observed in many plant species and organs (Byczkowska et al., 2013). DNA degradation could also be detected in the root tissue of *Nr* mutants exposed to 100 mM NaCl. In the wild type plants a controlled fragmentation of DNA into oligosomal units can be observed (DNA ladder), while lethal salt stress resulted in general DNA damage in *Nr* roots.

A growing body of evidence suggests that PCD associated with increased H_2O_2 production was mediated by proteases with caspase-like activity (Yakimova et al.; 2006; Poór et al., 2013). Proteolytic activity determined against the synthetic substrate azocasein was significantly enhanced in the roots exposed to lethal salt stress. Class-specific protease inhibitor E-64, an irreversible inhibitor of cysteine proteases reduced the protease activity by

more than 50 % both in the wild type and *Nr* tissue extracts, which suggests that cysteine proteases were mainly responsible for the increases in the proteolytic activity during salt stress. Since ethylene regulates the expression of distinct cysteine protease genes in various plant tissues, e.g. during seed germination in chickpea (Cervantes et al., 1994) and during the senescence of carnation petals (Jones et al. 2004), it is interesting that the protease activity increased to the same degree in the wild type and in the ethylene insensitive *Nr* mutants. Since the up-regulation of certain cysteine protease genes could be observed in ethylene insensitive transgenic petunia corollas, expressing a mutant *etr1-1* ethylene receptor (Jones at al., 2005), it can be presumed that specific cysteine proteases can be up-regulated in the absence of ethylene signalling in *Nr* roots. Proteases can be activated not only by ethylene but by nitric oxid, too. NO accumulated during Cd-induced PCD and promoted MPK 6-mediated caspase3-like activity which resulted in the execution of PCD in *Arabidopsis thaliana* seedlings (Ye et al., 2013).

The strength of the ionic stress caused by high salinity is strongly affected by ion disequilibrium, mainly by the K^+/Na^+ ratio in the tissues. Na^+ uptake into the cytosol is accompanied by a K^+ deficiency (Kim et al. 2014). In contrast to Monteiro et al. (2011) who did not find significantly enhanced Na^+ accumulation under moderate salt stress in the root and leaf tissues of *Nr* mutant in Micro-TOM background, we detected significantly increased Na^+ accumulation both in the wild type and the *Nr* root tissues at both Na^+ concentrations (Table 1). While wild type roots could maintain potassium content, K^+ uptake was inhibited or K^+ leaked out from the cells at much higher rate from the *Nr* roots under moderate salt stress. In contrast to wild type this resulted in a significant decrease in K^+/Na^+ ratio in the mutant tissues even under moderate salt stress. K^+ deficiency could influence the initiation of PCD by activating various PCD-associated enzymes comprising cysteine proteases (Demidchik et al., 2010). In recent study it was demonstrated that the overexpression of an antiapoptotic protein, BCL-2 significantly reduced the salt stress-induced K^+ efflux and repressed the expression of vacuolar processing enzyme, a plant caspase-like cysteine protease thus alleviated PCD symptoms (Kim et al., 2014).

5. Conclusion

The ethylene insensitive *Nr* tomato mutants in Ailsa Craig background were more sensitive to salt stress than the wild type plants and even at moderate salt stress the viability of

root tip cells decreased significantly as it was detected by the loss of fluorescein diacetate fluorescence and by the significantly increased electrolyte leakage from the tissues. These changes can be attributed to a stronger ionic stress due to the intensive K^+ loss, decreased K^+/Na^+ ratio as well as to the enhanced accumulation of superoxide anion radical and NO in the root tips at moderate salt stress. These reactive oxygen and nitrogen forms can induce more damaging oxidative and nitrosative stress that led to PCD at moderate salt stress in the root apices of *Nr* mutants.

Contributions

IT and PP designed the experiments, analyzed the data and wrote the manuscript. PP and PB conducted the experiments with fluorescence microscope, JK, PB, ZT and ÁSZ measured the activity of cysteine proteases and PCD induction. Figures were designed and created by PP.

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References

Albacete, A., Ghanem, M.E., Martínez-Andújar, C., Acosta, M., Sánchez-Bravo, J., Martínez, V., et al., 2008. Hormonal changes in relation to biomass partitioning and shoot growth impairment in salinized tomato (*Solanum lycopersicum* L.) plants. J. Exp. Bot. 59, 4119-4131.

Arasimowicz-Jelonek M., Floryszak-Wieczorek J., Deckert J., Rucińska-Sobkowiak R., Gzyl J., Pawlak-Sprada S., et al., 2012. Nitric oxide implication in cadmium-induced programmed cell death in roots and signaling response of yellow lupine plants. Plant Physiol. Biochem. 58, 124–134

Barry, C.S., McQuinn, R.P., Thompson, A.J., Seymour, G.B., Grierson, D., Giovannoni, J.J., 2005. Ethylene insensivity conferred by Green-ripe and Never-ripe 2 ripening mutants of tomato. Plant Physiol. 138, 267-275.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248-254.

Byczkowska, A., Kunikowska, A., Kaźmierczak, A., 2013. Determination of ACC-induced cell-programmed death in roots of *Vicia faba* ssp. minor seedlings by acridine orange and ethidium bromide staining. Protoplasma, 250, 121-128.

Cervantes, E., Rodríguez, A., Nicolás, G., 1994. Ethylene regulates the expression of a cysteine proteinase gene during germination of chickpea (*Cicer arietinum* L.). Plant Mol. Biol. 25, 207-215.

Chmielowska-Bąk, J., Gzyl, J., Rucińska-Sobkowiak, R., Arasimowicz-Jelonek, M., Deckert, J., 2014. The new insights into cadmium sensing. Front. Plant Sci. 5, 245. doi:10.3389/fpls.2014.00245

Delledonne, M., Zeier, J., Marocco, A., Lamb, C., 2001. Signal interactions between nitric oxide and reactive oxygen intermediates in the plant hypersensitive disease resistance response. Proc. Natl. Acad. Sci. U.S.A. *98*, 13454-13459.

Demidchik, V., Cuin, T. A., Svistunenko, D., Smith, S. J., Miller, A. J., Shabala, S., et al., 2010. *Arabidopsis* root K^+-efflux conductance activated by hydroxyl radicals: single-channel properties, genetic basis and involvement in stress-induced cell death. J. Cell Sci. 123, 1468-1479.

De Pinto, M.C., Locato, V., De Gara, L., 2012. Redox regulation in plant programmed cell death. Plant Cell Environ. 35, 234-244.

Guo, H., Ecker, J. R., 2004. The ethylene signaling pathway: new insights. Curr. Opin. Plant Biol. 7, 40-49.

Jones, M.L., 2004. Changes in gene expression during senescence. In: Noodén, L. D. (Ed.). (2004). Plant cell death processes. Academic Press, San Diego California pp. 51-67.

Jones, M.L., Chaffin, G.S., Eason, J.R., Clark, D.G., 2005. Ethylene-sensitivity regulates proteolytic activity and cysteine protease gene expression in petunia corollas. J. Exp. Bot. 56, 2733-2744.

Kalemba, E.M., Pukacka, S., 2014. Carbonylated proteins accumulated as vitality decreases during long-term storage of beech (*Fagus sylvatica* L.) seeds. Trees 28, 503-515.

Kamiyoshihara, Y., Tieman, D.M., Huber, D.J., Klee, H.J., 2012. Ligand-induced alterations in the phosphorylation state of ethylene receptors in tomato fruit. Plant Physiol. 160, 488-497.

Khan, M.I.R., Iqbal, N., Masood, A., Khan, N.A., 2012. Variation in salt tolerance of wheat cultivars: Role of glycinebetaine and ethylene.Pedosphere, 22, 746-754.

Kieber, J.J., 1997. The ethylene response pathway in *Arabidopsis.* Annu. Rev. Plant Physiol. Plant Mol. Biol. 48, 227-296.

Kim, Y., Wang, M., Bai, Y., Zeng, Z., Guo, F., Han, N., et al., 2014. Bcl-2 suppresses activation of VPEs by inhibiting cytosolic Ca^{2+} level with elevated K^+ efflux in NaCl-induced PCD in rice. Plant Physiol. Biochem. 80, 168-175.

Kubis, S.E., Castilho, A.M.M.F., Vershinin, A.V., Heslop-Harrison, J.S., 2003. Retroelements, transposons and methylation status in the genome of oil palm (*Elaeis guineensis*) and the relationship to somaclonal variation. Plant Mol. Biol. 52, 69-79.

Lanahan, M.B., Yen, H.C., Giovannoni, J.J., Klee, H.J., 1994. The *never ripe* mutation blocks ethylene perception in tomato. Plant Cell 6, 521-530.

Lehotai, N., Pető, A., Bajkán, Sz., Erdei, L., Tari, I., Kolbert, Zs., 2011. In vivo and in situ visualization of early physiological events induced by heavy metals in pea root meristem. Acta Physiol. Plant. 33, 2199-2207.

Lei, G., Shen, M., LI, Z.G., Zhang, B., Duan, K.X., Wang, N., et al., 2011. EIN2 regulates salt stress response and interacts with MA3 domain-containing protein ECIP1 in *Arabidopsis*. Plant Cell Environ. 34, 1678-1692.

Li, W., Liu, X., Khan, M. A., Yamaguchi, S., 2005. The effect of plant growth regulators, nitric oxide, nitrate, nitrite and light on the germination of dimorphic seeds of *Suaeda salsa* under saline conditions. J. Plant Res. *18*, 207-214.

Liu, D., Chen, X., Liu, J., Ye, J., Guo, Z., 2012. The rice ERF transcription factor OsERF922 negatively regulates resistance to *Magnaporthe oryzae* and salt tolerance. J. Exp. Bot. 63, 3899-3911.

Ma, H., Song, L., Shu, Y., Wang, S., Niu, J., Wang, Z., et al., 2012. Comparative proteomic analysis of seedling leaves of different salt tolerant soybean genotypes. J. Proteomics 75, 1529-1546.

Monteiro, C.C., Carvalho, R.F., Gratão, P.L., Carvalho, G., Tezotto, T., Medici, L.O., Peres, L.E.P., Azevedo, R.A., 2011. Biochemical responses of the ethylene-insensitive Never ripe tomato mutant subjected to cadmium and sodium stresses. Environ. Exp. Bot. 71, 306-320.

Overmyer, K., Brosché, M., Kangasjärvi, J., 2003. Reactive oxygen species and hormonal control of cell death. Trends Plant Sci. 8, 335-342.

Pan, Y., Seymour, G.B., Lu, C., Hu, Z., Chen, X., Chen, G., 2012. An ethylene response factor (ERF5) promoting adaptation to drought and salt tolerance in tomato. Plant Cell Rep. 31, 349-360.

Pető, A., Lehotai, N., Feigl, G., Tugyi, N., Ördög, A., Gémes, K., et al., 2013. Nitric oxide contributes to copper tolerance by influencing ROS metabolism in *Arabidopsis*. Plant Cell Rep. 32, 1913-1923.

Petrov, V., Hille, J., Mueller-Roeber, B., Gechev, T.S., 2015. ROS-mediated abiotic stress-induced programmed cell death in plants. Front. Plant Sci. 6.doi: 10.3389/fpls.2015.00069, http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4332301/

Poór, P., Kovács, J., Szopkó, D., Tari, I., 2013. Ethylene signaling in salt stress-and salicylic acid-induced programmed cell death in tomato suspension cells. Protoplasma, 250, 273-284.

Poór, P., Borbély, P., Kovács, J., Papp, A., Szepesi, Á., Takács, Z., Tari, I., 2014. Opposite extremes in ethylene/nitric oxide ratio induce cell death in suspension culture and root apices of tomato exposed to salt stress. Acta Biol. Hung. 65, 428-438.

Poór, P., Laskay, G., Tari, I., 2015. Role of nitric oxide in salt stress-induced programmed cell death and defense mechanisms. In: Khan, M.N., Mobin, M., Mohammad F., Corpas, F.J. (eds) Nitric Oxide Action in Abiotic Stress Responses in Plants. Springer International Publishing, Switzerland, pp. 193-219.

Rossano, R., Larocca, M., Riccio, P. (2011). 2-D zymographic analysis of Broccoli (*Brassica oleracea* L. var. Italica) florets proteases: Follow up of cysteine protease isotypes in the course of post-harvest senescence. J. Plant Physiol. 168, 1517-1525.

Quinet, M., Ndayiragije, A., Lefèvre, I., Lambillotte, B., Dupont-Gillain, C.C., Lutts, S., 2010. Putrescine differently influences the effect of salt stress on polyamine metabolism and ethylene synthesis in rice cultivars differing in salt resistance J. Exp. Bot. 61, 2719-2733.

Tari, I., Poór, P., Gémes, K., 2011. Sublethal concentrations of salicylic acid decrease the formation of reactive oxygen species but maintain an increased nitric oxide production

in the root apex of the ethylene-insensitive *Never* ripe tomato mutants. Plant Signal Behav. 6,1263-1266.

Trobacher, C.P., 2009. Ethylene and programmed cell death in plants. Botany, 87, 757-769.

Yang, S.F., Hoffman, N.E., 1984. Ethylene biosynthesis and its regulation in higher plants. Annu. Rev. Plant Physiol. 35, 155-189.

Yakimova E.T., Kapchina-Toteva V.M., Laarhoven L.J., Harren F.M., Woltering E.J., 2006. Involvement of ethylene and lipid signalling in cadmium-induced programmed cell death in tomato suspension cells. Plant Physiol. Biochem. 44, 581–589

Ye, Y., Li, Z., Xing, D., 2013. Nitric oxide promotes MPK6-mediated caspase-3-like activation in cadmium-induced *Arabidopsis thaliana* programmed cell death. Plant Cell Environ 36, 1–15.

Wang, H., Liang, X., Wan, Q., Wang, X., Bi, Y., 2009. Ethylene and nitric oxide are involved in maintaining ion homeostasis in *Arabidopsis* callus under salt stress. Planta, 230, 293-307.

Wang, H., Liang, X., Huang, J., Zhang, D., Lu, H., Liu, Z., Bi, Y., 2010. Involvement of ethylene and hydrogen peroxide in induction of alternative respiratory pathway in salt-treated *Arabidopsis* calluses. Plant Cell Physiol. 51, 1754-1765.

Wang, Y., Loake, G.J., Chu, C., 2013. Cross-talk of nitric oxide and reactive oxygen species in plant programmed cell death. Front. Plant Sci. 4, 314, doi: 10.3389/fpls.2013.00314, http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3744911/

Wi, S.J., Jang, S.J., Park, K.Y. 2010. Inhibition of biphasic ethylene production enhances tolerance to abiotic stress by reducing the accumulation of reactive oxygen species in *Nicotiana tabacum*. Mol. Cells 30, 37-49.

Zhang, Z., Wang, J., Zhang, R., Huang, R., 2012. The ethylene response factor AtERF98 enhances tolerance to salt through the transcriptional activation of ascorbic acid synthesis in *Arabidopsis*. Plant J. 71, 273-287.

Zhong, S., Lin, Z., Grierson, D., 2008. Tomato ethylene receptor–CTR interactions: visualization of NEVER-RIPE interactions with multiple CTRs at the endoplasmic reticulum.J. Exp. Bot. 59, 965-972.

Figure 1. Changes in ethylene production in root apices of wild type and ethylene receptor mutant *Never ripe* tomato after exposure to 100 or 250 mM NaCl for one- (A), six- (B) and 24 hours (C). (Means ± SD, n=6). Bars with different letters are significantly different at 0.05 levels (Duncan's multiple range test).

Figure 2. Changes in superoxide ($O_2^{\cdot-}$) production in root apices of wild type and ethylene receptor mutant *Never ripe* tomato after exposure to 100 or 250 mM NaCl for one- (A), six- (B) and 24 hours (C). (Means ± SD, n=6). Bars with different letters are significantly different at 0.05 levels (Duncan's multiple range test).

Figure 3. Changes in hydrogen peroxide (H_2O_2) production in root apices of wild type and ethylene receptor mutant *Never ripe* tomato after exposure to 100 or 250 mM NaCl for one- (A), six- (B) and 24 hours (C). (Means ± SD, n=6). Bars with different letters are significantly different at 0.05 levels (Duncan's multiple range test).

Figure 4. Changes in nitric oxide (NO) production in root apices of wild type and ethylene receptor mutant *Never ripe (Nr)* tomato after exposure to 100 or 250 mM NaCl for one- (A), six- (B) and 24 hours (C). (Means ± SD, n=6). Bars with different letters are significantly different at 0.05 levels (Duncan's multiple range test).

Figure 5. Changes in peroxynitrite ($ONOO^-$) production in root apices of wild type and ethylene receptor mutant *Never ripe* tomato after exposure to 100 or 250 mM NaCl for one- (A), six- (B) and 24 hours (C). (Means ± SD, n=6). Bars with different letters are significantly different at 0.05 levels (Duncan's multiple range test).

Figure 6. Changes in viability of root apices of wild type and ethylene receptor mutant *Never ripe* tomato after exposure to 100 or 250 mM NaCl for one- (A), six- (B) and 24 hours (C). (Means ± SD, n=6). Bars with different letters are significantly different at 0.05 levels (Duncan's multiple range test).

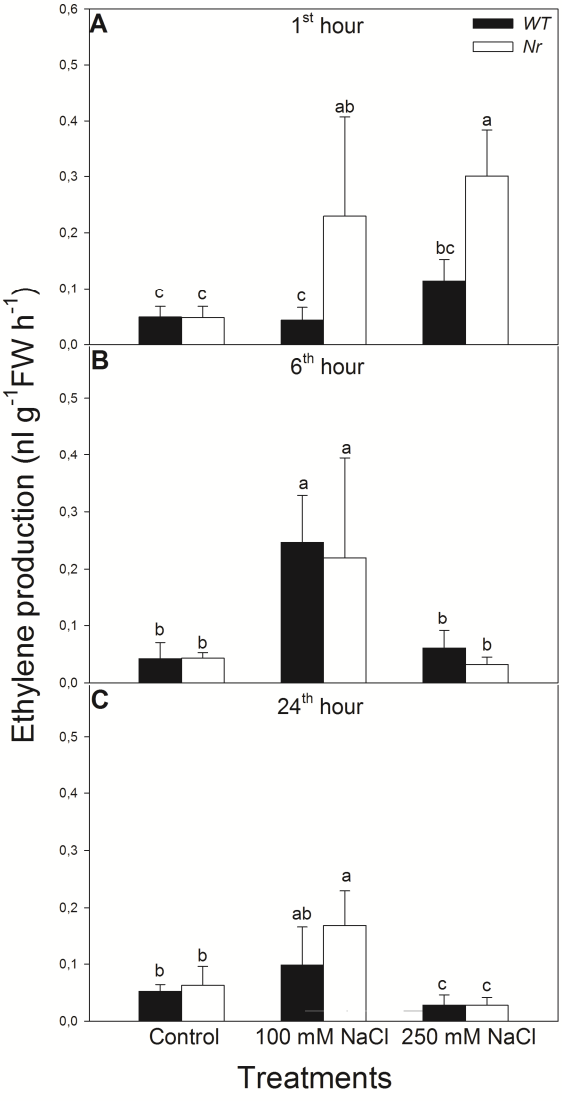
Figure 7. Changes in electrolyte leakage (EL) (A) and in DNA degradation determined by agarose gel electrophoresis (B) in the roots of wild type and ethylene receptor mutant *Never ripe* tomato after exposure to 100 or 250 mM NaCl for 24 hours. (Means ± SD, n=6). Part A: Bars with different letters are significantly different at P=0.05 level (Duncan's multiple range test). Part B: L (DNA ladder); C (Control); 100 (100 mM NaCl); 250 (250 mM NaCl); WT: wild type; *Nr*: *Never ripe* mutant.

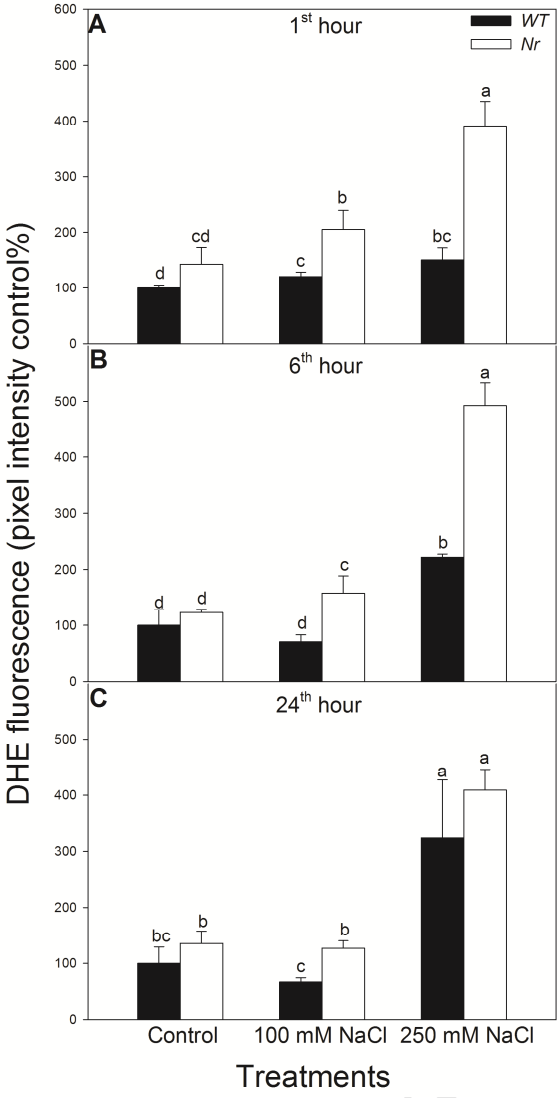
Figure 8. Changes in protein content (A) and azocaseinolytic activity (B) in the roots of wild type and ethylene receptor mutant *Never ripe* tomato after exposure to 100 or 250 mM NaCl for 24 hours. (Means ± SD, n=6). Bars with different letters are significantly different at 0.05 levels (Duncan's multiple range test).

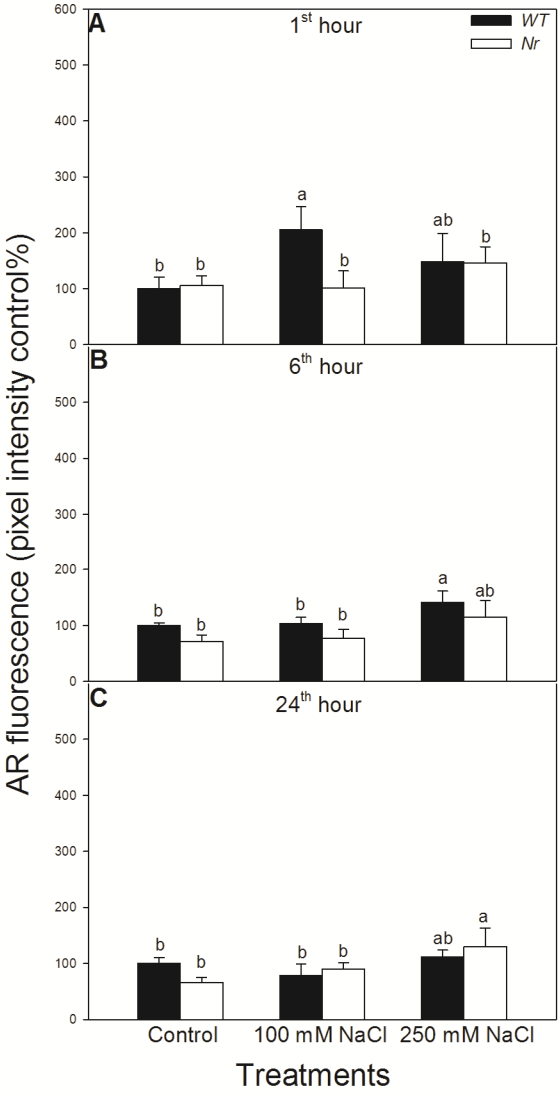
Table 1. Changes in the intracellular K^+ and Na^+ contents and the K^+/Na^+ ratio in the roots of wild type and ethylene receptor mutant *Never ripe* tomato after exposure to 100 or 250 mM NaCl for 24 hours.

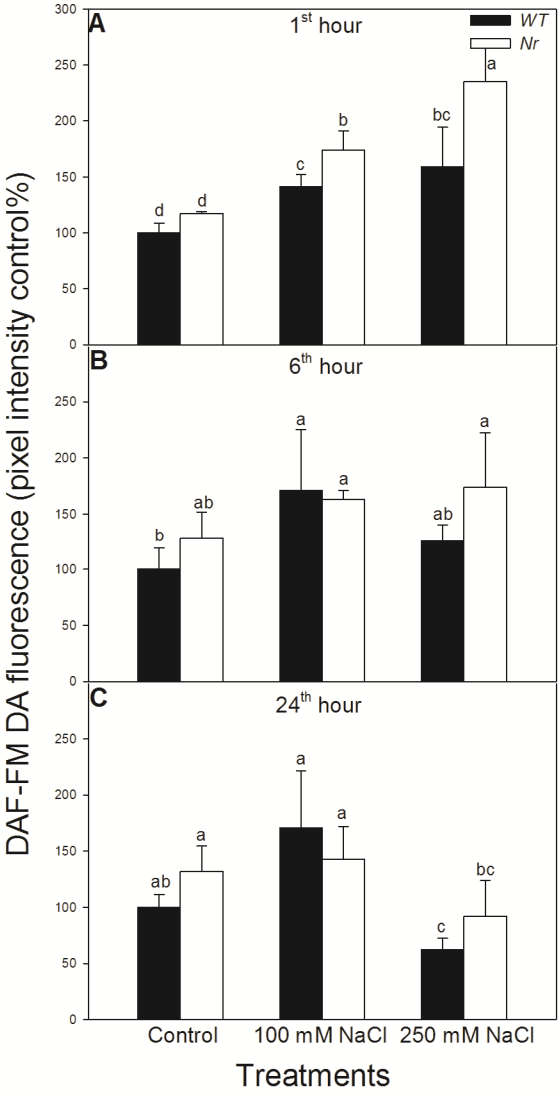
Elements (mg gDW^{-1})	Treatments					
	Control		100 mM NaCl		250 mM NaCl	
	WT	*Nr*	WT	*Nr*	WT	*Nr*
K^+	$23.80+2.50^{b}$	$17.60+3.75^{b}$	$39.44+7.33^{a}$	$12.43+3.13^{c}$	$9.66+1.29^{c}$	$6.03+0.70^{c}$
Na^+	$14.16+0.14^{d}$	$12.80+1.75^{d}$	$25.33+1.73^{b}$	$20.62+0.30^{c}$	$27.93+0.94^{a}$	$26.16+1.36^{ab}$
K^+/Na^+	$1.68+0.17^{a}$	$1.36+0.12^{b}$	$1.55+0.18^{ab}$	$0.60+0.16^{c}$	$0.35+0.06^{d}$	$0.23+0.01^{d}$

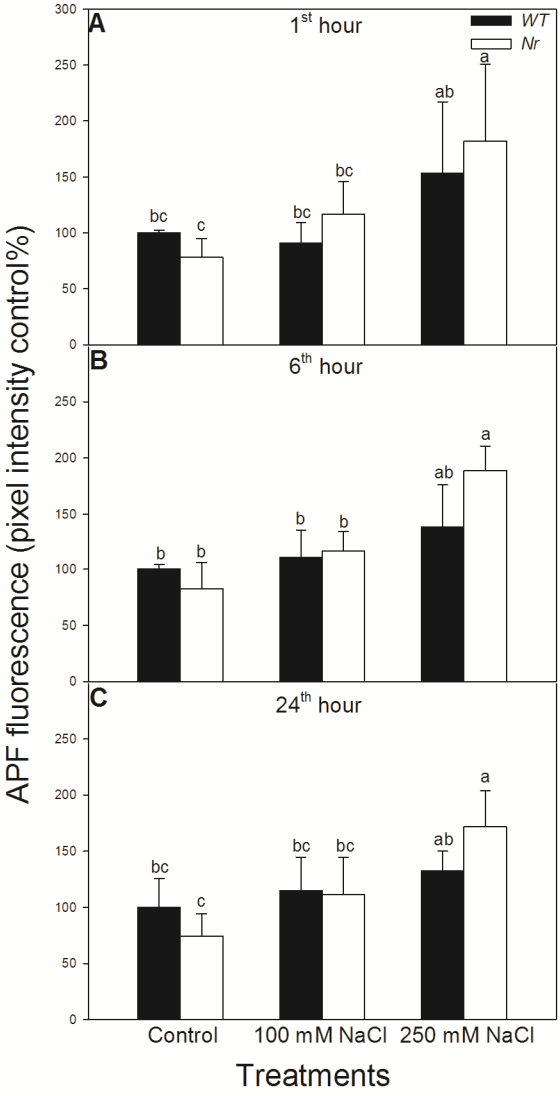
(Means ± SD, n=6). Bars with different letters are significantly different at 0.05 levels (Duncan's multiple range test).

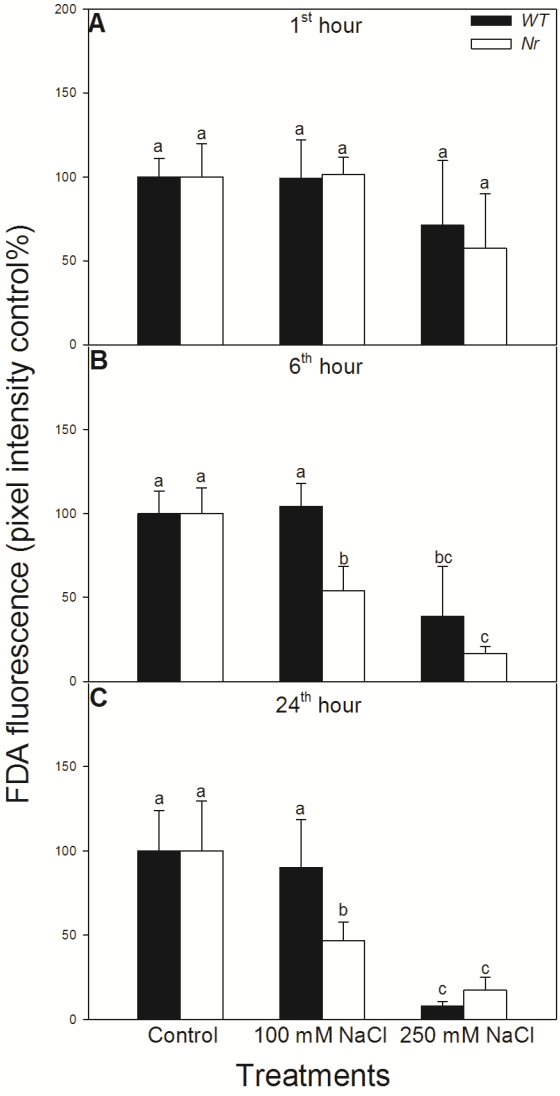


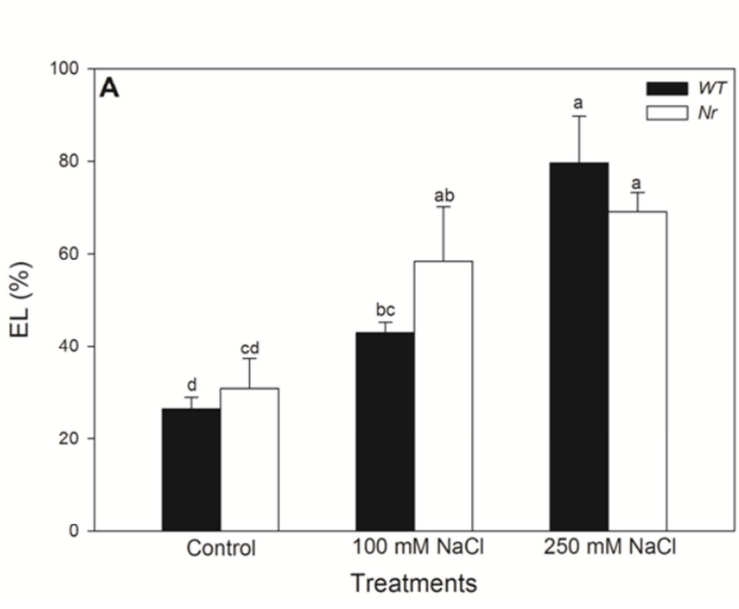


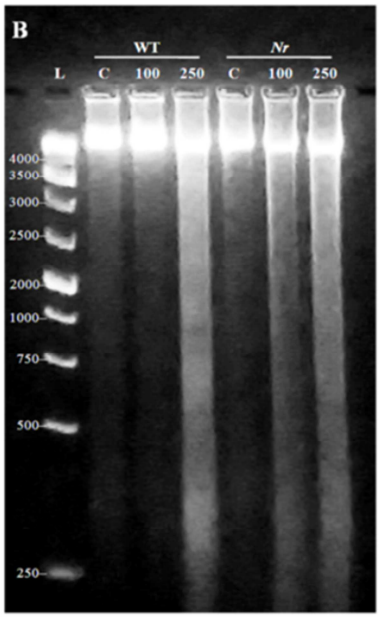


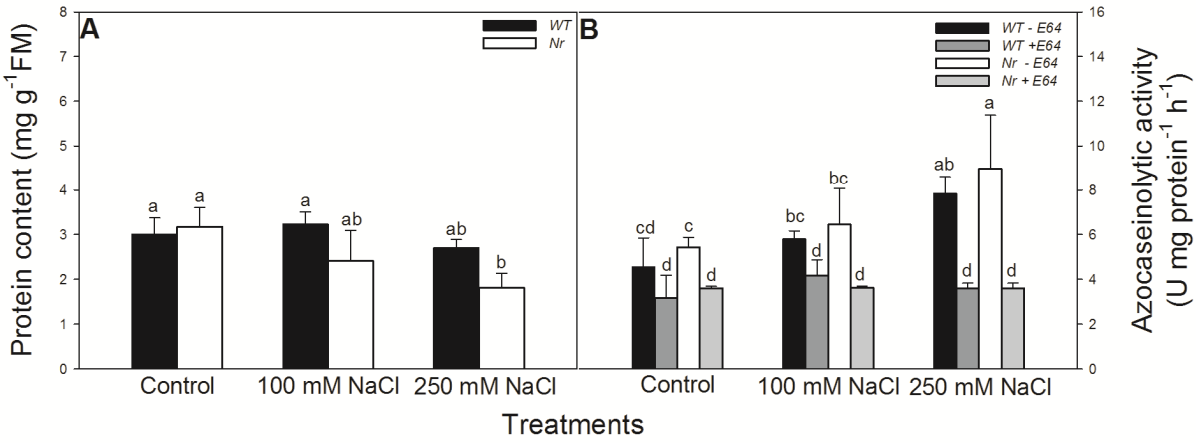












Highlights

- *Never ripe* gene encodes a mutant ethylene receptor in tomato.
- *Never ripe* mutants are more sensitive to salt stress than the wild types.
- The mutants showed higher superoxide and NO accumulation in the root tips.
- Root tip cells in *Nr* plant exhibited programmed cell death at moderate salt stress.
- Due to high K^+ loss from roots the mutants were subjected to stronger ionic stress.

Contributions

IT and PP designed the experiments, analyzed the data and wrote the manuscript. PP and PB conducted the experiments with fluorescence microscope, JK, PB, ZT and ÁSZ measured the activity of cysteine proteases and PCD induction. Figures were designed and created by PP.