



Research article

H₂O₂ homeostasis in wild-type and ethylene-insensitive *Never ripe* tomato in response to salicylic acid treatment in normal photoperiod and in prolonged darkness

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ABSTRACT

Ethylene proved to be an important modulator of salicylic acid (SA) signalling pathway. Since SA may regulate both the production and scavenging of hydrogen peroxide (H₂O₂), which show light-dependency, the aim of this study was to compare H₂O₂ metabolism in the leaves of SA-treated wild-type (WT) tomato (*Solanum lycopersicum* L. cv. Ailsa Craig) and in ethylene receptor *Never-ripe* (*Nr*) mutants grown in normal photoperiod or in prolonged darkness. H₂O₂ accumulation was higher in the WT than in the mutants in normal photoperiod after 1 mM SA treatment, while *Nr* leaves contained more H₂O₂ after light deprivation. The expression of certain superoxide dismutase (SOD) genes and activity of the enzyme followed the same tendency as H₂O₂, which was scavenged by different enzymes in the two genotypes. Catalase (CAT, EC 1.11.1.6) activity was inhibited by SA in WT, while the mutants maintained enhanced enzyme activity in the dark. Thus, in WT, CAT inhibition was the major component of the H₂O₂ accumulation elicited by 1 mM SA in a normal photoperiod, since the expression and/or activity of ascorbate (APX, EC 1.11.1.11) and guaiacol peroxidases (POD, EC 1.11.1.7) were induced in the leaves. The absence of APX and POD activation in mutant plants suggests that the regulation of these enzymes by SA needs functional ethylene signalling. While the block of ethylene perception in *Nr* mutants was overwritten in the transcription and activity of certain SOD and CAT isoenzymes during prolonged darkness, the low APX and POD activities led to H₂O₂ accumulation in these tissues.

1. Introduction

The plant hormone, salicylic acid (SA) plays an important role in the signal transduction pathways of abiotic and biotic stress responses (Vlot et al., 2009). During this process SA interacts with other principal defence hormones such as jasmonic acid (JA) and ethylene (ET) making the hormonal homeostasis and signalling cross-talk crucial for the fine regulation of defence mechanisms (Koornneef and Pieterse, 2008). In the case of biotic stress SA is generally implicated in the defence against biotrophic pathogens, whereas JA/ET mediate plant responses to insects or necrotrophic pathogens (Glazebrook, 2005).

SA and ET are involved in both synergistic and antagonistic interactions in several physiological processes. SA was found to inhibit the ET synthesis in tomato (Li et al., 1992) but to promote ET accumulation

in *Arabidopsis* (Rao et al., 2002) and in *Solanum chilense* (Gharbi et al., 2016) in response to O₃ exposure and salt stress, respectively. Moreover, ET induced the accumulation of SA in tomato plant infected with the necrotrophic pathogen *Xanthomonas campestris* (O'Donnell et al., 2001).

The rapid production and accumulation of reactive oxygen species (ROS), such as singlet oxygen, superoxide anion radical, hydroxyl radical and hydrogen peroxide, are generated by SA and ET in response to stress. ROS, especially H₂O₂, can act together with SA or ET in a self-amplifying feedback loop where SA and/or ET induce H₂O₂ accumulation, which then enhances the accumulation of these hormones (Wi et al., 2010; Xia et al., 2015). The biotic and abiotic stress responses of plants are often accompanied by biphasic ROS (Apel and Hirt, 2004; Wi et al., 2010), H₂O₂ (Takács et al., 2017) or ET production (Wi et al.,

Abbreviations: AC, Ailsa Craig; ACC, 1-aminocyclopropane-1-carboxylic acid; APX, ascorbate peroxidase; CAT, catalase; EL, electrolyte leakage; ET, ethylene; HR, hypersensitive response; JA, jasmonic acid; *Nr*, *Never ripe*; POD, guaiacol dependent peroxidase; ROS, reactive oxygen species; SA, salicylic acid; SAR, systemic acquired resistance; SOD, superoxide dismutase

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2010). In the case of defence responses, it was found that increases in SA levels are preceded by apoplastic H_2O_2 bursts mediated by plasma membrane-localized NADPH oxidases and by extracellular peroxidases (PODs) (Mammarella et al., 2015) but both abiotic and biotic stress factors can also generate ROS in other compartments such as chloroplasts and peroxisomes (Apel and Hirt, 2004).

A number of reports indicated that SA not only plays a pro-oxidant role, but also has an antioxidant role in concert with glutathione (GSH), and controls cellular redox homeostasis through the regulation of antioxidant enzyme activities under stress conditions (Herrera-Vásquez et al., 2015; Xia et al., 2015). SA may increase the activity of superoxide dismutase (SOD, EC 1.15.1.1), the main scavenger of superoxide radicals, which catalyses the conversion of superoxide to H_2O_2 in different cell compartments (Rao et al., 1997). Among the isoforms of SOD, Mn-SOD is localized in the mitochondria and peroxisomes, Fe-SOD in the chloroplast and Cu/Zn-SOD in the chloroplasts, peroxisomes, cytosol and apoplast. SA inhibits the activity of certain catalase isoenzymes (CAT, EC 1.11.1.6), cytosolic ascorbate peroxidase (APX, EC 1.11.1.11) and guaiacol peroxidases (POD, EC 1.11.1.7), the main scavengers of H_2O_2 in plant cells (reviewed by Horváth et al., 2007; Rivas-San Vicente and Plasencia, 2011; Herrera-Vásquez et al., 2015).

Biphasic ROS production is also accompanied by a biphasic shift in cell redox potential in tissues treated with SA. An initial oxidative phase, characterized by a transient increase in ROS levels and a decline in GSH reducing power, is followed by a reductive phase characterized by increased GSH levels and reducing power. These temporal phases determine the sequential activation of redox-regulated processes, among them the transcription of defence genes (Herrera-Vásquez et al., 2015). SA-promotes the redox modification of specific Cys residues and the monomerisation of the oligomeric form of the NPR1 (NON-EXPRESSOR OF PR GENES 1) transcription coactivator, a central compound of SA signalling, which is transported to the nucleus and interacts with certain TGA transcription factors (TGA2 and TGA3), the regulators of SA-dependent late genes (e.g. *PATHOGENESIS RELATED 1*, *PR1* gene) (Pape et al., 2010). Class II TGA transcription factors (TGA2/5/6) are also essential regulators in SA-induced gene expression, but they mediate the NPR1-independent induction of early defence genes including antioxidant enzymes (Blanco et al., 2009).

It was found that ET could also regulate the ROS metabolism by modulating antioxidant enzymes. Pre-treatment with the ET precursor 1-aminocyclopropane-1-carboxylic acid (ACC) enhanced SOD, APX and POD activities in *Agrostis stolonifera* under heat stress (Larkindale and Huang, 2004). Moreover, treatment with 1-methylcyclopropene, which blocks the effects of ET by inhibiting the ethylene receptor, decreased the POD and CAT activities in plum (Kan et al., 2011).

ET is sensed by multiple receptors (e.g. ETR1, ERS1 in *Arabidopsis*) localized to the endoplasmic reticulum and Golgi membranes. Genetic analysis has shown that these receptors function as negative regulators of the ET signalling pathway and actively suppress ET responses in the absence of ET. CTR1, a Ser/Thr protein kinase, acts downstream of the receptors and is also a negative regulator of ET signalling. Once ET is bound, suppression is cancelled, permitting the response to occur through the activation of positive regulators, including ETHYLENE INSENSITIVE2 (EIN2), a member of the NRAMP metal-ion transporter family, and the transcription factors ETHYLENE INSENSITIVE3 (EIN3), EIN3-like (EIL) and ERFs, which directly regulate the primary and secondary ethylene response genes, respectively. EIN3 directly up-regulates *ERF1* expression by binding to the primary ethylene response elements (PERE) of the *ERF1* promoter (Cheng et al., 2013).

ET receptor and signalling mutants make it possible to analyse not only the molecular steps of signalling but also the physiological functions of ET (Poór et al., 2015). In tomato there are seven ET receptors, LeETR 1, 2, 4 to 7, and *Never ripe* (*Nr*), five of which were shown to bind ET with high affinity (Kamiyoshihara et al., 2012). LeETR3 of tomato is referred to as *Nr* for historical reasons, and shows high homology with the *Arabidopsis* ET receptor ETR1 (Zhong et al., 2008). The *Nr* mutant

was described as a dominant mutation due to a single base substitution in the N terminal coding region. It shows insensitivity to ET not only during fruit ripening but in vegetative tissues as well, in the triple response, in leaf petiole epinasty, in senescence of petals and flower abscission. Nevertheless, *Nr* plants maintained high ET production after pathogen infection, indicating that the mutation did not affect ET biosynthesis (Lanahan et al., 1994).

The illumination of plant tissues has been shown to have a strong influence on hormone-regulated defences. Plants infected in the dark showed reduced systemic acquired resistance (SAR) (Karpiński et al., 2003) and lesion formation in response to avirulent pathogens (Zeier et al., 2004). The accumulation of SA, SA-mediated oxidative stress (Chandra-Shekara et al., 2006; Poór et al., 2017), ET biosynthesis (Rodrigues et al., 2014) and signalling are also dependent on the presence of light (Liebsch and Keech, 2016).

Light may either stimulate or inhibit ET production, depending on the plant species, tissue and developmental phase (Bassi and Spencer, 1983). It is also well known that keeping leaf tissues in the dark induced the accumulation of ET, the breakdown of chlorophyll and carbohydrates or the recycling of chloroplast proteins and activated dark-induced senescence (Lim et al., 2007). Since the light-driven electron transport chain in the chloroplasts also generates ROS, the production and physiological role of various forms of ROS may differ in illuminated or dark environments (Xing et al., 2013).

The allocation of resources between growth and defence under stress conditions is critical for the survival of photosynthesizing plants. Thus the activation of growth or plant defence against pathogens and insects needs fine interaction between SA and ET signalling (Ballaré, 2014). These processes are controlled by light, sensed by red light receptors, phytochromes, which interact with both SA- and ET-induced signalling and oxidative stress.

The aim of this study was to elucidate how the SA-dependent oxidative stress is modulated in WT and, in the absence of ET signalling, in ET receptor mutant *Nr* tomato plants grown under a 12/12 h light/dark photoperiod or in prolonged darkness. Since SA proved to have different effects on H_2O_2 homeostasis and on the activation of antioxidant enzymes in the presence or absence of light in WT tomato plants (Poór et al., 2017), the question arose of whether the SA-dependent H_2O_2 metabolism was controlled by the same or by different series of antioxidant isoenzymes located in different compartments in WT plants and *Nr* mutants under the two types of light conditions.

2. Materials and methods

2.1. Plant material and growth conditions

Wild-type and ET receptor mutant *Nr* tomato plants (*Solanum lycopersicum* L. cv. Ailsa Craig) were grown in a controlled environment with 24 °C/22 °C day/night temperature, 55–60% relative humidity, 12/12 h day/night cycle, and 200 $\mu\text{mol m}^{-2} \text{s}^{-1}$ photon flux density (F36W/GRO lamps, Sylvania, Germany) in plastic boxes (40 cm length, 30 cm width, 20 cm depth, 12 seedlings per box) filled with 20 L of nutrient solution containing 2 mM $\text{Ca}(\text{NO}_3)_2$, 1 mM MgSO_4 , 0.5 mM KCl, 0.5 mM KH_2PO_4 and 0.5 mM Na_2HPO_4 at pH 6.2. The concentrations of micronutrients were 0.001 mM MnSO_4 , 0.005 mM ZnSO_4 , 0.0001 mM $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}$, 0.01 mM H_3BO_4 and 0.02 mM Fe(III)-EDTA. The culture medium was changed twice a week. Eight-week-old plants were treated by adding 0.1 mM or 1 mM SA to the nutrient solution for 24 h, half of the plants remaining under the same 12/12 h light/dark period and conditions and half of them being placed in a darkroom at the same temperature and humidity for the next 24 h. Samples were prepared in three replicates from the second, fully expanded young leaves after 24 h. The experiments were repeated 3–4 times.

2.2. Determination of salicylic acid content

The extraction and determination of methanol-soluble free and bound SA were performed on 0.5 g of leaf tissue as described by Poór et al. (2017) following the procedure of Pál et al. (2005). The organic phases containing free and bound SA were evaporated to dryness under a vacuum and resuspended in 1 mL of the HPLC initial mobile phase. Free and bound SA were separated using high-performance liquid chromatography (HPLC) on a reverse-phase column (Supelcosil ABZ Plus, 5 μ m 5 μ ; 150 mm \times 4.6 mm) at 25 °C using a 2690 separation module equipped with a W474 scanning fluorescence detector (WATERS, Milford, MA, USA) and monitored with excitation at 305 nm and emission at 407 nm.

2.3. Measurement of ethylene production

Samples of 0.5 g leaf tissue were taken after SA treatment and were incubated in gas-tight flasks fitted with rubber stoppers. After 1 h 2.5 mL of the gas was removed from the tubes with a gas-tight syringe and injected into the gas chromatograph. The production of ET was measured with a Hewlett-Packard 5890 Series II gas chromatograph equipped with a flame ionization detector and a column packed with activated alumina, as described by Poór et al. (2015).

2.4. Determination of H₂O₂ levels and the integrity of the plasma membrane

The H₂O₂ level was measured spectrophotometrically as described by Takács et al. (2016). After the homogenization of 0.2 g of leaf tissue with 1 mL of ice-cold, 0.1% trichloroacetic acid, the samples were centrifuged at 10 000 g for 20 min at 4 °C. The reaction mixture contained 0.25 mL of 10 mM phosphate buffer (pH 7.0), 0.5 mL of 1 M potassium iodide (KI) and 0.25 mL of the supernatant. The absorbance of the samples was measured after 10 min at 390 nm. The amount of H₂O₂ was calculated using a standard curve prepared from H₂O₂ stock solution. 3,3'-diaminobenzidine (DAB) staining was used to detect the production of H₂O₂ *in situ*, which was carried out according to Thordal-Christensen et al. (1997). Detached leaves from SA-treated tomato plants were incubated for 2 h in darkness in 2 mg/mL DAB solution at room temperature. After incubation the leaves were immersed in 96% (v/v) ethanol to eliminate the chlorophyll content completely and then they were incubated in 50% (v/v) glycerol solution. To detect the intensity of the dye color digital camera (Sony Cyber-shot DSC-H9, Sony Co., Tokyo, Japan) was used.

Disintegration of cell membranes was determined by the measurement of electrolyte leakage. 0.1 g of fresh leaf tissues was incubated in 25 mL of double distilled water at 25 °C for 2 h. The conductivity in the bathing solution was determined (C1) with conductivity meter (Radelkis, Hungary). Then, the samples were heated at 95 °C for 40 min and the total conductivity (C2) of the cooled samples was measured. Relative EL was expressed as EL (%) = (C1/C2) \times 100 (Kovács et al., 2016).

2.5. RNA extraction and expression analysis with real-time RT-PCR

Gene expression analysis was performed following the description of Takács et al. (2016) using quantitative real-time reverse transcription-PCR (qRT-PCR; Piko Real-Time qPCR System, Thermo Scientific). Tomato genes were mined from the Sol Genomics Network (SGN; <http://solgenomics.net/>, accessed 17 January 2018) and National Center for Biotechnology Information (NCBI; <http://www.ncbi.nlm.nih.gov/>, accessed 17 January 2018) databases. Primers were designed using NCBI and Primer 3 softwares (<http://frodo.wi.mit.edu/>, accessed 17 January 2018) and are listed in Supplementary Table 1. The PCR reaction consisted of 10 ng cDNA template, 400 nM each of forward and reverse primers, 5 μ L of Maxima SYBR Green qPCR Master Mix (2X) (Thermo Scientific) and nuclease-free water in a total volume of 10 μ L.

The qRT-PCR was conducted at 95 °C for 7 min, followed by 40 cycles at 95 °C for 15 s and annealing extension at 60 °C for 60 s. A melting curve analysis of the product was performed (increasing the temperature from 55 to 90 °C, 0.2 °C s⁻¹) to determine the specificity of the reaction. Data analysis was made using PikoReal Software 2.2 (Thermo Scientific). Tomato 18S rRNA and elongation factor-1 α subunit genes were used as the reference genes and the 2^(- $\Delta\Delta C_t$) formula was used to calculate data from the qRT-PCR. Each reaction was repeated at least three times.

2.6. Extraction and analysis of antioxidant enzymes

To analyse the enzyme activities, 0.5 g leaf tissue was homogenised on ice in 1 mL of 100 mM phosphate buffer (pH 7.0) containing 1 mM phenylmethylsulfonyl fluoride and 1% (w/v) polyvinyl-pyrrolidone and the extracts were centrifuged at 12 000 g for 20 min at 4 °C. APX was extracted in the presence of 1 mM ascorbate (AsA). The supernatant was used for measuring enzyme activity with a dual-beam spectrophotometer (KONTRON, Milano, Italy) as described by Poór et al. (2017). The enzyme activities were expressed as U mg⁻¹ protein. SOD (EC 1.15.1.1) activity was determined by measuring the ability of the enzyme to inhibit the photochemical reduction of nitroblue tetrazolium (NBT) in the presence of riboflavin in the light. One enzyme unit (U) of SOD corresponds to the amount of enzyme causing 50% inhibition of NBT reduction in the light. CAT (EC 1.11.1.6) activity was determined from the absorbance change at 240 nm based on the amount of decomposed H₂O₂ (ϵ_{240} = 39.4 mM⁻¹ cm⁻¹). One unit of CAT is defined as the amount of enzyme necessary to decompose 1 μ mol min⁻¹ H₂O₂. APX (EC 1.11.1.11) activity was measured by monitoring the decrease in AsA content at 290 nm (ϵ_{290} = 2.8 mM⁻¹ cm⁻¹). One unit of APX activity is defined as the amount of enzyme necessary to oxidize 1 μ mol min⁻¹ AsA. POD (EC 1.11.1.7) activity was measured at 470 nm (ϵ_{470} = 26.6 mM⁻¹ cm⁻¹). The amount of enzyme producing 1 μ mol min⁻¹ of oxidised guaiacol was defined as one U. The soluble protein concentration was determined according to Bradford (1976) using bovine serum albumin as a standard. All chemicals were purchased from Sigma-Aldrich, Darmstadt, Germany.

2.7. Native PAGE and enzyme activity staining

Leaf samples weighing 0.5 g were crushed to a fine powder in a mortar under liquid N₂, after which soluble proteins were extracted by resuspending the powder in two volumes of 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM phenylmethylsulfonyl fluoride. For APX, 2 mM AsA was added to the enzyme extraction buffer. The homogenate was centrifuged at 12 000 g for 20 min at 4 °C.

The electrophoretic separation of SOD, APX and POD isoenzymes was performed with native polyacrylamide gel electrophoresis (PAGE) using an omniPAGE electrophoresis system (Cleaver Scientific Ltd., Rugby, Warwickshire, UK). Electrophoresis was performed at 4 °C for 1–3 h at a constant voltage of 120 V using 25 mM Tris and 192 mM glycine solution (pH 8.3) as a running buffer. For SOD and APX, 4% stacking and 10% resolving polyacrylamide gels were used, whereas for POD, 3% and 6% stacking and resolving gels were used, respectively. Samples were mixed with 62.5 mM Tris-HCl buffer (pH 6.8), containing 10% (v/v) glycerol and 0.025% (w/v) bromophenol blue before loading onto the gels. Equal amounts (30 μ g) of protein were loaded for the SOD, APX and POD determinations and after electrophoresis the gels were stained for enzyme activities according to well-established protocols, as indicated.

SOD was stained according to the method described by Monteiro et al. (2011). The APX and POD activities were detected according to Kim et al. (2015) and García-Limones et al. (2002), respectively.

2.8. Statistical analysis

The results are expressed as means \pm S.E. After analyses of

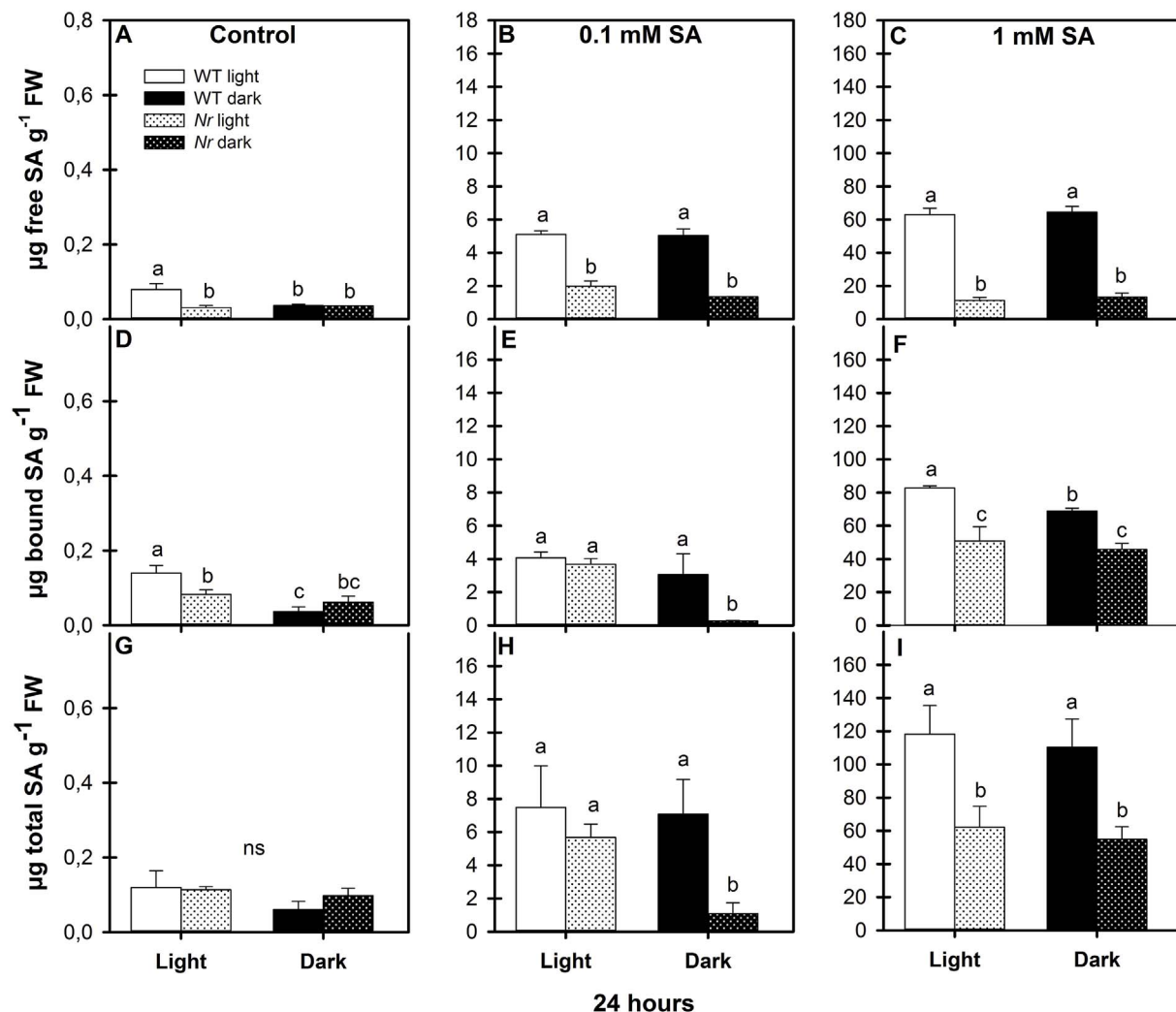


Fig. 1. Changes in free, bound and total methanol-soluble salicylic acid (SA) content in the leaves of wild type (WT, open columns) and ethylene receptor mutant *Never ripe* (*Nr*, latticed columns) tomato plants treated with 0.1 mM or 1 mM SA for 24 h in the presence or absence of light (□ Light, ■ Dark). Control: A, D, G; 0.1 mM SA: B, E, H; 1 mM SA: C, F, I. Results are means \pm SE from at least three independent experiments. Data with different letters indicate significant differences ($P \leq 0.05$, $n = 3$).

variance (ANOVA) a multiple comparison followed by Tukey's test was performed with SigmaPlot version 12 software (SYSTAT Software Inc. SPSS). The means were treated as significant if $P \leq 0.05$. All the experiments were carried out at least three times. In each treatment at least three samples were measured.

3. Results

3.1. Effects of SA treatment on endogenous SA content and ET production

To detect the exogenous SA-induced physiological responses in the leaves of WT and *Nr* mutants, the accumulation of free and bound (as well as total) methanol-soluble SA was measured in tomato plants treated with 0.1 or 1 mM exogenous SA in an illuminated or dark environment. Under control growth conditions, the free SA content was about $0.1 \mu\text{g g}^{-1}$ fresh mass in illuminated WT leaves and less than $0.05 \mu\text{g g}^{-1}$ fresh mass in prolonged darkness (Fig. 1A). While the free and bound SA contents were lower in *Nr* leaves in the light, they were equal to those of WT samples in the dark (Fig. 1A, D). Free SA contents were significantly increased to 50-fold by 0.1 mM SA and to 600-fold by 1 mM SA treatment compared with the control in WT under both environmental conditions (Fig. 1B and C). However, the free SA content increased only slightly in *Nr* leaves after SA treatment and, unexpectedly, was always lower than in the corresponding WT leaves

(Fig. 1B and C). In accordance with our earlier findings, free SA contents were not significantly different in the WT (Poór et al., 2017) or in the mutant plants after SA treatments in normal photoperiod or in prolonged darkness. Unlike free SA, the amount of bound and total SA in *Nr* mutants after 0.1 mM SA treatment was similar to that in WT in the light, but these fractions were significantly lower in mutant plants in prolonged darkness (Fig. 1E, H). In the presence of 1 mM SA, the fractions of bound and total SA in *Nr* leaves were much lower than in WT under the same conditions, but there were no significant differences in the total SA contents between light and dark conditions in either WT or *Nr* leaves (Fig. 1F, I).

The control leaves of *Nr* plants exhibited higher ET production than WT in both environments (Supplementary Fig. 1A). In the presence of 0.1 mM SA, ET production did not change significantly in the two genotypes under a normal photoperiod, but in the dark the WT leaves emitted more ET and the *Nr* leaves less ET than their respective controls (Supplementary Fig. 1B). In the case of treatment with 1 mM SA, there was no significant change in ET production of WT plants in the light but a significant decrease was observed in *Nr* leaves. In prolonged darkness this resulted in similar tendencies in ET production after 1 mM SA treatment as under control conditions (Supplementary Fig. 1).

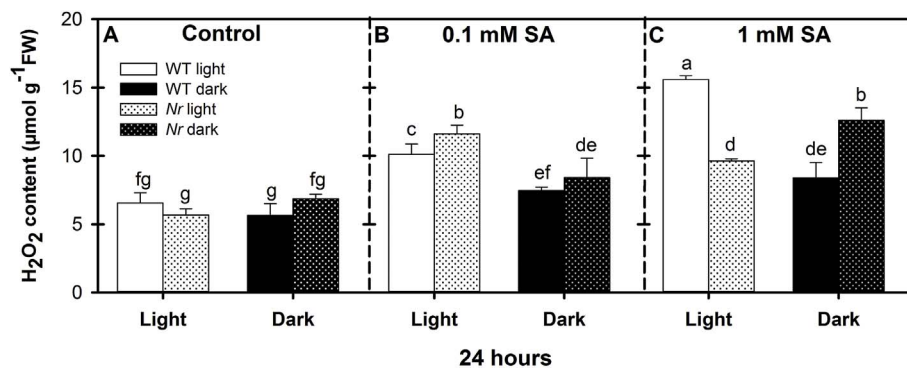


Fig. 2. Changes in H₂O₂ content in the leaves of wild type (WT, open columns) and ethylene receptor mutant *Never ripe* (*Nr*, latticed columns) tomato plants treated with 0.1 mM or 1 mM SA for 24 h in the presence or absence of light (□ Light, ■ Dark). Control: A; 0.1 mM SA: B; 1 mM SA: C. Results are means ± SE from at least three independent experiments. Data with different letters indicate significant differences ($P \leq 0.05$, $n = 3$).

3.2. Effect of SA treatment on hydrogen peroxide content and electrolyte leakage

To investigate the effect of ET signalling on SA-induced H₂O₂ accumulation, the H₂O₂ content in the leaves was recorded. There were no significant differences between the control of WT and *Nr* mutants under either light or dark conditions (Fig. 2A). However, H₂O₂ accumulated in response to both SA treatments in the light, in both genotypes increasing with the SA concentration in WT plants, but reaching a maximum at 0.1 mM SA in *Nr* plants. In prolonged darkness there was a gradual increase in the H₂O₂ content of the tissues as a function of SA concentration in both genotypes. After 1 mM SA treatment *Nr* leaves contained less than 62% of the H₂O₂ content of WT in a normal photoperiod, but much higher amount, 150% of the respective WT in prolonged darkness (Fig. 2B and C and Supplementary Fig. 2). Since H₂O₂ is a major component of oxidative stress caused by SA, electrolyte leakage (EL), which indicates the loss of plasma membrane integrity, was determined. It was found that EL followed the tendency obtained for H₂O₂ accumulation. In the leaves of illuminated WT 1 mM SA caused significant, 40% increase in EL compared to untreated control, while *Nr* mutants displayed only 17% increment (Fig. 3). Conversely, in the absence of light, EL was significantly lower in WT than in *Nr*, which suggested that *Nr* plants were more sensitive to SA under prolonged darkness than the WT, while WT plants were more sensitive than *Nr* in normal photoperiod.

3.3. Effect of SA treatment on antioxidant enzyme activities and on the expression of their coding genes

The SA-induced H₂O₂ metabolism is mediated by various antioxidant enzymes. In order to determine whether antioxidant enzyme activity and gene expression were differentially regulated by 0.1 mM or 1 mM SA in the ET receptor mutant *Nr* and WT in the presence or absence of light, the activities and transcript levels of genes encoding SOD (*SiMnSOD*, *SiFeSOD*, *SiCu/ZnSOD*), CAT (*SiCAT1*, *SiCAT2*, *SiCAT3*) and APX (*SiAPX1*, *SiAPX2*) together with the activity of POD were

determined.

3.3.1. Superoxide dismutase

Three SOD genes from the tomato genome, the mitochondrial MnSOD, the chloroplastic FeSOD and Cu/ZnSOD, were used in the experiments. The transcripts levels of these genes were not significantly different in WT and *Nr* plants under a normal photoperiod. However, *SiMnSOD* was down-regulated, the expression of *SiCu/ZnSOD* was induced and the expression of the *SiFeSOD* gene remained at a constant level in the leaves of WT controls after 24 h of darkness. Surprisingly, all the SOD coding genes were suppressed in *Nr* mutants in the dark (Fig. 4A, D, G). No significant differences were observed in the presence of 0.1 mM SA compared to the control in either WT or *Nr* plants, except that *SiFeSOD* and *SiCu/ZnSOD* showed down-regulation in prolonged darkness in WT (Fig. 4B, E, H). In contrast, a very strong induction of *SiMnSOD* and *SiCu/ZnSOD* transcription was observed after 1 mM SA treatment in illuminated WT leaves, while the transcription of *SiFeSOD* remained constant (Fig. 4C, F, I). Unexpectedly, one chloroplastic enzyme, *SiCu/ZnSOD* was up-regulated in prolonged darkness to a greater extent in *Nr* leaves than in WT after 1 mM SA treatment (Fig. 4I).

In spite of the differences in gene expression, SOD activity did not differ in WT and *Nr* plants under control conditions in the presence or absence of light (Fig. 5A). The activity of SOD remained constant in *Nr* leaves under a normal photoperiod after 0.1 mM SA treatment, but decreased in WT leaves in both environments (Fig. 5B). However, SOD activity increased significantly after 1 mM SA treatment in illuminated WT leaves, but was significantly higher in *Nr* than in WT leaves in prolonged darkness (Fig. 5C). The changes followed similar pattern as H₂O₂ in leaf tissues, the specific activity in *Nr* extracts was 66% of WT in normal photoperiod and 140% in prolonged darkness.

Native PAGE analysis of SOD indicated the presence of five bands in both WT and *Nr* leaf extracts, although the first, less mobile band was hardly detectable (Fig. 5D). The first band could be identified as MnSOD, since it was not inhibited by either KCN or H₂O₂, the second band was characterized as FeSOD since it was negatively affected by H₂O₂, and the third, fourth and fifth bands were identified as Cu/

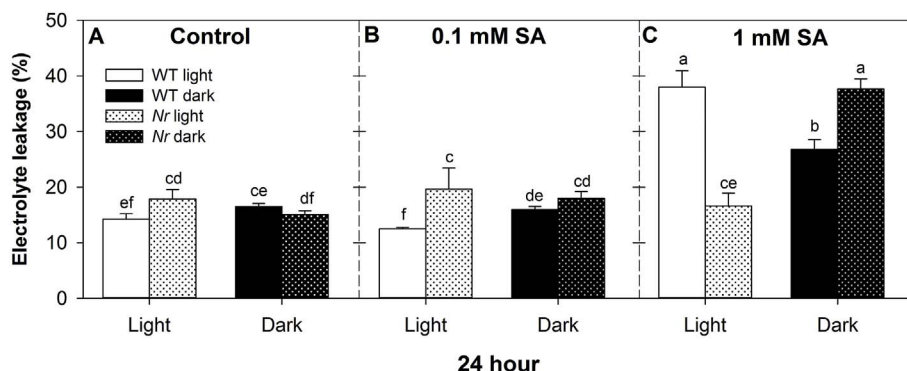


Fig. 3. Changes in the electrolyte leakage from the leaves of wild type (WT, open columns) and ethylene receptor mutant *Never ripe* (*Nr*, latticed columns) tomato plants treated with 0.1 mM or 1 mM SA for 24 h in the presence or absence of light (□ Light, ■ Dark). Control: A; 0.1 mM SA: B; 1 mM SA: C. Results are means ± SE from at least three independent experiments. Data with different letters indicate significant differences ($P \leq 0.05$, $n = 5$).

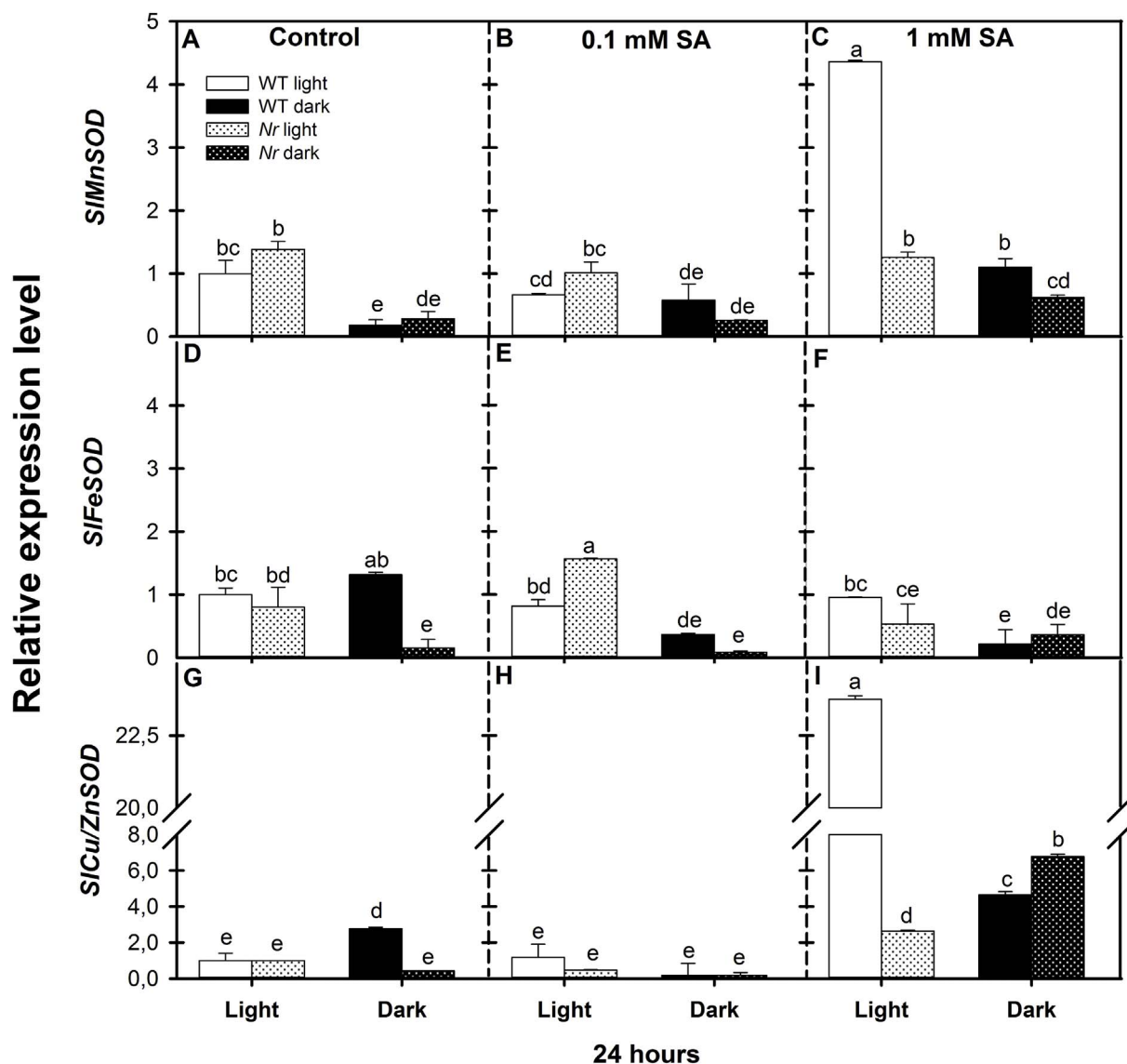


Fig. 4. Changes in relative expression levels of *SIMnSOD*, *SIFeSOD* and *SICu/ZnSOD* genes in the leaves of wild type (WT, open columns) and ethylene receptor mutant *Never ripe* (*Nr*, latticed columns) tomato plants treated with 0.1 mM or 1 mM SA for 24 h in the presence or absence of light (□ Light, ■ Dark). Control: A, D, G; 0.1 mM SA: B, E, H; 1 mM SA: C, F, I. Results are means \pm SE from at least three independent experiments. Data with different letters indicate significant differences ($P \leq 0.05$, $n = 3$).

ZnSODs, since they were inhibited by both inhibitors (data not shown). Based on the native PAGE, the Cu/ZnSODs (I, II, III) were the main SOD isoforms in WT and *Nr* leaves showing results similar to those observed for total SOD activity (Fig. 5A, B, C). Under dark conditions, the activity of SOD isoforms in *Nr* leaves was higher than in WT after 1 mM SA treatment (Fig. 5D).

3.3.2. Catalase

All the *SICAT* genes were less strongly expressed in the *Nr* leaves than in WT control plants in a normal photoperiod (Fig. 6A, D, G). In addition, *SICAT2* expression decreased significantly in the dark compared to illuminated samples in the case of WT (Fig. 6D). In the presence of SA, the transcript levels of *SICAT1* were suppressed, but the expression of *SICAT2* and *SICAT3* exhibited constant expression in WT leaves under a normal photoperiod. At the same time, the expression of CAT isoenzymes was low in *Nr* leaves and displayed only small changes after SA treatments in the light (Fig. 6). In darkness, SA inhibited the expression of the CAT genes or caused moderate changes in their transcript levels in WT, while in *Nr* leaves a significant induction was observed in the relative transcript levels of *SICAT1* at both

concentrations of SA and of *SICAT3* after treatment with 1 mM SA (Fig. 6B, C, I).

In accordance with previous findings SA caused a concentration-dependent, about 68% inhibition in CAT activity in the light, which was not so pronounced (48%) in *Nr* leaves, while the inhibiting effect of SA could only be observed in WT plants in prolonged darkness. Unexpectedly, SA inhibition was not manifested in *Nr* tissues in the dark, since the enzyme activity increased as a function of SA concentration (Fig. 7).

3.3.3. Ascorbate peroxidase

Besides CAT and POD, APX is the most important H_2O_2 scavenging enzyme in plant cells. In the dark, the levels of *SIAPX1* and *SIAPX2* transcripts in control leaves were suppressed both in WT and *Nr* compared to those in illuminated plants (Fig. 8A, D). SA up-regulated the APX genes and caused 2.6- and 3.1-fold higher expression of *SIAPX1* and *SIAPX2* transcripts, respectively, in WT leaves at the 1 mM concentration under a normal photoperiod, while the expression of *SIAPX* genes did not change at this SA concentration in illuminated *Nr* leaves compared to the control. In addition, the transcript levels of *SIAPX*

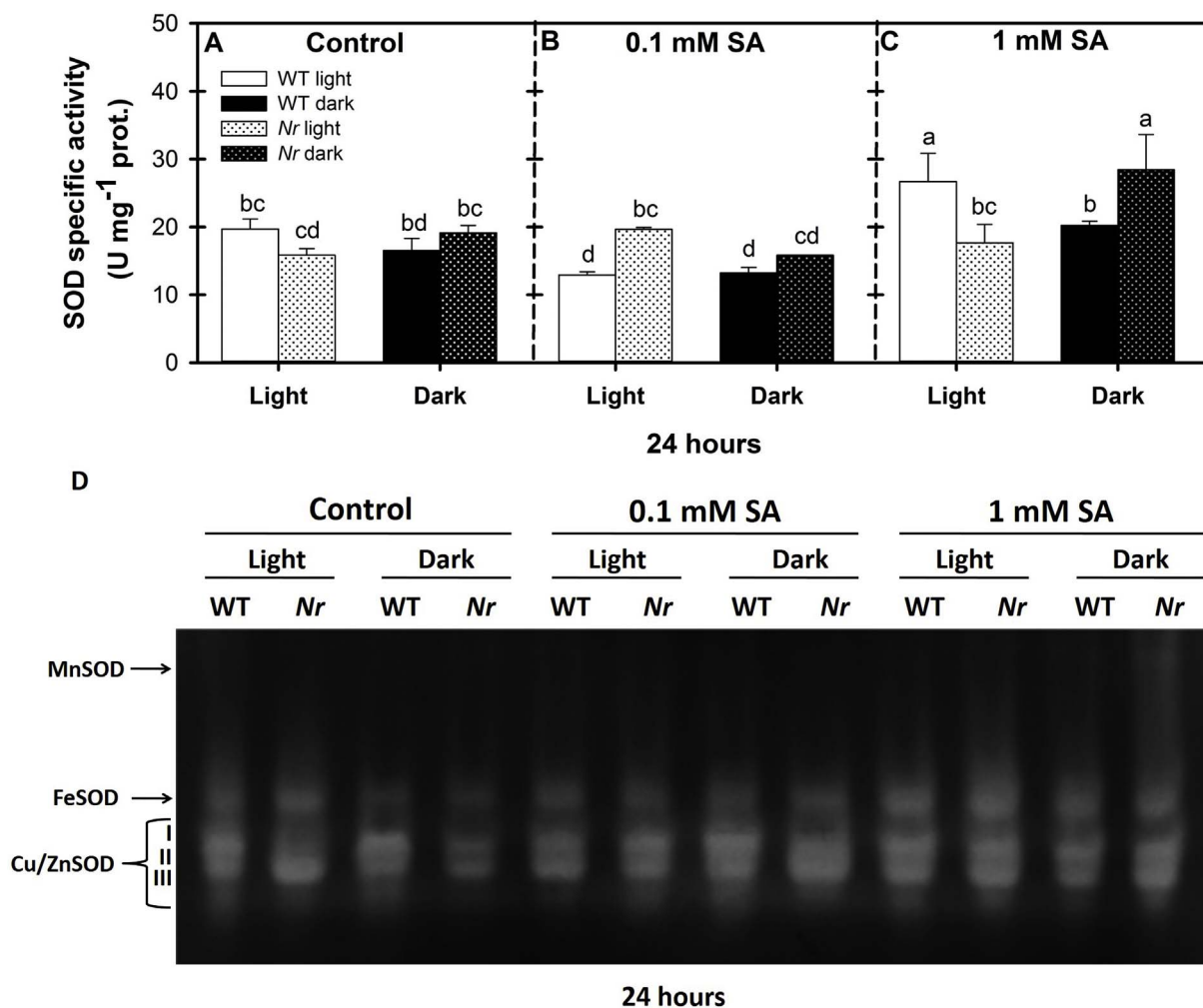


Fig. 5. Specific activity of superoxide dismutase (SOD) in the leaves of wild type (WT, open columns) and ethylene receptor mutant *Never ripe* (*Nr*, latticed columns) tomato plants treated with 0.1 mM or 1 mM SA for 24 h in the presence or absence of light (□ Light, ■ Dark). Control: A; 0.1 mM SA: B; 1 mM SA: C. Results are means \pm SE from at least three independent experiments. Data with different letters indicate significant differences ($P \leq 0.05$, $n = 3$). (D) Native PAGE analysis of SOD activity in the leaves of WT and *Nr* mutants. Separation of isoenzymes and activity staining were performed as described by Monteiro et al. (2011) using 30 μ g of protein per lane.

genes remained at the control level in the dark in both genotypes (Fig. 8).

APX activity, in correlation with the expression of APX genes, was generally higher in WT than in *Nr* leaves under both environmental conditions (Fig. 9A). Both SA treatments induced APX activity in WT and more moderately in *Nr* leaves in the light, while the enzyme activities remained at the control level in the dark (Fig. 9B and C).

Native PAGE analysis of APX indicated two isoforms in both WT and *Nr* leaves (Fig. 9D). The intensity of the bands was, in general, well correlated with the total enzyme activity of APX, assayed in the same samples (Fig. 9A, B, C). Increased APX bands were detected after SA treatment in both WT and *Nr* leaves in the light, but much lower activities were found in the dark (Fig. 9D).

3.3.4. Guaiacol dependent peroxidase

POD, the other important H_2O_2 metabolizing enzyme, was also determined (Fig. 10) and the results clearly showed that SA caused a concentration-dependent increase in POD activity in illuminated WT leaves, which was significantly lower in a prolonged dark period (Fig. 10B and C). On the other hand, the POD activity in the leaves of *Nr* mutants was substantially lower than in WT plants and the enzyme activity changed only slightly after SA treatment (Fig. 10B and C).

As far as the POD isoenzyme profile was concerned (Fig. 10D), two distinct isoforms were observed on the gels. The intensity of the POD

bands in different samples generally correlated well with the enzyme activities assayed previously in the same samples (Fig. 10A, B, C). The POD isoforms exhibited higher activity in WT leaves as a function of SA concentration, whereas the activity was always lower in *Nr* leaves.

4. Discussion

ET receptor *Nr* mutants of tomato make it possible to analyse the role of ET signalling and the functions of ET in SA-induced H_2O_2 production and metabolism. In previous work it was found that treating WT tomato (*Solanum lycopersicum* cv. Ailsa Craig) with 0.1 or 1 mM SA increased the free SA content in the leaves to 5–6 μ g g⁻¹ and ~60 μ g g⁻¹ fresh mass, respectively, which corresponds to the SA levels in the tissues in the systemic leaves or in tissues surrounding penetrating pathogens during hypersensitive response (HR) (Poór et al., 2017). Thus, the lower concentration of SA could lead to abiotic stress acclimation or SAR during biotic stress, while the higher concentrations can initiate cell death and necrotic lesions in leaf tissues. Unexpectedly, *Nr* plants showed lower basic SA contents, and exogenous SA treatment resulted in much lower free SA accumulation in the mutant leaves than in WT. These differences were not dependent on the presence of light. In spite of the low endogenous SA content in *Nr* leaves, ROS production and scavenging in the two genotypes were comparable, suggesting that SA may induce oxidative stress by diverse mechanisms.

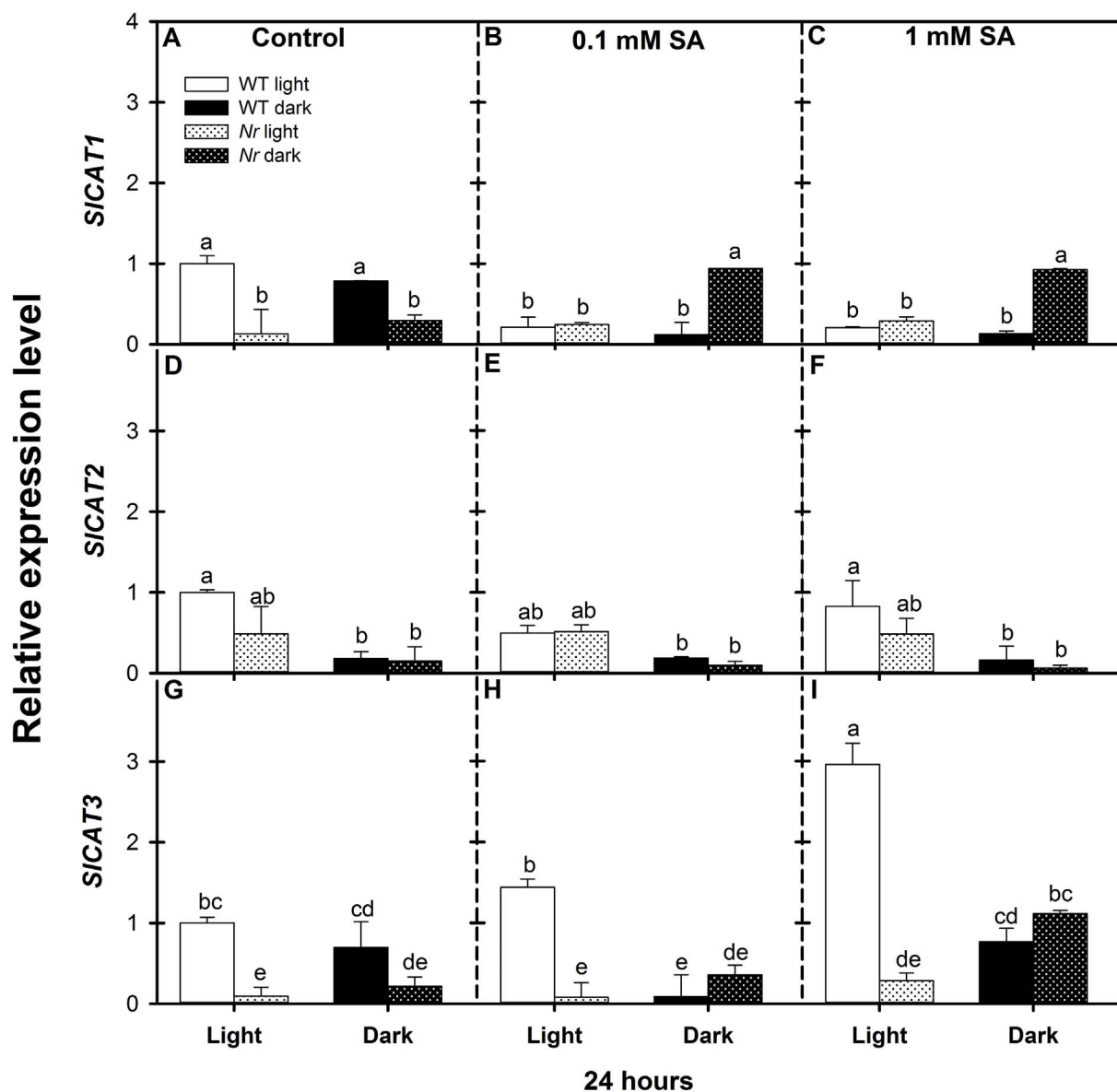


Fig. 6. Changes in relative expression levels of *SICAT1*, *SICAT2* and *SICAT3* genes in the leaves of wild type (WT, open columns) and ethylene receptor mutant *Never ripe* (*Nr*, latticed columns) tomato plants treated with 0.1 mM or 1 mM SA for 24 h in the presence or absence of light (□ Light, ■ Dark). Control: A, D, G; 0.1 mM SA: B, E, H; 1 mM SA: C, F, I. Results are means \pm SE from at least three independent experiments. Data with different letters indicate significant differences ($P \leq 0.05$, $n = 3$).

In accordance with the results of Lanahan et al. (1994) the present data confirmed that ET biosynthesis was not impaired in *Nr* plants. ET may either inhibit or promote its own biosynthesis. The control of ET synthesis by ET in vegetative tissues occurs via autoinhibition, while autocatalytic ET production can be observed during certain stress

conditions, senescence and fruit ripening (Rodrigues et al., 2014). Not surprisingly, in the absence of negative feedback due to the mutation of the most effective ET receptor, ET accumulation was significantly higher in *Nr* plants under both environmental conditions. Since in the present work dark treatment reduced the ET emanation from the tissues

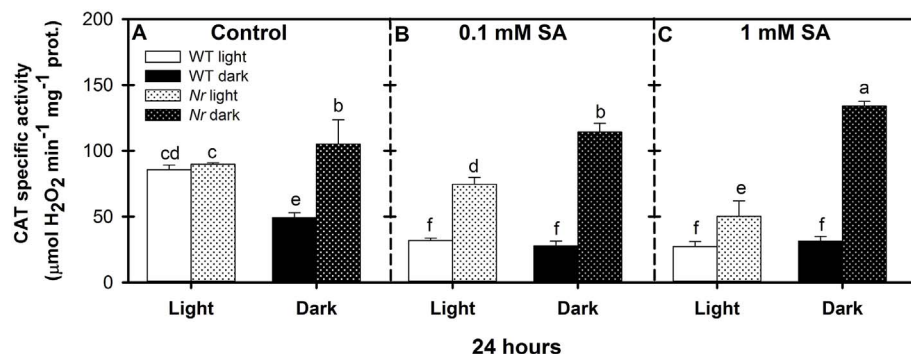


Fig. 7. Specific activity of catalase (CAT) in the leaves of wild type (WT, open columns) and ethylene receptor mutant *Never ripe* (*Nr*, latticed columns) tomato plants treated with 0.1 mM or 1 mM SA for 24 h in the presence or absence of light (□ Light, ■ Dark). Control: A; 0.1 mM SA: B; 1 mM SA: C. Results are means \pm SE from at least three independent experiments. Data with different letters indicate significant differences ($P \leq 0.05$, $n = 3$).

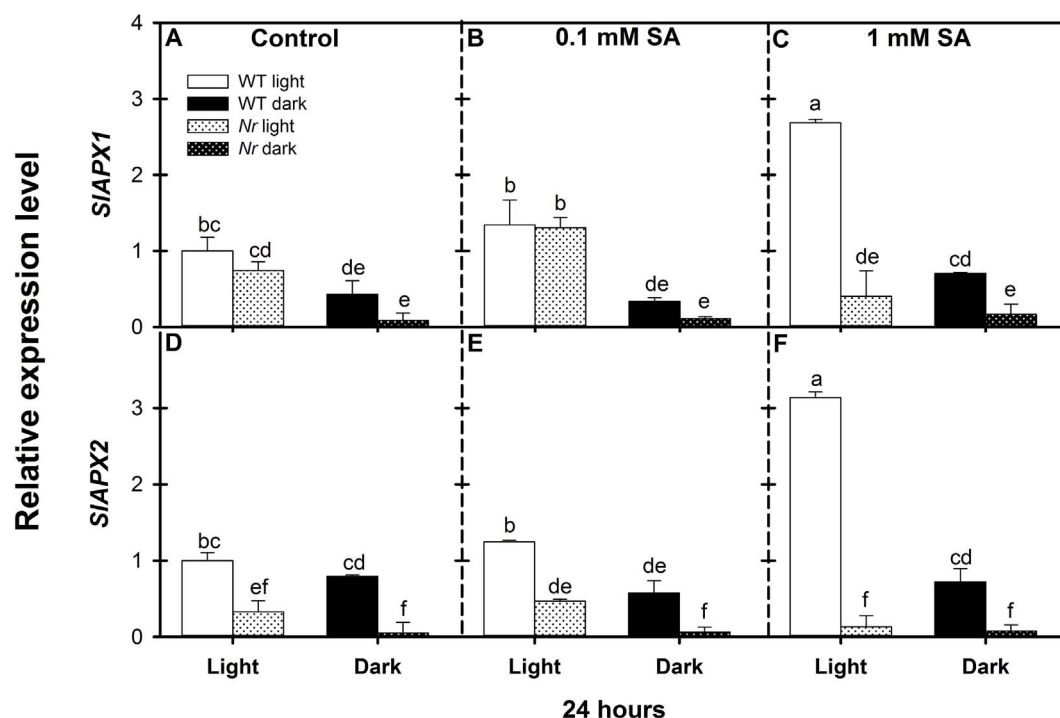


Fig. 8. Changes in relative expression levels of *SIAPX1* and *SIAPX2* genes in the leaves of wild type (WT, open columns) and ethylene receptor mutant *Never ripe* (*Nr*, latticed columns) tomato plants treated with 0.1 mM or 1 mM SA for 24 h in the presence or absence of light (□ Light, ■ Dark). Control: A, D; 0.1 mM SA: B, E; 1 mM SA: C, F. Results are means \pm SE from at least three independent experiments. Data with different letters indicate significant differences ($P \leq 0.05$, $n = 3$).

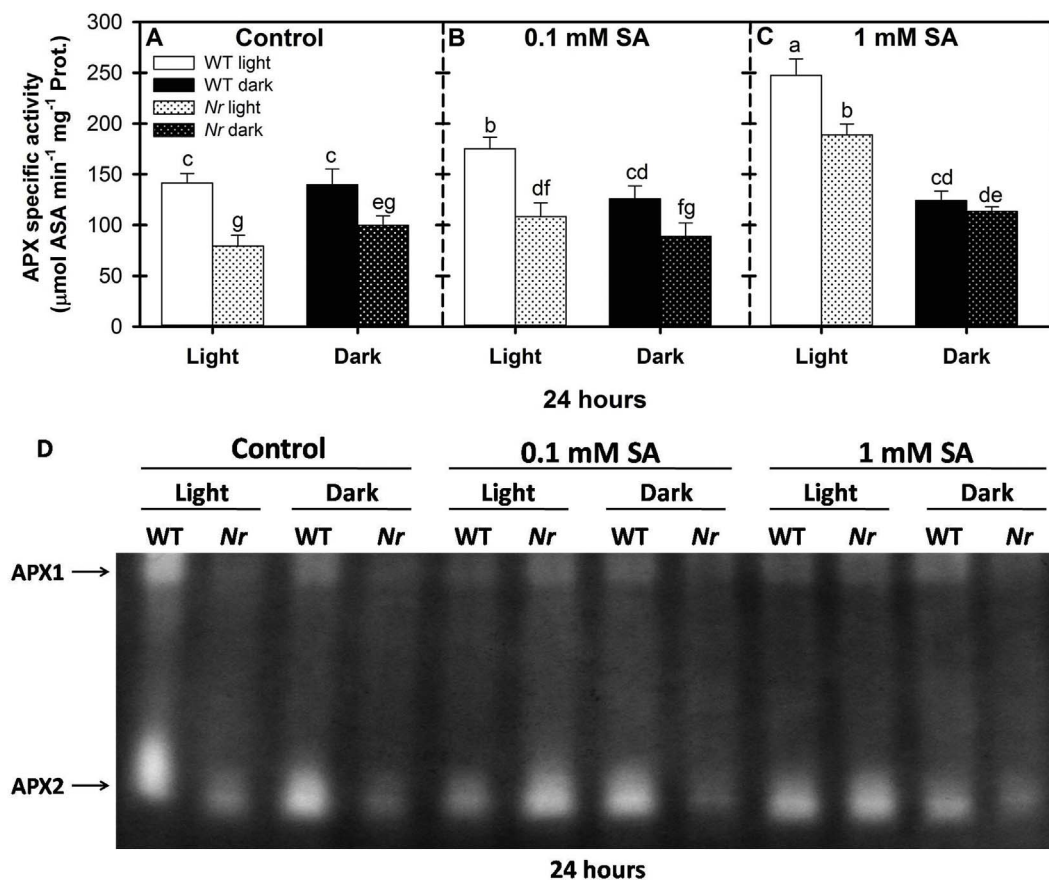


Fig. 9. Specific activity of ascorbate peroxidase (APX) in the leaves of wild type (WT, open columns) and ethylene receptor mutant *Never ripe* (*Nr*, latticed columns) tomato plants treated with 0.1 mM or 1 mM SA for 24 h in the presence or absence of light (□ Light, ■ Dark). Control: A; 0.1 mM SA: B; 1 mM SA: C. Results are means \pm SE from at least three independent experiments. Data with different letters indicate significant differences ($P \leq 0.05$, $n = 3$). (D) Native PAGE analysis of APX activity in leaves of WT and *Nr* tomato plants. Separation of isoenzymes and activity staining were performed as described by Kim et al. (2015) using 30 μg of protein per lane.

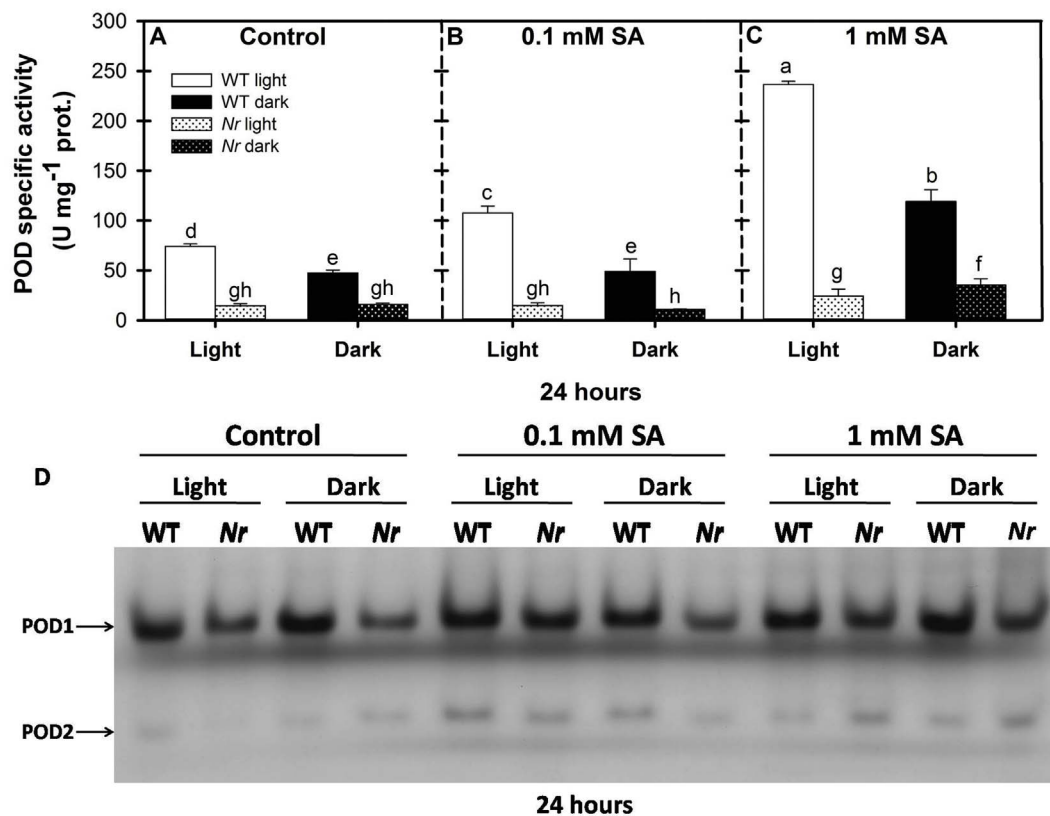


Fig. 10. Specific activity of guaiacol peroxidase (POD) in the leaves of wild type (WT, open columns) and ethylene receptor mutant *Never ripe* (*Nr*, latticed columns) tomato plants treated with 0.1 mM or 1 mM SA for 24 h in the presence or absence of light (□ Light, ■ Dark). Control: A; 0.1 mM SA: B; 1 mM SA: C. Results are means \pm SE from at least three independent experiments. Data with different letters indicate significant differences ($P \leq 0.05$, $n = 3$). (D) Native PAGE analysis for POD activity from leaves of wild type and ethylene receptor mutant *Never ripe* tomato plants. Separation of isoenzymes and activity staining were performed as described by García-Limones et al. (2002) using 30 μ g of protein per lane.

compared to corresponding light samples, it can be concluded that the lack of ET-induced negative feedback in *Nr* plants can be modulated by the illumination conditions and SA.

The first 24 h after stress stimuli is particularly important in plant responses where H_2O_2 plays a central role in the action of various hormones, such as SA and ET (Van Aken and Van Breusegem, 2015). In a previous study on tomato it was demonstrated that a sublethal concentration of SA increased the H_2O_2 accumulation under a normal photoperiod while high concentrations caused biphasic H_2O_2 accumulation, which was much more moderate during prolonged dark period. The H_2O_2 metabolism was also controlled by different antioxidant enzymes in WT plants in the presence or absence of light (Poór et al., 2017).

Although the function of ET in ROS production during stress reactions is well known, the SA-induced antioxidant mechanisms in conjunction with ET signalling and plant illumination have not yet been fully elucidated. It was revealed that the tomato ERF transcription factor, TERF1, is the key factor in the activation of ROS scavenging in ET signalling. The transcription of TERF1 is regulated by the upstream, EIN3-like transcription factors, LeEIL3 and LeEIL4 and can also be induced by reagents that generate ROS. The ectopic expression of TERF1 in tobacco promoted the expression of CAT and glutathione peroxidase, reduced ROS accumulation and increased the oxidative stress tolerance of plants (Zhang et al., 2016). The EIN3 (Mur et al., 2013) and ERFs may be also targets of the crosstalk between ET/SA signalling pathways (Broekgaarden et al., 2015).

In the present experiments H_2O_2 accumulation was not significantly different in the two genotypes under control conditions. SA stimulated H_2O_2 accumulation in WT tissues as a function of concentration, but much more so in the light, than in the dark. SA-induced increases in H_2O_2 were also observed in *Nr* plants. The most interesting tendencies

could be detected at 1 mM SA concentration. While the inhibition of ET signalling by the *Nr* mutation decreased the SA-induced H_2O_2 content in the light compared to WT, it resulted in pronounced H_2O_2 accumulation in prolonged darkness. This suggests that not only the SA and ET levels but also their signalling pathways are dependent on the presence or absence of light (Chandra-Shekara et al., 2006; Fukao et al., 2012) and also raises the possibility that H_2O_2 -producing and -scavenging processes and enzymes are regulated differently in WT and *Nr* plants. H_2O_2 accumulation exhibited strong correlation with the electrolyte leakage, a good marker of plasma membrane damage. These results demonstrated that the *Nr* mutants were more tolerant to SA-induced oxidative stress than WT in normal photoperiod but they proved to be more sensitive during prolonged darkness.

The elevated H_2O_2 level caused by exogenous SA treatment may act not only as a toxic compound inducing oxidative stress and HR, but also as a signalling molecule regulating antioxidant enzyme activity and gene expression (Van Aken and Van Breusegem, 2015). Five distinct SOD isoforms, one MnSOD (I), one FeSOD (II) and three Cu-ZnSODs (III-IV), were detected in the leaf tissues of both genotypes. This corresponds with the results of Monteiro et al. (2011), who characterized also five SOD isoforms in the leaves of WT and *Nr* mutants in a Micro-Tom background, though these authors found an extra MnSOD band in the root extract and demonstrated organ- and time-dependent alterations in enzyme activities in extracts prepared from salt- and Cd-stressed plants. In the present experiments 1 mM SA increased the activity of SOD in WT plants in both environments, but the changes were more pronounced under a normal photoperiod. The concentration-, time- and organ-specific activation of SOD by SA was observed earlier in tomato (Tari et al., 2015) and tobacco (Horváth et al., 2007), and it was also demonstrated that darkness had an inhibitory effect on SOD activity in WT plants (Camejo et al., 2007).

At the same time, the present results confirmed that ET signalling has a great influence on the SA-induced activation of SOD. While SA treatment did not increase the activity of SOD in illuminated *Nr* leaves, the total activity, the activity of *SlCu/ZnSODs* and the expression of chloroplastic *SlCu/ZnSOD* were significantly induced by 1 mM SA during prolonged dark period in the mutants. Considering that *SlCu/ZnSODs* are the predominant isoforms, the total SOD activity showed a good correlation with these bands. Furthermore, the increased SOD activity of WT leaves in the light and that of *Nr* in the dark could explain the increased H_2O_2 content in the leaves. It seems reasonable that active ET signalling is necessary for the SA-induced expression of *SlCu/ZnSOD*, but the prolonged dark treatment may partially substitute for the activation of ET-induced transcription factors without the activation of the upstream components in ET signalling.

The high H_2O_2 levels induced by SA treatments may be partially generated by SOD, but the equilibrium between H_2O_2 production and scavenging may be perturbed in the absence of H_2O_2 -inactivating enzymes such as CAT, APX and POX. It is well known that CAT activity is inhibited by SA at both the gene expression and protein level (Agarwal et al., 2005; Horváth et al., 2007; Tari et al., 2015). The present results demonstrated that both concentrations of SA inhibited the CAT activity in WT leaves under both environmental conditions and in illuminated *Nr* leaves. However, in prolonged darkness SA still increased the CAT enzyme activity in *Nr* leaves, suggesting that some SOD and CAT isoenzymes are controlled in a similar way in the dark in mutant plants. In parallel with enzyme activities, the expression of two peroxisomal CAT genes, *SlCAT1* and *SlCAT3*, were also up-regulated in the mutants by 1 mM SA in darkness. It was found by other authors that the expression of wheat CAT isoenzymes was regulated by the circadian rhythm and modulated by darkness (Luna et al., 2005). Han et al. (2015) observed that the use of 1-methylcyclopropene, which interacts with ET receptors and thereby inhibits ET action, increased CAT activity after 24 h in bitter melon in the dark, which was also supported by our experiments.

In agreement with the results of Kanazawa et al. (2000), who found that APX activity decreased during dark-induced senescence, the two important H_2O_2 scavenging enzymes, APX and POD, exhibited strong light dependence and parallel changes after SA treatment in both genotypes. APX activity was basically lower in the *Nr* mutant and the activity of the enzyme increased as a function of SA concentration in illuminated WT and *Nr* leaves. However, the enzyme activity remained constant in the dark in both genotypes. The expression of the *SlAPX1* and *SlAPX2* genes displayed tendencies similar to those observed for enzyme activities: both APX genes were highly expressed after 1 mM SA treatment in the presence of light, but their expression was down-regulated during a prolonged dark period. Irrespective of the illumination, the transcription of the *SlAPX1* and *SlAPX2* genes was significantly lower in *Nr* leaves, suggesting that active ET signalling is necessary for APX expression.

It was found earlier that the POD activity in the cytosol and cell wall increased in a dose-dependent manner after pre-treatment with SA (Rao et al., 1997; Ananieva et al., 2004). The present results confirmed that SA caused a concentration-dependent activation of POD in illuminated WT leaves. Moreover, the partial activation of PODs could contribute to the decomposition of H_2O_2 in the dark in WT, which is particularly important in the absence of CAT and APX induction in the SA-mediated protective actions in WT plants. Interestingly, the effect of SA on POD induction was significantly less pronounced in *Nr* plants. Monteiro et al. (2011) also observed lower POD activities in *Nr* leaves compared to WT plants, suggesting that ET has significant control over the activity of PODs. In the present work, the activity of POD was very low in *Nr* mutants and was only slightly elevated after SA treatment in the dark.

5. Conclusion

It can be concluded that H_2O_2 accumulation in WT leaves in response to 1 mM SA treatment is mainly determined by increased SOD

and decreased CAT activities in the light. In these plants APX and POD proved to be main participants of H_2O_2 scavenging and as a net result, H_2O_2 content was much higher in the WT than in *Nr* leaves. In a prolonged dark period the SOD and CAT activities and the expression of *SlCu/ZnSOD* and *SlCAT3* genes were activated in *Nr* mutants exposed to 1 mM SA but the absence of APX and POD activation led to a significant accumulation of H_2O_2 in these samples. These results suggest that the SA-induced H_2O_2 metabolism is regulated by different enzymes in WT and ET signalling mutant plants, which is modified by the presence or absence of light. Thus, *Nr* mutants seem to be more tolerant to SA-induced oxidative stress than WT plants in normal photoperiod, but they are more sensitive in prolonged darkness.

Contributions

ZT, PP, PB and CZ performed and analysed the experiments, GSz was responsible for the analysis of salicylic acid and its derivatives. PP, ZT and IT designed the experiments, wrote and provided critical review of the manuscript.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.plaphy.2018.02.026>.

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