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Prolonged dark period modulates the oxidative burst and enzymatic antioxidant systems in the leaves of salicylic acid-treated tomato

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ABSTRACT

Salicylic acid (SA) is an important plant growth regulator playing a role in the hypersensitive reaction (HR) and the induction of systemic acquired resistance. Since the SA-mediated signalling pathways and the formation of reactive oxygen species (ROS) are light-dependent, the time- and concentration-specific induction of oxidative stress was investigated in leaves of tomato plants kept under light and dark conditions after treatments with 0.1 mM and 1 mM SA. The application of exogenous SA induced early superoxide- and H_2O_2 production in the leaves, which was different in the absence or presence of light and showed time- and concentration-dependent changes. 1 mM SA, which induced HR-like cell death resulted in two peaks in the H_2O_2 production in the light but the first, priming peak was not detected in the dark. Unlike 0.1 mM SA, 1 mM SA application induced NADPH oxidase activity leading to increased superoxide production in the first hours of SA treatments in the light. Moreover, SA treatments inhibited catalase (CAT) activity and caused a transient decline in ascorbate peroxidase (APX), the two main enzymes responsible for H_2O_2 degradation, which led to a fast H_2O_2 burst in the light. Their activity as well as the expression of some isoenzymes of SOD and APX increased only from the 12th h in the illuminated samples. The activity of NADPH oxidase and expression *SlRBOH1* gene encoding a NADPH oxidase subunit was much lower in the dark. In spite of low CAT and APX activity after SA treatments in the dark, the activation of guaiacol-dependent peroxidase (POD) could partially substitute H_2O_2 scavenging activity of these enzymes in the dark, which reduced the ROS burst and development of lesion formation in the leaves.

1. Introduction

Light is the most important energy source for biomass production and it is required for growth and developmental processes in plant kingdom (Janda et al., 2014). Light may also control the plant defence mechanisms and excess of light energy under stress conditions leads to oxidative stress, which may contribute to the initiation of cell death in tissues (Karpinski et al., 2003; Kangasjärvi et al., 2012; Ballaré, 2014). Plants are able to sense the amount of photons, the intensity and quality of light as well as the changes in light/dark cycles (Chen et al., 2004). Hence, the absence of light (i.e. prolonged darkness) can alter the light-dependent activation of plant responses and can turn up new signalling and regulation pathways of defence.

Salicylic acid (SA) is a natural phenolic compound, which accumulates under abiotic and biotic stress and controls physiological and biochemical functions in plants (Khan et al., 2015). The effect of SA depends on plant species and developmental stage, on the mode of application and concentration of SA or on environmental conditions (Raskin, 1992; Horváth et al., 2007; Hayat et al., 2010; Khan et al., 2013).

The amount of endogenous SA in unstressed plant tissues may change from 10 to 200 ng g^{-1} fresh mass to as high as 37.19 μg g^{-1} g fresh weight (FW), the latter was detected in rice (Silverman et al., 1995). Changes in free and glucosylated SA (bound SA) can be detected upon exposure of plants to abiotic stresses. Increases in free SA were found in mustard plants (from ~2 to ~5 μg g^{-1} FW) under heat stress (Dat et al., 1998) and in tobacco leaves exposed to UV-C light (from ~300 ng to 4 μg g^{-1} FW) (Catinot et al., 2008). It was found that upon ozone-exposure SA accumulated in the leaf tissues of wild type Col-0, an O_3-sensitive, Cvi-0 *Arabidopsis* genotypes, while NahG plants

Abbreviations: APX, ascorbate peroxidase; AsA, ascorbic acid; CAT, catalase; HR, hypersensitive response; ROS, reactive oxygen species; NADPH oxidase, nicotinamide adenine dinucleotide phosphate-oxidase; NBT, nitro blue tetrazolium; PM, plasma membrane; POD, guaiacol-dependent peroxidase; SA, salicylic acid; SAR, systemic acquired resistance; SOD, superoxide dismutase

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expressing salicylate hydroxylase gene failed to increase SA concentrations. The accumulation of reactive oxygen species (ROS), defence gene expression and severity of lesion formation correlated with SA levels in these genotypes upon ozone exposure (Rao and Davis, 1999).

SA-induced defence reactions can also protect plants against many pathogens, including fungi, bacteria and viruses. Pathogens can be classified into biotrophs, hemibiotrophs and nectrotrophs on the basis of their lifestyle, which may predict the response of plants to the infection and hormone signalling pathways initiated by the pathogen invasion. Thus the salicylate response is involved in resistance to many biotrophs (*Pseudomonas syringae*, *Xanthomonas campestris*) and some necrotrophs (*Botrytis cinerea*) in tomato (Thaler et al., 2004).

SA plays a role in the formation of local and systemic acquired resistance (SAR) and in the initiation of cell death in the tissues surrounding the invading pathogens (Li et al., 2014). SA levels can increase more than 40-fold, up to 75 μg g^{-1} FW after tobacco mosaic virus (TMV) infection in the immediate vicinity of hypersensitive response (HR), at the site of pathogen penetration and up to 1.5 μg g^{-1} FW in non-infected leaves (Enyedi et al., 1992). Interestingly, 10–15 day after infection SA accumulation proved to be an important component of susceptible disease response of tomato to *Xanthomonas campestris* cv. *vesicatoria* infection. In this work the authors found 22.06 μg g^{-1} FW SA in the leaves of UC828 tomato genotype, this SA accumulation led to an enhanced necrotic cell death in the infected tissues (O'Donnell et al., 2001).

The application of exogenous SA may enhance the resistance to abiotic and to biotic stresses and the development of the resistance may depend on the illumination since the accumulation of SA and the initiation of SA-mediated signalling pathways are light-dependent (Genoud et al., 2002; Chandra-Shekara et al., 2006). Plants infected in the dark showed reduced lesion formation in response to an avirulent pathogen (Zeier et al., 2004). In addition, SAR development in response to the infection is completely lost in the absence of light (Karpinski et al., 2003). Nevertheless, light availability is particularly important during the early stages of the plant-pathogen interaction (Griebel and Zeier, 2008).

There is close correlation between ROS production and changes in SA content (Xu and Brosche, 2014). SA-induced biotic stress tolerance depends on the accumulation of superoxide radical ($\cdot O_2^-$) and hydrogen peroxide (H_2O_2), that are essential mediators of the HR and cell death induction and they could contribute to cellular redox homeostasis through the regulation of the expression and activity of antioxidant enzymes (Saruhan et al., 2012; Janda and Ruelland, 2015). To overcome oxidative stress, plants have fast acting defence systems such as non-enzymatic antioxidants and antioxidant enzymes to alleviate the oxidative damage (Mittler et al., 2011).

Several studies reported that SA treatments induced the accumulation of ROS by modulating the activity of some antioxidant enzymes including superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX) (Khan et al., 2014). SODs belong to a group of metallo-enzymes, which are the main scavengers of superoxide by catalyzing the conversion of $\cdot O_2^-$ to H_2O_2. Mn-SOD is localized to mitochondria and peroxisomes, Fe-SODs function inside the chloroplast peroxisomes and isoforms of Cu/Zn-SOD can be found in the chloroplasts, in the cytosol as well as in peroxisomes and apoplast (Fernández-Ocaña et al., 2011). It was also found that SA induced the activity of Cu/Zn-SOD in tobacco plants (Horváth et al., 2007). Most of H_2O_2 in plant cells is eliminated by CAT and APX. SA can bind directly to the CAT enzyme and it can inhibit the activity of certain isoenzymes (Horváth et al., 2002). At the same time SA induces the activity of APXs and guaiacol-peroxidases (POD), which also catalyze the decomposition of H_2O_2 to water (Horváth et al., 2007; Rivas-San Vicente and Plasencia, 2011; Tari et al., 2015; Chen et al., 2016). However, the timing and the role of the early activation/inactivation of various isoforms of these antioxidant enzymes under SA-induced ROS wave are less known.

Formation of ROS is strictly coupled to light-driven electron transport chain in the chloroplasts under abiotic- or biotic stress (Bailey-Serres and Mittler, 2006; Xing et al., 2013). However, a flavoenzyme NADPH oxidase, localized to the plasma membrane (PM), also known as respiratory burst oxidase homologue (RBOH), can translocate electrons from cytosolic NADPH to oxygen, leading to the generation of $\cdot O_2^-$ in the apoplast (Sagi and Fluhr, 2006). The activation of NADPH oxidases mediates the progression of ROS signals from cell to cell under pathogen infection (Miller et al., 2009; Dubiella et al., 2012) and the enzyme is also activated by SA treatment (Kawano et al., 2004). Two RBOH genes, *SlRBOH1* and *SlWFI1*, were identified in tomato and it was found that *SlRBOH1* plays a role in apoplastic H_2O_2 production and in the induction of stomatal closure (Li et al., 2015). The apoplastic ROS burst mediated by NADPH oxidase can also communicate with ROS production in chloroplasts, because under high light conditions chloroplasts adopt a position adjacent to the PM (Shapiguzov et al., 2012). Moreover, the activity of NADPH oxidase can contribute to the first, priming oxidative burst after pathogen infection (Jiménez-Quesada et al., 2016).

Thus, the regulation of defence mechanisms and cell death in plant tissues seems to be different in light and dark conditions, although many pathogens are active in the dark. It was also found in many cases that HR was suppressed or delayed after pathogen infection in the dark (Chandra-Shekara et al., 2006; Grimmer et al., 2012), but the role of SA and SA induced signalling in this process remained unclear. There is also little information about the effects of different concentrations of SA on the expression and activity of antioxidant enzymes in the light or in the dark, especially of those, which participate in $\cdot O_2^-$ and H_2O_2 metabolism.

The aim of this study was to elucidate the time- and concentration-specific induction of oxidative defence responses elicited by SA in tomato leaves under normal photoperiod and extended dark conditions. The question is whether the SA-induced $\cdot O_2^-$ and H_2O_2 metabolism are mediated by different enzymes in the presence or absence of light. In this study we would like to reveal the changes in the activity of most important ROS producing and ROS-scavenging enzymes as a function of time in plants grown under normal photoperiod or under prolonged darkness. These results broaden our knowledge about the SA generated oxidative stress and defence in the first particularly important 24 h after SA accumulation in the presence or absence of light.

2. Materials and methods

2.1. Plant materials and growth conditions

Seeds of tomato plants (*Solanum lycopersicum* L. cv. Ailsa Craig) were germinated at 26 °C for three days in the dark, and the seedlings were subsequently transferred to perlite for two weeks. The plants were then placed into plastic boxes (40 cm length, 30 cm width, 20 cm depth, 12 seedlings per box) filled with 20 L of modified Hoagland nutrient solution as described earlier (Poór et al., 2011). The culture medium was changed twice a week. Plants were grown in a controlled environment condition under 200 μmol m^{-2} s^{-1} photon flux density (F36W/GRO lamps, OSRAM SYLVANIA, Danvers, MA, USA), with 12/12-h light/dark period, a day/night temperatures of 24/22 °C and a relative humidity of 55–60% for eight weeks.

Tomatoes were treated with 0.1 mM or 1 mM salicylic acid (SA) supplied in the nutrient solution, which was presumed to adjust SA content to the levels that found in systemically induced leaves or in tissues exposed directly to pathogen infection. Half of the plants remained for 24 h under the growing light/dark cycle (light samples) or half of them were put into prolonged darkness (dark samples) at 25 °C. The experiments were conducted from 9 a.m. and were repeated three times. The samples were prepared from the second, fully expanded young leaves in three replicates 1; 3; 6; 12; 24 h after the different SA treatments.

2.2. Salicylic acid extraction and analytical procedures

The extraction and determination of free and bound SA were performed as described by Pál et al. (2005). Briefly, 0.5 g of leaf tissues was ground in liquid nitrogen in the presence of quartz sand. The tissue powder was transferred to a centrifuge tube and mixed with 2 mL of 70% methanol containing 250 ng *ortho*-anisic acid (oANI) (used as internal standard) and 25 μg *para*-hydroxy-benzoic acid (pHBA). The extract was centrifuged at 10.000*g* for 20 min. The pellet was resuspended in 2 mL of 90% methanol, vortexed, and centrifuged as above. The methanol content was evaporated at room temperature under vacuum. One mL of 5% (w/v) trichloroacetic acid (TCA) was added to the residual aqueous phase and the mixture was centrifuged at 15.000*g* for 10 min. The supernatant was gently partitioned twice against 3 mL of 1:1 (v/v) mixture of ethyl acetate/cyclohexane and free phenolic acids accumulated in he upper organic layers. The aqueous phases containing the methanol-soluble bound phenolics were acid hydrolyzed. 250 ng oANI, 25 μg pHBA, and 1.3 mL 8 N HCl were added to the aqueous phase and incubated for 60 min at 80 °C before partitioning twice as above. Conjugated forms of SA were hydrolysed by boiling for 30 min in acidified phosphate buffer and the samples were then treated in the same way as in case of free SA. The organic phases were evaporated to dryness under vacuum and resuspended in 1 mL of the HPLC initial mobile phase. Free and bound SA were separated by high-performance liquid chromatography (HPLC) on reverse-phase column (Supelcosil ABZ Plus, 5 μ; 150 × 4.6 mm) at 25 °C (WATERS, Milford, MA, USA) and monitored with UV/VIS detector (W474 scanning fluorescence detector, Waters, USA) with excitation at 305 nm and emission at 407 nm.

2.3. Determination of the H_2O_2 level

The H_2O_2 level was measured spectrophotometrically as described by Horváth et al. (2015) with some modifications. After homogenisation of 200 mg leaf tissues with 1 mL 0.1% ice-cold TCA, 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 H_2O_2 was calculated using a standard curve prepared from increasing concentration of H_2O_2.

2.4. Histochemical detection of superoxide production

NBT staining was used to detect the production of superoxide radical ($\cdot O_2^-$) *in situ,* which was carried out according to Wohlgemuth et al. (2002). Detached leaves from SA-treated tomato plants were incubated for 1 h in the dark in 50 mM sodium phosphate buffer (pH 7.6) containing 0.1 % nitro blue tetrazolium (NBT). 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 colour digital camera (Sony Cyber-shot DSC-H9, Sony Co., Tokyo, Japan) was used.

2.5. Native PAGE analysis of NADPH oxidase activity

NADPH oxidase activity was analysed using omniPAGE electrophoresis system (Cleaver Scientific Ltd., Rugby, Warwickshire, UK) as described by Carter et al. (2007). 0.5 g of leaf samples were crushed to a fine powder in a mortar under liquid N_2 and then soluble proteins were extracted by resuspending the powder in 1 mL of 50 mM sodium phosphate buffer (pH 6.8) containing 0.5% Triton X-100. The homogenate was centrifuged at 12.000*g* for 20 min at 4 °C. Crude leaf protein extracts were mixed with 62.5 mM Tris-HCl buffer (pH 6.8), containing 10% (v/v) glycerol and 0.025% (w/v) bromophenol blue and equal amounts of protein (30 μg) were loaded onto the gel. Electrophoresis was performed at 4 °C for 1–3 h and at a constant voltage of 120 V using 25 mM Tris and 192 mM glycine solution (pH 8.3) as running buffer and 4% stacking and 10% separating polyacrylamide gels. After electrophoresis, the gels were incubated in 0.5 mg mL^{-1} *p*-nitro blue tetrazolium chloride (NBT) in 50 mM Tris (pH 7.4) and 134 μM NADPH until bands were detected. Diphenyleneiodonium chloride (DPI), the inhibitor of NADPH oxidase was applied at 50 μM concentration for the inhibition of NADPH oxidase activity (data not shown).

2.6. Determination of antioxidant enzyme activities

To analyse the enzyme activities, 0.2 g leaf tissue was homogenised on ice in 1 mL of 100 mM phosphate buffer (pH 7.0) containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1% (w:v) polyvinyl-polypirrolidone (PVPP). The extraction of ascorbate peroxidase (APX) occurred in the presence of 1 mM ascorbate (AsA). The homogenate was centrifuged at 12.000*g* for 20 min at 4 °C and the supernatant was used for enzyme activity assays including superoxide dismutase (SOD), catalase (CAT), APX and guaiacol-dependent peroxidase (POD). All absorbances were measured by spectrophotometer (KONTRON, Milano, Italy). 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 NBT in the presence of riboflavin in the light. One enzyme unit (U) of SOD corresponds to the amount of enzyme causing a 50% inhibition of NBT reduction in the light. CAT (EC 1.11.1.6) activity was determined by following the consumption of H_2O_2 (ε_{240} = 39.4 mM^{-1} cm^{-1}) at 240 nm for 3 min at 25 °C. One unit of CAT is defined as the amount of enzyme necessary to decompose 1 μmol min^{-1} H_2O_2. APX (EC 1.11.1.11) activity was determined by monitoring the decrease of AsA content at 290 nm (ε_{290} = 2.8 mM^{-1} cm^{-1}) for 3 min at 25 °C. One unit of APX activity is defined as the amount of enzyme necessary to oxidize 1 μmol min^{-1} AsA (Tari et al., 2015). POD (EC 1.11.1.7) activity was measured following the increase of absorbance at 470 nm due to guaiacol oxidation (ε_{470} = 26.6 mM^{-1} cm^{-1}). The amount of enzyme producing 1 μmol min^{-1} of oxidised guaiacol was defined as one U (Horváth et al., 2015). Soluble protein concentration was determined according to Bradford (1976) using bovine serum albumin as a standard.

2.7. RNA extraction, expression analyses by qRT-PCR

Quantitative real-time reverse transcription-PCR (qRT-PCR; Piko Real-Time qPCR System, Thermo Scientific) was used to detect the expression pattern of the selected tomato genes mined from Sol Genomics Network (SGN; http://solgenomics.net/;) and National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/) databases (Horváth et al., 2015). Primers were designed using NCBI and Primer 3 software (http://frodo.wi.mit.edu/) and listed in Supplementary Table S1 (in the online version at DOI: 10.1016/j.jplph.2017.03.013). The PCR reaction consisted of 10 ng cDNA template, 400–400 nM forward and reverse primers, 5 μL of Maxima SYBR Green qPCR Master Mix (2X) (Thermo Scientific), and nuclease-free water in a total volume of 10 μL. After the PCR (denaturation at 95 °C for 7 min, followed by 40 cycles of denaturation 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 occurred by PikoReal Software 2.2 (Thermo Scientific). Tomato 18S rRNA and elongation factor-1α subunit genes were applied as the reference genes and $2^{(-\Delta\Delta Ct)}$ formula was used to calculate data from the qRT-PCR. Each reaction was repeated at least three times.

2.8. Statistical analysis

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

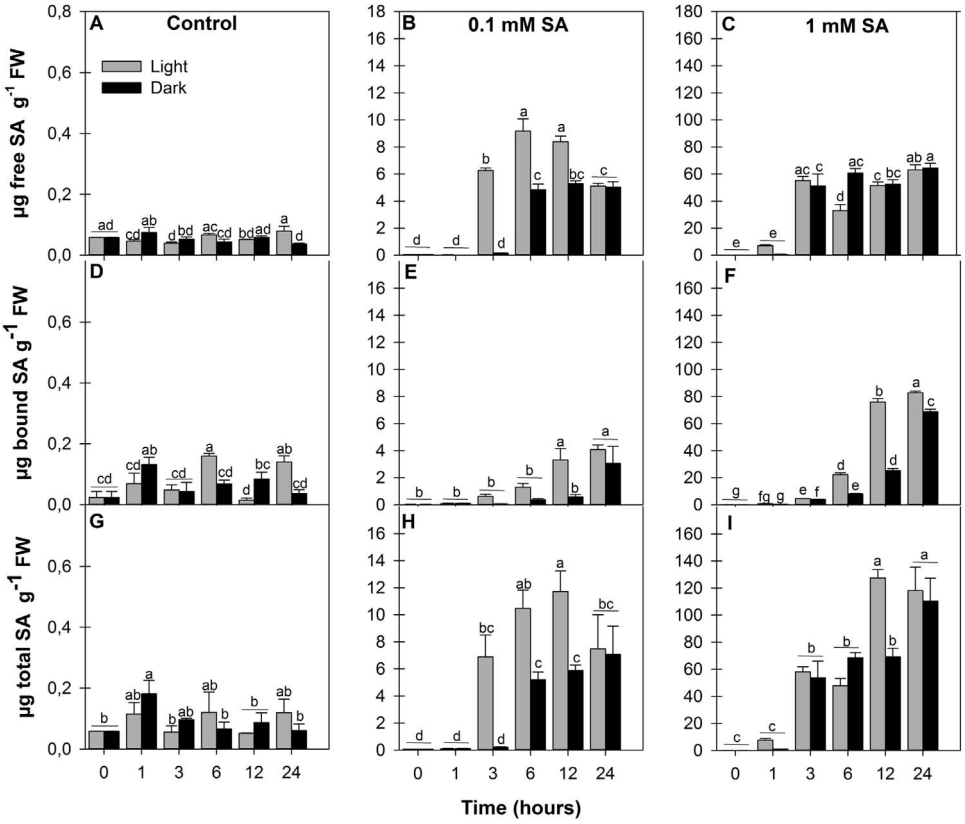

Fig. 1. Changes in free, bound and total salicylic acid (SA) content as a function of time in leaves of tomato plants in the presence or absence of light (Light, Dark): control (A, D, G), 0.1 mM SA treatment (B, E, H), 1 mM SA treatment (C, F, I). Means + SE, n = 3. Data with different letters indicate significant differences at $P \leq 0.05$ level (Tukey test) in each time point.

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

3. Results

3.1. Effect of SA treatments on endogenous SA contents in tomato leaves under light and dark conditions

To examine the role of illuminated and dark environment on SA accumulation, free, bound and total endogenous SA contents were determined upon different SA treatments in tomato leaves. Free SA content was 0.06 + 0.01 µg g^{-1} FW in the illuminated control leaves, which did not change significantly in darkness until 24 h (Fig. 1A). In contrast to free SA, bound SA content was lower after 6 h in the dark compared to the illuminated leaves (Fig. 1D). Under the control conditions, total SA levels were between 0.08–0.20 µg g^{-1} FW (Fig. 1G). Free and total SA contents increased more than 100 fold upon the treatment with 0.1 mM SA (Fig. 1B, E, H) in tomato leaves. The maximum in free and total SA contents was reached between 6 and 12 h of the treatment in the light. In darkness, SA levels increased later and the maximum was significantly lower after 6 h but SA content was not different after 24 h (Fig. 1B, E, H). In contrast to 0.1 mM SA, treatment with 1 mM SA increased free SA levels ~1000 fold and the total SA levels ~1300 fold compared to the control (Fig. 1C, F, I). The maximum of free SA content was detected after 3 h upon 1 mM SA treatment and it did not show pronounced differences under dark condition compared to the light (Fig. 1C). The highest level of bound SA was found after 12 h in the light and after 24-h in the dark (Fig. 1F). However, the total SA content did not show significant differences between light and dark samples after 24 h (Fig. 1I).

3.2. Effect of SA on oxidative burst in tomato leaves under light and dark conditions

H_2O_2 is the key molecule in SA-mediated signalling, thus H_2O_2 content was determined in the leaves of tomato after SA treatments. Upon control condition a slightly increased H_2O_2 content was observed in the light compared to the dark (Fig. 2A).

Both SA treatments increased H_2O_2 content in concentration- and time-dependent manner, but it was significantly lower in the dark (Fig. 2B, C). 0.1 mM SA increased H_2O_2 level from the 6th h in the light but it did not change in the dark until 24 h (Fig. 2B). However, H_2O_2 levels was significantly enhanced upon 1 mM SA treatments already from the first hours of the SA application and resulted in two peaks at 3 and 24 h (Fig. 2C). Nevertheless, 1 mM SA increased H_2O_2 levels only from the 6th h in the dark, which had only one maximum (Fig. 2C).

NBT has been generally used to detect the superoxide radical ($\cdot O_2^-$) production *in situ*. After incubation of intact leaves in NBT solution, higher $\cdot O_2^-$ accumulation was found after 1 mM SA treatment from the first hour compared to 0.1 mM SA treatment in the light. In darkness, 1 mM SA-treated leaves displayed lower levels of $\cdot O_2^-$ compared to the illuminated samples (see Supplementary Fig. S1 in the online vesion at DOI: 10.1016/j.jplph.2017.03.013).

3.3. Effect of SA on enzyme activities and expression of their coding genes under light and dark conditions

In order to investigate the production of H_2O_2 and $\cdot O_2^-$ in the leaves of SA-treated tomato, the putative enzymatic sources of superoxide anion radical was determined. PM-localized NADPH oxidase is one of the key players in this process. Both SA treatments induced $\cdot O_2^-$

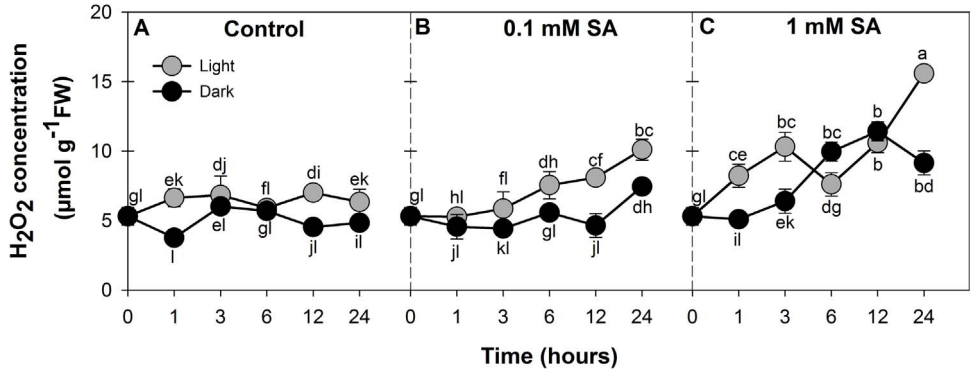

Fig. 2. Changes in H_2O_2 content as a function of time in leaves of tomato plants in the presence or absence of light (▒ Light, ■ Dark): control (A), 0.1 mM SA treatment (B), 1 mM SA treatment (C). Means + SE, n = 3. Data with different letters indicate significant differences at $P \leq 0.05$ level (Tukey test) in each time point.

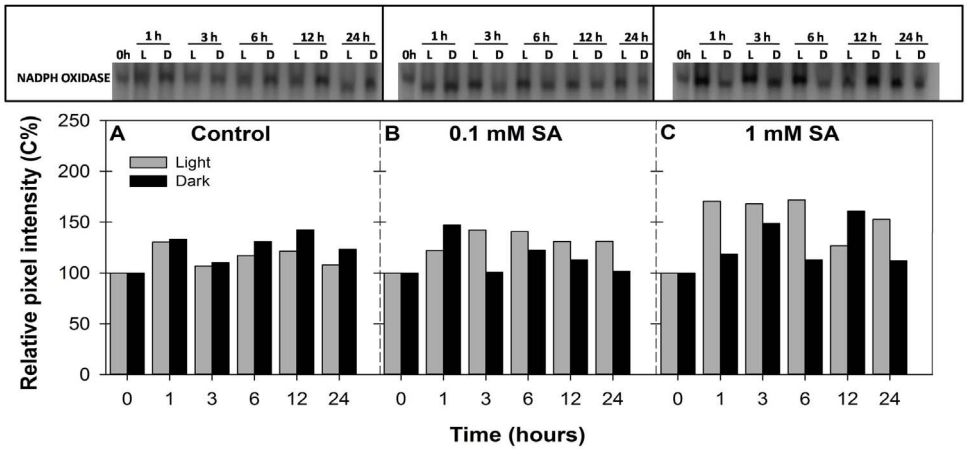

Fig. 3. Activity and relative pixel intensity of NADPH oxidase on non-denaturing gel as a function of time in leaves of tomato plants in the presence or absence of light (▒ Light, ■ Dark): control (A), 0.1 mM SA treatment (B), 1 mM SA treatment (C). Relative pixel intensity of lane profile after application of different SA concentrations was assessed. Data are from a single representative experiment, repeated three times.

production by NADPH oxidase activity as an early event. 0.1 mM SA induced the activity of the enzyme from the 3rd h, whereas 1 mM SA increased it from the 1st h (Fig. 3). In the light at 1 mM SA concentration the enzyme activity was permanently high during the first 6 h and at 24 h that manifested itself in a double-peak. However, dark treatment delayed this increment and the enzyme activities were much lower (Fig. 3C).

The transcript levels of *SlRBOH1* were also elevated by SA treatments from the first to 6th hours and at 24 h in the light, showing two peaks in the gene expression (Fig. 4B, C). Interestingly, 1 mM SA also induced the expression of *SlRBOH1* under the dark condition at 1st and 12th h, as well (Fig. 4C).

Although there were no pronounced changes in total SOD activity of the tissues, the activity of the enzyme, which catalyzes the dismutation of $\cdot O_2^-$ into molecular oxygen and H_2O_2, was induced slightly by both SA treatments in 24 h samples in the light, but the enzyme activity was generally lower at the analysed time points at 1 mM SA in the dark (Fig. 5).

The transcript levels of mitochondrial *SlMnSOD* as well as plastidial *SlFeSOD* and *SlCu/ZnSOD* genes were also analysed as a function of time after SA treatments. Among the three *SOD* genes, about a threefold induction was found in the transcript level of *SlFeSOD* in the control leaves after 6 h and 5-fold after 12 h in dark environment (Fig. 6D). Around 2.6, 4.5 and 3.3-fold induction for *SlMnSOD*, *SlFeSOD* and *SlCu/*

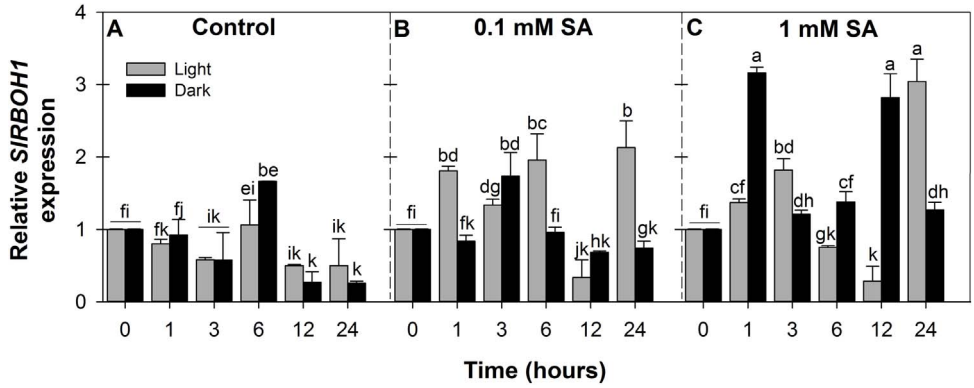

Fig. 4. Changes in the relative transcript levels of *SlRBOH1* as a function of time in leaves of tomato plants in the presence or absence of light (▒ Light, ■ Dark): control (A), 0.1 mM SA treatment (B), 1 mM SA treatment (C). Means + SE, n = 3. Data with different letters indicate significant differences at $P \leq 0.05$ level (Tukey test) in each time point.

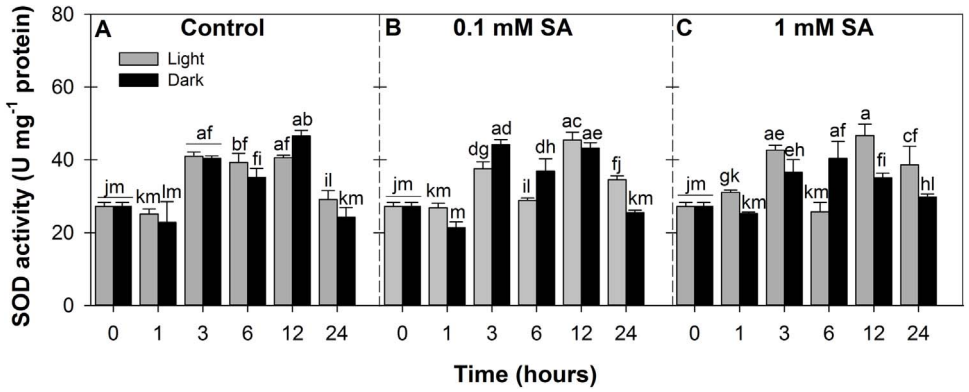

Fig. 5. Specific activity of superoxide dismutase (SOD) as a function of time in leaves of tomato plants in the presence or absence of light (□ Light, ■ Dark): control (A), 0.1 mM SA treatment (B), 1 mM SA treatment (C). Means + SE, n = 3. Data with different letters indicate significant differences at $P \leq 0.05$ level (Tukey test) in each time point.

ZnSOD transcripts, respectively, were detected in the dark at 3 h after 0.1 mM SA treatment, whereas their expression was kept constant or decreased compared to respective controls in the light (Fig. 6B, E, H). In contrast to the effect of 0.1 mM SA, *SlMnSOD* transcripts increased from the 12th h to 4.5 fold in illuminated leaves upon 1 mM SA treatment (Fig. 6C). The most characteristic changes were observed in the expression of *SlCu/ZnSOD*, which was induced by 15, 9, and 23-fold from the 6 th to 24th h in the light after 1 mM SA treatment. Similar tendencies in the expression of *SlCu/ZnSOD* were shown in the dark, but the gene was expressed to much lower extent (Fig. 6I). The expression of *SlFeSOD* decreased upon SA treatments, but was kept almost constant in the light, but decreased in the dark by 1 mM SA (Fig. 6F).

The activity of CAT, which catalyzes the decomposition of H_2O_2 to water and oxygen, displayed a diurnal rhythm. In the dark the enzyme activity showed similar tendencies until 6 h than in the light, however, the activity decreased after 12 h in control leaves (Fig. 7A). SA exerted a clear inhibition on CAT activity both in light and dark conditions and the tendencies were similar under normal photoperiod and dark environment (Fig. 7B, C).

The expression of three tomato *CAT* genes was also analysed. *SlCAT3* was only transiently up-regulated 5-fold after 3 h under control

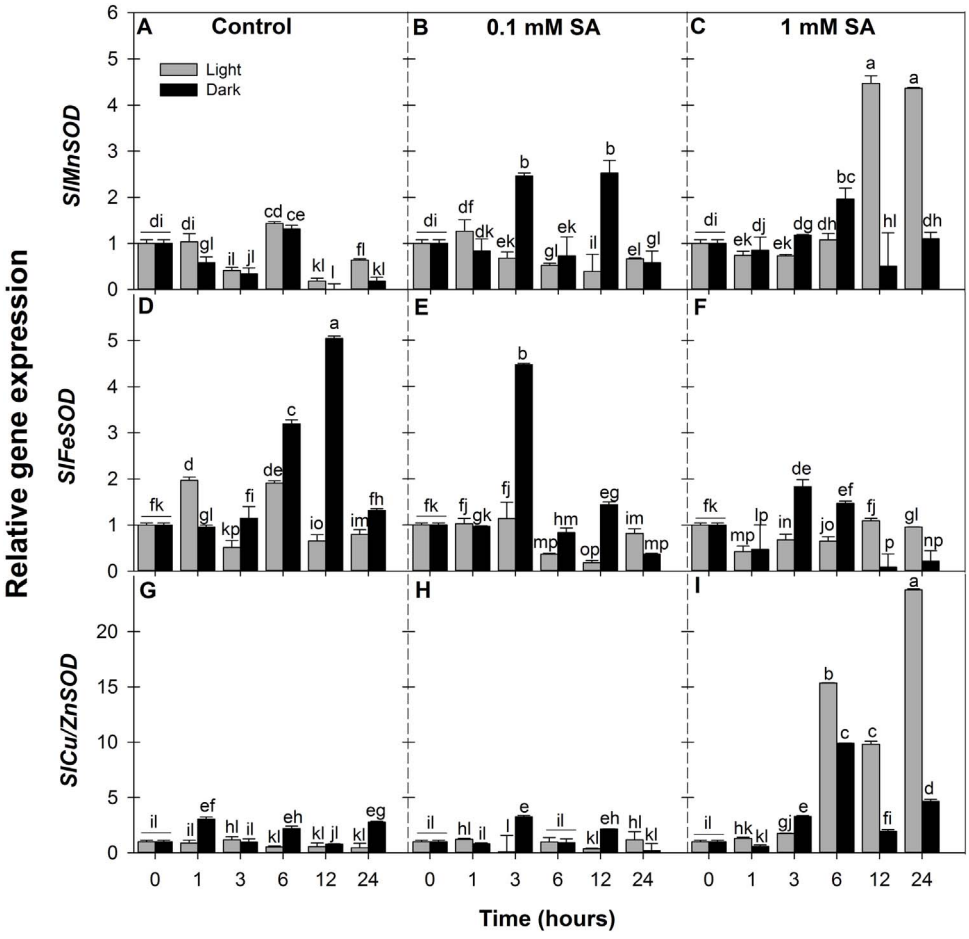

Fig. 6. Changes in the relative transcript levels of *SlMnSOD*, *SlFeSOD* and *SlCu/ZnSOD* as a function of time in leaves of tomato plants in the presence or absence of light (□ Light, ■ Dark): control (A, D, G), 0.1 mM SA treatment (B, E, H), 1 mM SA treatment (C, F, I). Means + SE, n = 3. Data with different letters indicate significant differences at $P \leq 0.05$ level (Tukey test) in each time point.

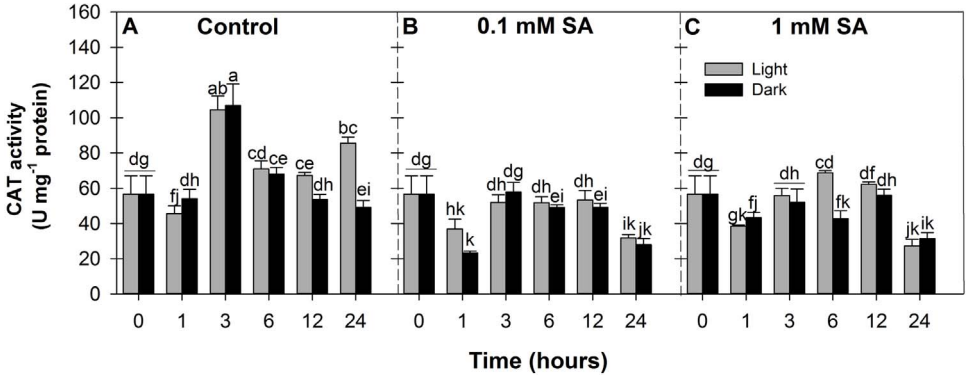

Fig. 7. Specific activity of catalase (CAT) as a function of time in leaves of tomato plants in the presence or absence of light (Light, Dark): control (A), 0.1 mM SA treatment (B), 1 mM SA treatment (C). Means + SE, n = 3. Data with different letters indicate significant differences at $P \leq 0.05$ level (Tukey test) in each time point.

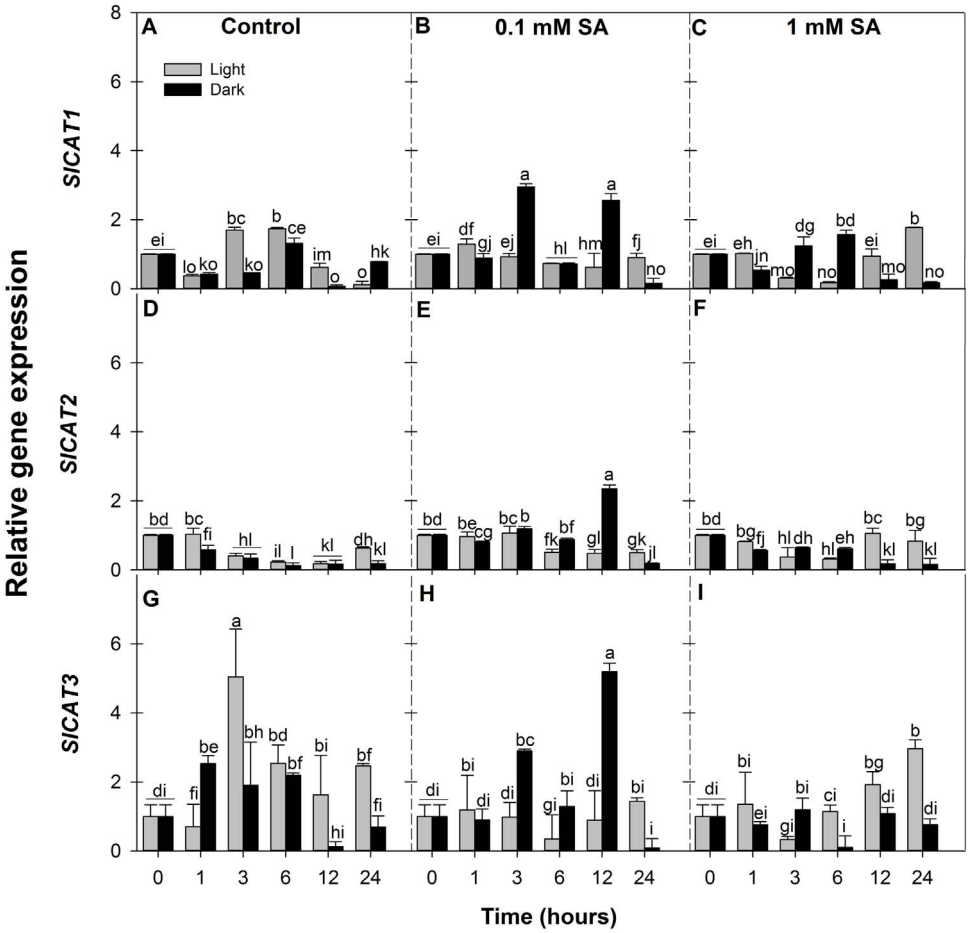

Fig. 8. Changes in the relative transcript levels of *SlCAT1*, *SlCAT2* and *SlCAT3* as a function of time in leaves of tomato plants in the presence or absence of light (Light, Dark): control (A, D, G), 0.1 mM SA treatment (B, E, H), 1 mM SA treatment (C, F, I). Means + SE, n = 3. Data with different letters indicate significant differences at $P \leq 0.05$ level (Tukey test) in each time point.

conditions in the light and the relative expression remained high after 24 h. Up-regulation of *SlCAT3* occurred earlier in the absence of light (Fig. 8G), whereas no change (*SlCAT2)* or decreases (*SlCAT1*) were observed in the expression of these latter isoenzymes in control leaves in the dark compared to light (Fig. 8A, D). *SlCAT1*, *SlCAT2* and *SlCAT3* transcripts accumulated 3- and 5-fold at 3 and/or 12 h after 0.1 mM SA treatment in darkness, while they remained around the control level or decreased in the illuminated leaves (Fig. 8B, E, H). Expression profiles of CAT genes upon 1 mM SA treatment were kept almost constant (*SlCAT1* and *SlCAT2*) or decreased (*SlCAT3*) in light and dark compared to the respective controls (Fig. 8C, F, I).

Activity of APX, which catalyzes H_2O_2 detoxification using AsA as co-substrate, exhibited the most light-dependent activity after SA treatments. In the first hours of both treatments the activity of APX was reduced, however it was enhanced by ~20–70% in the light upon 0.1 mM and 1 mM SA treatments from 12th h, while the activity was not changed in the dark (Fig. 9B, C).

The transcript levels of APX coding genes (*SlAPX1* and *SlAPX2*) exhibited diurnal fluctuations and decreased under control conditions after 3 h (Fig. 10A, D). Upon 0.1 mM SA treatment *SlAPX1* and *SlAPX2* transcripts increased to ~2 and 3-fold, respectively in darkness at 3 and 12 h, whereas small increases were observed in the light (Fig. 10B, E). In illuminated leaves, however, the transcripts of APXs accumulated 3-fold from 6, to 24 h after 1 mM SA treatment, and in darkness they

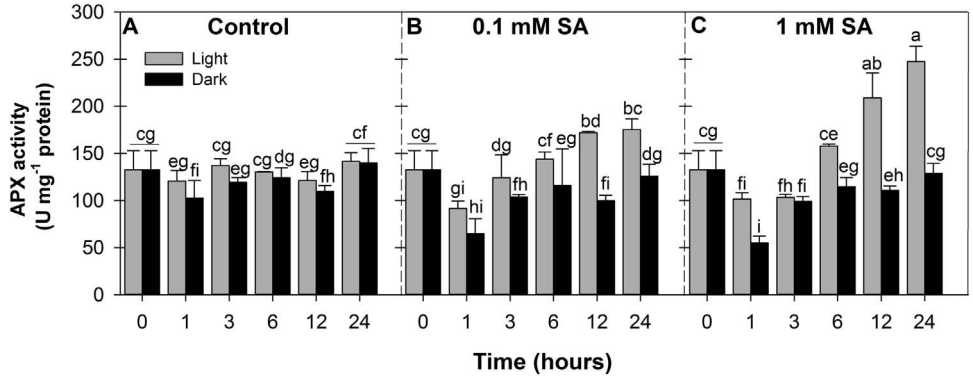

Fig. 9. Specific activity of ascorbate peroxidase (APX) as a function of time in leaves of tomato plants in the presence or absence of light (Light, Dark): control (A), 0.1 mM SA treatment (B), 1 mM SA treatment (C). Means + SE, n = 3. Data with different letters indicate significant differences at $P \leq 0.05$ level (Tukey test) in each time point.

reached 3 fold levels at 3 and returned to the basal level at 12 and 24 h (Fig. 10C, F).

POD activity, which also plays a role in H_2O_2 detoxification, was higher in the leaves upon treatment with 1 mM SA (see Supplemental Fig. S2 in the online version at DOI: 10.1016/j.jplph.2017.03.013). Under control conditions, POD activity displayed slight increase as a function of time and the enzyme activity was almost similar in the illuminated and the dark-treated leaves (see Supplemental Fig. S2A in the online version at DOI: 10.1016/j.jplph.2017.03.013). After 24 h, both SA treatments induced the activity of POD, but significantly higher increment could be observed upon 1 mM SA treatment, which was much higher in illuminated leaves (see Supplemental Fig. S2B, C in the online version at DOI: 10.1016/j.jplph.2017.03.013).

4. Discussion

Depending on the concentration, SA may control various biochemical and molecular biological activities in plant tissues (Hayat et al., 2010). Previous studies proved that in most of plant species endogenous SA acted as a signalling molecule in 0.1–10.0 μg g^{-1} FW concentration range (Chen et al., 2016). However, plants usually suffered from oxidative stress that can induce cell death, when the endogenous concentration of SA exceeded a species-specific limit or exogenous SA was applied at higher, than 1 mM concentration for tomato (Poór et al., 2013). In the present work we found that these endogenous SA concentrations in the leaves can be achieved by the application of 0.1 or 1 mM SA, respectively, through the root system in hydroponic medium. It was also found that the concentrations of free or bound SA increased earlier in the light than in the dark at 0.1 mM SA, while free SA concentrations increased simultaneously in light and dark-grown plants after 1 mM SA treatment but the total and free SA content did not differ in the light and dark after 24 h of SA treatment. Gharbi et al. (2016) also found that exogenously applied SA at 0.01 mM enhanced the endogenous SA levels by about 100-fold in tomato plants. SA at appropriate concentration can activate defence responses, including activation of ROS scavenging enzymes and pathogenesis related (PR) gene expression. In accordance with this, it was demonstrated by our earlier experiments that applying 0.1 mM SA potentiated salt tolerance mechanism while pre-treatment with 1 mM SA induced cell death in tomato exposed to subsequent salt stress (Poór et al., 2011).

In the present study, we demonstrated that these SA treatments could affect $\cdot O_2^-$ and H_2O_2 metabolism differently in the presence or absence of light in tomato leaves (Fig. 11). Our results confirmed the

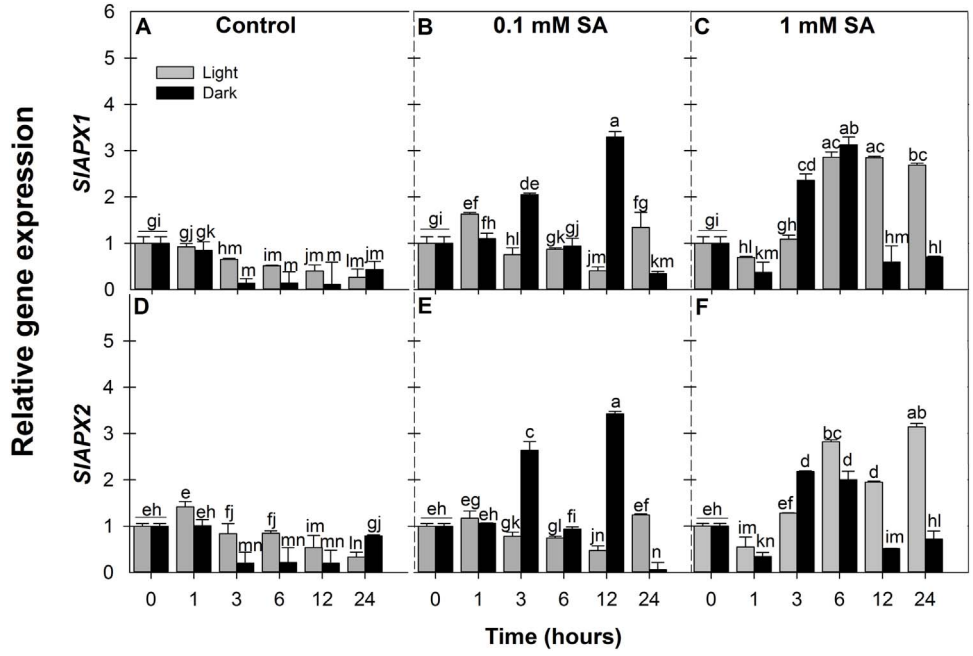

Fig. 10. Changes in the relative transcript levels of *SlAPX1* and *SlAPX2* as a function of time in leaves of tomato plants in the presence or absence of light (Light, Dark): control (A, D), 0.1 mM SA treatment (B, E), 1 mM SA treatment (C, F). Means + SE, n = 3. Data with different letters indicate significant differences at $P \leq 0.05$ level (Tukey test) in each time point.

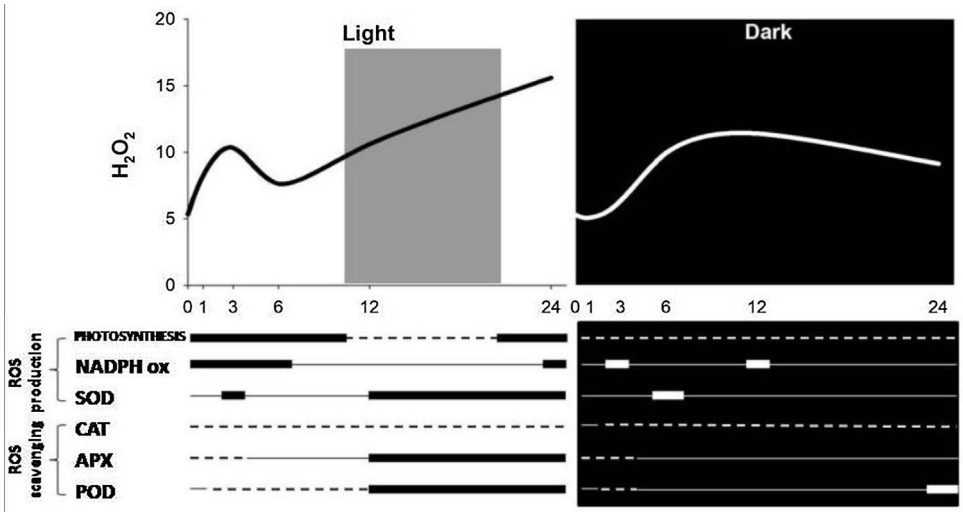

Fig. 11. Changes in H_2O_2 and enzymatic activities which play role in H_2O_2 metabolism after 1 mM SA treatment as a function of time compared in the light and dark. Thick lines show high (▬) or slight (▬) increases, dashed line shows decreases or inhibition in the enzyme activities as a function of time (ROS induced by photosynthesis; NADPH ox: NADPH oxidase; SOD: superoxide dismutase; CAT: catalase; APX: ascorbate peroxidase; POD: guaiacol-dependent peroxidase).

observation that H_2O_2 accumulation of young leaf tissues was stimulated in the light by SA, but it was delayed and the maximum was lower in the prolonged dark period. Moreover, 1 mM SA induced higher H_2O_2 levels in the illuminated leaves than 0.1 mM SA and in these leaves two peaks were detected, which cannot be observed in the plants kept in prolonged darkness. The lack of the first, priming burst of H_2O_2 in the dark at lethal concentration of SA suggests that the early source of H_2O_2 depends on the presence of light. Van Aken and Van Breusegem (2015) concluded that this early peak in H_2O_2 accumulation originated indirectly from PM localized NADPH oxidases or from other energy organelles, such as chloroplasts and mitochondria.

Several years ago it was found that a biphasic ROS burst elicited by biotic stress preceded cell death and the first maximum of ROS was measured within minutes after the initial stimulus and the second, more intense burst occurred several hours later (Dat et al., 2000; Van Aken and Van Breusegem, 2015). The first maximum is a priming burst that sets up the cell for cell death. Similar kinetics of ROS accumulation was found in tomato exposed to lethal salt stress (Takács et al., 2017) and biphasic ethylene production was also observed in tobacco leaf discs treated with H_2O_2, which led to severe necrosis and death of tissues (Wi et al., 2010).

Plant NADPH oxidases (Rbohs) localized to PM produce apoplastic ROS by reducing molecular oxygen to $\cdot O_2^-$ in the apoplast using cytoplasmic NADPH as reductant. There are 10 Rboh coding genes in *Arabidopsis* genom and the isoforms have been shown to participate in different processes. They are responsible for the fast moving ROS signal in the apoplast mediating SAR in several abiotic and biotic stresses (Jaspers and Kangasjärvi, 2010). A respiratory burst oxidase homologue was also found in tomato (SlRBOH1) that could be activated by Ca^{2+} and Ca^{2+}-dependent phosphorylation (Sagi and Fluhr, 2006).

The SA-induced activation of NADPH oxidase and $\cdot O_2^-$ production were well established in illuminated leaves (Agarwal et al., 2005), but it was not investigated in the dark. NADPH oxidase activity was induced from the 1st h upon 1 mM and from the 3rd h upon 0.1 mM SA treatment in the presence of light. In contrast to this, delayed activation of NADPH oxidase was measured at higher SA concentration under dark condition, which confirmed the importance of light in the activation of enzyme protein. The expression of *SlRBOH1* gene, encoding a subunit of NADPH oxidase was enhanced and exhibited two maxima in the light at both SA concentrations. An early increase in its expression was observed in the dark at 1 mM SA but the enzyme activity increased later. This suggests that while *SlRBOH1* gene was up-regulated by both SA treatments in illuminated samples, the enzyme activity increased at greater extent at cell death-inducing SA concentration only in the light. H_2O_2 in the cell wall can also be generated through the action of SOD on NADPH oxidase-produced $\cdot O_2^-$ or by the action of other enzymes, such as peroxidases, oxalate oxidase and amine oxidases (Jiménez-Quesada et al., 2016). It was found in our earlier work that terminal oxidation of the apoplastic putrescine and spermidine by diamine- and polyamine oxidases, respectively, was enhanced significantly by sub-lethal, 0.1 mM SA treatment, while it remained lower at 1 mM SA. This suggests that terminal oxidation of polyamines plays a minor role in the induction of cell death by SA in this system (Takács et al., 2016).

NADPH oxidase-mediated ROS can also communicate with ROS production in the chloroplast (Miller et al., 2009). Inhibition of the photosynthetic electron transport under stress conditions leads to excess excitation energy and the formation of ROS, which can affect the redox homeostasis in the chloroplast and cytoplasm under pathogen attack and abiotic stress. Thus, illumination of tissues and as a consequence, ROS generated by chloroplasts can be modulator of cell death.

It was also found that the positive control of HR involves interplay of chloroplastic and SA-dependent signals (Genoud et al., 2002). SA increased or did not affect the photosynthetic activity in tomato in 0.001–0.1 mM concentration interval but caused chronic photoinhibition and inhibited the photosynthetic electron transport at 1 mM in our system (Poór et al., 2011). This means that ROS production by the photosynthetic apparatus increased at lethal SA concentration. Since all genes encoding antioxidant enzymes and enzymes involved in the synthesis of non-enzymatic antioxidants are localized in the nucleus, plastid-associated signals such as changes in the redox status of photosynthetic electron transport chain, increased production of singlet oxygen ($^1O_2$) and enhanced production of the reduced forms of oxygen ($\cdot O_2^-$ and H_2O_2) have to be transmitted to the nucleus (Mittler et al., 2011). This retrograde signalling in combination with SA signal mediates defence gene activation or potentiates genetically encoded cell death pathway.

Plants possess scavenging systems that keep ROS formation below damaging level. The first step is the dismutation of $\cdot O_2^-$ into molecular oxygen and H_2O_2 by SOD isoenzymes. In contrast to other plants total SOD activity was not significantly affected by SA in the early phase of application and was only slightly activated by SA from the 12th h in the light. Each SOD isoenzymes seems to be regulated independently due to the oxidative stress within the subcellular compartments (Attia et al., 2009) but these differences can be masked at tissue level. In our system, the expression of the chloroplastic Cu/Zn-SOD and mitochondrial Mn-

SOD coding genes were increased already from the 6–12 h of 1 mM SA treatment in the light samples while there were no major changes at 0.1 mM SA. This suggests that the oxidative stress may activate the expression of chloroplastic Cu/Zn-SOD and mitochondrial Mn-SOD. Similar results were found in the activity of Cu/Zn-SOD, Mn-SOD and Fe-SOD under oxidative stress in methyl viologen-treated *Arabidopsis* plants (Alscher et al., 2002). In accordance with our results, high light induced a significant increase in the expression and activity of various SOD isoforms in *Arabidopsis* (Attia et al., 2009). Interestingly, the expression of *SlFeSOD* proved to be higher in the dark in 3-h-samples treated with SA and after 6 h in the control leaves.

The H_2O_2 produced by SOD is then eliminated by other scavenging enzymes such as CAT, APX and POD. Among the main groups of catalases Class I. catalases are highly expressed in leaves, they are light-dependent and are involved in the removal of H_2O_2 generated by photorespiration. Class II catalases are mainly found in vascular tissues and the members belonging to Class III group are localized to peroxisomes. The expression of certain CAT isoenzymes is regulated by circadian rhythm (Luna et al., 2005). A putative circadian regulation can be observed in relative transcript abundance of *SlCAT1* and *SlCAT3* in control leaves whereas *SlCAT2* shows a rapid decline as a function of time. Moreover, CAT protein is susceptible to photoinactivation leading to a decline in enzyme activities. It is well known that CAT activity is inhibited by SA (Agarwal et al., 2005; Horváth et al., 2007; Tari et al., 2015). Interestingly both SA treatments inhibited the activity of CAT enzyme from the 1st h in our system and it was independent of the illumination. Moreover, both SA treatments decreased the expression of *SlCAT1* and *SlCAT3* coding genes at 3 and 6 h in the light but unexpectedly, the expression of all three genes were elevated in the dark upon 0.1 mM SA treatment.

The other H_2O_2 scavenging enzyme, APX exhibited the highest light-dependency after SA treatments. In the first hours APX activity was inhibited by 1 mM SA, which can contribute in parallel with the inhibition of CAT activity to the first peak of H_2O_2 accumulation. Later, APX was activated in correlation with the relative transcript levels of *SlAPX1* and *SlAPX2* genes encoding cytosolic isoenzymes from the 6th h in the illuminated leaves at 1 mM SA. Interestingly, total activity of APX did not change at the same time under the dark condition. Earlier it was found that SA increased not only APX activity but also AsA content in many plants, which is the cosubstrate for APX in catalyzing the transformation of H_2O_2 into H_2O as part of the AsA/glutathione cycle (Khan et al., 2015). Similar results were found in AsA accumulation in tomato plants primed with SA under salt stress (Tari et al., 2015). To the best of our knowledge the light dependency of SA effect on the activity of APX has not been revealed until now. In contrast to APX, POD activity increased upon 1 mM SA treatment later, from the 12th h in the light and at 24 h in both environments. Thus, POD activity can contribute to the decomposition of H_2O_2 in the dark, which is particularly important in the absence of CAT and APX induction in SA-mediated protective actions.

5. Conclusion

It can be concluded that the early $\cdot O_2^-$- and H_2O_2 production of the leaves exposed to sublethal and lethal concentration of SA was different and the activities and expression levels of antioxidant enzymes displayed time- and SA concentration-dependent differences in the absence or presence of light.

The features associated with a HR, such as biphasic oxidative burst, activation of NADPH oxidase and thus the potentiation of cell death pathway, were activated only in the light upon 1 mM SA treatment. In these samples 1 mM SA induced NADPH oxidase activity, the expression *of SlRBOH1* gene and $\cdot O_2^-$ production after the first hours of SA treatment. Interestingly, the expressions of *SlMnSOD, SlCu/ZnSOD, SlCAT3, SlAPX1* and *SlAPX2* were much higher at 1 mM SA concentration in the light. This suggests that the expressions of these genes are regulated by oxidative stress but the enzyme proteins are individually regulated by ROS or other factors.

At the same time, SA treatments inhibited CAT activity from the first hour and decreased the activity of APX at 1 h, which was compensated by the induction of APX activity as well as by the expression of APX genes only from the 6th h, in the light. Moreover, 1 mM SA inhibited the photosynthetic electron transport in the same system in tomato leaves (Poór et al., 2011), which contributes to the higher ROS production in illuminated leaves.

Nevertheless, APX showed the most light-dependent activity after SA treatment, which was reduced in the dark but the activity of POD was induced under the same condition after 24 h in darkness. This observation suggests that POD can substitute APX activity in H_2O_2 decomposition in the dark. Tissues kept in prolonged darkness failed to exhibit HR-like responses, the activation of NADPH oxidases, they exhibited only one H_2O_2 peak with much moderate maximum and a greatly reduced antioxidant defence response. In accordance with this, SA induced cell death was reduced in these tissues (Takács et al., 2016).

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