Interaction between polyamines and ethylene in the response to salicylic acid under normal photoperiod and prolonged darkness

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Abstract

The impact of salicylic acid (SA) on ethylene (ET) production and polyamine (PA) metabolism was investigated in wild type (WT) and ET receptor mutant *Never ripe* (*Nr*) tomato leaves under normal photoperiod and prolonged darkness. *Nr* displayed higher ET emanation compared to WT under control conditions and after SA treatments, but the ET signalling was blocked in these tissues. The accumulation of PAs was induced by 1 mM but not by 0.1 mM SA and was higher in WT than in *Nr* leaves. Upon 1 mM SA treatment, which caused hypersensitive response, illuminated leaves of WT showed high spermine (Spm) content in parallel with an increased expression of S-adenosylmethionine decarboxylase and Spm synthase (*SlSPMS*) suggesting that this process depended on the light. In *Nr*, however, Spm content and the expression of the *SlSPMS* gene were very low independently of the light conditions and SA treatments. This suggests that Spm synthesis needs functional ET perception. In WT leaves 1 mM SA enhanced putrescine (Put) synthesis by increasing the expression of Put biosynthesis genes, arginine and ornithine decarboxylases under darkness, while they were down-regulated in *Nr*. The activities of diamine (DAO) and polyamine oxidases (PAO), however, were generally higher in *Nr* compared to the WT after SA treatments. In *Nr* both SA applications increased the expression of *SlPAO1* under normal photoperiod, while *SLPAO2* was down-regulated in the dark suggesting a diverse role of PAOs in PA catabolism. These results indicated that ET could modulate the SA-induced PA metabolism in light-dependent manner.

Key words:

Ethylene, Illuminated and dark samples, *Never ripe* mutant, Polyamines, Salicylic acid, Tomato

Abbreviations:

AC	Ailsa Craig
ACC	1-aminocyclopropane-1-carboxylic acid
ACS	ACC synthase
ACO	ACC oxidase
ADC	Arginine decarboxylase
CTR	Constitutive triple response 1
DAO	Diamine oxidase
dcSAM	Decarboxylated SAM
EIN	Ethylene insensitive
ER	Endoplasmic reticulum
ET	Ethylene
Nr	*Never ripe* tomato (ethylene receptor mutant)
ODC	Ornithine decarboxylase
PAs	Polyamines
PAO	Polyamine oxidase
Put	Putrescine
SA	Salicylic acid
SAM	S-adenosylmethionine
SAMDC	S-adenosylmethionine decarboxylase
Spd	Spermidine
SPDS	Spermidine synthase
Spm	Spermine
SPMS	Spermine synthase

1. Introduction

Plant signalling pathways are now recognized as being highly networked, thus the plant hormone ethylene (ET) and polyamines (PAs) are in interaction with other plant growth regulators such as salicylic acid (SA) during stress acclimation (Arif *et al.* 2020; Nehela and Killiny, 2020;). SA is a phytohormone required for plant defence against biotrophic pathogens, whereas jasmonate and ET participate in defence reactions initiated by herbivores, insects or necrotrophic pathogens (Yang *et al.* 2015).

Beyond stress responses, the gaseous phytohormone ET regulates several phases of plant growth and development. ET is synthesized from S-adenosylmethionine (SAM) in two enzymatic steps. The conversion of SAM to 1-aminocyclopropane-1-carboxylic acid (ACC) and then the oxidation of ACC to ethylene is catalysed by ACC synthase (ACS, EC 4.4.1.14) and ACC oxidase (ACO, EC 1.14.17.4), respectively, which is a general biosynthetic pathway of ET in all higher plants (Sun *et al.* 2017).

ET perception is mediated by various ET receptors. Seven ET receptors (LeETR1-7, including Never ripe (Nr) have been identified in tomato (Kamiyoshihara *et al.* 2012). The protein of tomato Nr shows high homology to ETR3, the ET receptor in *Arabidopsis* (Zhong *et al.* 2008), but from a historical point of view, SlETR3 has been named as “Never ripe” in the literature. This mutant shows insensitivity to ET not only in the fruit ripening but also in leaf petiole epinasty, in the triple response and in the senescence of petals and flowers. At the same time, *Nr* mutants are able to produce ET. This was observed under pathogen infection confirming that *Nr* plants are not impaired in ET biosynthesis (Lanahan *et al.* 1994). ETR1-7 proteins are localized in the endoplasmic reticulum (ER) membranes together with other downstream elements of the ET signalling pathway, such as the negative regulator, Raf-like serine/threonine kinase CONSTITUTIVE TRIPLE RESPONSE 1 (CTR1) and the positive regulator ETHYLENE INSENSITIVE 2 (EIN2) proteins. The downstream transcription factors EIN3, EIN3-LIKE 1 (EIL1) and ET response factors (ERFs) are nuclear proteins. The ET and polyamine (PA) biosynthetic pathways may compete for their common precursor, SAM, which is the substrate of ACS and SAM decarboxylase (SAMDC, EC 4.1.1.50) in the synthesis of ACC and longer chain PAs, spermidine (Spd) and spermine (Spm), respectively (Majumdar *et al.* 2017).

PAs are low-molecular-weight organic polycations that can regulate a wide range of physiological processes, including growth and development, as well as abiotic and biotic stress responses. They can stabilize membranes, scavenge free radicals, inhibit proteases and may

activate several antioxidant enzymes (Pál and Janda 2017). The diamine putrescine (Put), the triamine Spd and tetramine Spm are the most important PAs in plants. PA biosynthetic pathway begins with the decarboxylation of ornithine, by ornithine decarboxylase (ODC, EC 4.1.1. 17), which directly produces Put whereas the pathway through arginine decarboxylase (ADC, EC 4.1.1.19) is a multi-step process. It has been revealed recently that plants can use ADC and arginase/agmatinase (ARGAH) enzyme system as a third route for Put synthesis (Patel *et al.* 2017). From Put, Spd and Spm are produced by the addition of aminopropyl groups, transferred from decarboxylated SAM (dcSAM), which is synthetized from SAM by SAM decarboxylase (SAMDC). The final steps of the Spd and Spm synthesis are catalysed by Spd synthase (SPDS, EC 2.5.1.16) and Spm synthase (SPMS, EC 2.5.1.22) enzymes (Moschou *et al.* 2008a), respectively.

The relationship between ET and PAs in plants exposed to various abiotic and biotic stresses have been examined by several authors, but the currently available data is contradictory (Pál and Janda 2017). Infection with citrus exocortis viroid enhanced ET production in purple passion (*Gynura aurantiaca*) and tomato plants, which was accompanied by a decline in ODC activity leading to a decrease in Put levels. Furthermore, the reduction in Put concentration cannot be observed in the presence of ET biosynthesis inhibitors or modulators that prevented the ET signalling, supporting also that ET exerted an inhibitory effect on ODC activity (Jimenez-Bremont *et al.* 2014). Moreover, the transcripts of *ACS6* were downregulated in Put accumulating, transgenic *Arabidopsis* leaves overexpressing *ADC* (*35S:AtADC2*) (Alcazar *et al.* 2005). Transgenic tomato line expressing the yeast *SPDS* exhibited high susceptibility to the fungus *Botrytis cinerea.* These leaves contained a relatively high level of Spd and showed an attenuated expression of ET biosynthetic and signalling genes (Nambeesan *et al.* 2012) supporting a fine control between ET and PA level.

In contrast to these observations, there was no direct antagonistic interaction between PA and ET biosynthetic pathways in rice (Quinet *et al.* 2010). Others observed that high Spd and Spm levels in transgenic tomato fruits overexpressing yeast *SAMDC* and *SPDS* genes were accompanied by the accumulation of *ACS* transcripts and increased ET production (Nambeesan *et al.* 2010). Similar changes were found in the leaves of transgenic *Arabidopsis* overexpressing *SPMS* (*35S:AtSPMS-9*) (Gonzalez *et al.* 2011). The biosynthetic interaction between PAs and ET is often considered as competitive (Pandey *et al.* 2009). However, it was found that the availability of SAM was not rate-limiting *in vivo* for ET or Spd/Spm biosynthesis and that both pathways could run simultaneously (Mehta *et al.* 2002).

125 Nevertheless, more data from other mutant plants such as ET receptor or signalling
126 mutants are needed to prove the relationship between ET and PA biosynthesis.
127 PAs accumulation was accompanied by the induction of PA catabolism, which is
128 regulated mainly by two classes of amine oxidases. The copper-dependent diamine oxidases
129 (DAOs, EC 1.4.3.6) show a high affinity for Put and have a low preference for Spd and Spm.
130 The other group, the flavin-containing polyamine oxidases (PAOs, EC 1.5.3.3) may catalyse
131 the terminal oxidation of Spd and Spm but certain isoenzymes participate in the back-
132 conversion pathway, converting Spm to Spd and Spd to Put. Catabolism of PAs contributes to
133 stress signalling through the generation of H_2O_2 and nitric oxide (NO). Various roles of DAO
134 and PAO have been found in plant growth and development, in response to abiotic stresses, and
135 in defence responses against phytopathogens through H_2O_2 signalling pathways (Moschou *et*
136 *al.* 2008a).
137 Thus the accumulation of free PAs and their further oxidation by DAOs and PAOs plays
138 a significant role in plant defence reactions against viruses, bacterial and fungal pathogens (Pál
139 and Janda 2017). Exogenous Spm application could activate a subset of hypersensitive response
140 (HR)-specific genes (Takahashi *et al*. 2004) and defence responses in *Arabidopsis* during
141 cucumber mosaic virus infection (Mitsuya *et al.* 2009).
142 In *Arabidopsis*, AtCuAO3, the enzyme localizing in peroxisomes, was responsive to
143 several stimuli such as SA, flagellin and methyl jasmonate, but not to wounding or to the ET
144 precursor ACC (Planas-Portell *et al.* 2013). Despite of increased PA degradation, exogenous
145 SA application could improve the accumulation of free PAs (Put, Spd and Spm) in the fruits of
146 tomato (Zhang *et al.* 2011) and both SA and Spm have been found to promote plant resistance
147 to several pathogens (Raju *et al.* 2009).
148 SA can also inhibit the synthesis of ET by inhibiting the conversion of SAM to ACC
149 catalysed by ACC synthase and then ACC oxidation to ET by ACO (Vanderstraeten and Van
150 Der Straeten 2017). In contrast to this, it was observed that SA induced the ET production in
151 *Solanum lycopersicum* cv. Ailsa Craig (Takács *et al.* 2018) and in *Solanum chilense* and the
152 expression of *ACO* was also up-regulated by SA in the latter species (Gharbi *et al.* 2016).
153 Previously we found that 0.1 and 1 mM SA caused distinct changes in PA metabolism
154 (Takács *et al.* 2016); however, SA and PAs crosstalk and the modulation of PA metabolisms
155 by SA are almost unknown in plants defective in active ET signalling.
156 Light is not only an energy source but also one of the most significant environmental
157 factors for plant growth and development. Light conditions have been shown to strongly

158 influence hormone-regulated defences. In the dark, a reduced lesion formation was found in
159 response to non-host and avirulent pathogens. In addition, systemic acquired resistance (SAR)
160 development was completely lost in the absence of light (Zeier *et al.* 2004). SA accumulation
161 and the SA-regulated signalling (Genoud *et al.* 2002), as well as ET levels and signalling, were
162 also modulated by the presence or absence of light (Fukao *et al*. 2012). It was found by other
163 authors that prolonged darkness caused significant ET emission, chlorophyll- and
164 carbohydrates breakdown or recycling of chloroplast proteins (Lim *et al.* 2007). The sensing of
165 ET by ET receptors is also controlled by light conditions (Wilson *et al.* 2014). These data show
166 that significant differences can be observed in the hormonal responses of plants if an SA-
167 inducing pathogen attack occurs in illuminated plants or in the dark.
168 Our current information about the ET-mediated effects on the transcription and activity
169 of PA metabolic enzymes in the presence of SA is incomplete and to the best of our knowledge
170 the ET, PA and SA interactions, which are common under biotic stress, has not been
171 investigated and compared under normal photoperiod or under darkness in detail.
172 In this study, SA was applied in hydroponic culture through the roots at 0.1 and 1 mM
173 concentrations, since it was previously observed that the lower concentration did not induce
174 HR, a special type of cell death, while 1 mM resulted in HR-like necrotic spots in wild type
175 (WT) tomato leaves in this experimental system (Takács *et al.* 2016). Our experiments are
176 aimed to investigate the role of ET signalling in SA-induced PA metabolism in WT and in ET
177 receptor mutant *Nr* tomato under different light conditions. These results can reveal new
178 information about the SA-mediated defence reactions, the role of ET and PA interaction in this
179 process under normal photoperiod and in the dark.
180
181 **2. Materials and methods**
182 **2.1. Plant materials and growth conditions**
183 Seeds of tomato *Solanum lycopersicum* cv. Ailsa Craig wild type plants (WT) and ethylene-
184 insensitive *Nr* mutants were germinated in perlite. 14-day-old seedlings were transferred to
185 plastic boxes containing modified Hoagland solution (Takács *et al.* 2018). The nutrient solution
186 was changed two times a week. Tomatoes were grown in controlled condition at 24/22 ^{o}C under
187 a 12/12-h light/dark period. Light intensity was 200 μmol m^{-2} s^{-1} (F36W/GRO lamps, OSRAM
188 SYLVANIA, Danvers, MA, USA) and relative humidity was maintained at 55–60 % for 8
189 weeks.

Plants were treated with 0.1 mM or 1 mM SA for 24 h via the hydroponic solution when they were 8 weeks old. One group of tomatoes remained under the normal light/dark cycle (light samples) and the second group was treated under prolonged darkness at the beginning of the 12-h light period (dark samples) at 24 ^{o}C. Samples were prepared from fully expanded young leaves at least in five replicates. All experiments were started from 9 a.m. to 9 a.m. of the next day and were repeated 3-4 times in independent experiments.

2.2. Measurement of ethylene production

The ET production was measured in six replicates with gas chromatograph (Hewlett-Packard 5890 Series II) equipped with a flame ionization detector and alumina column (Takács *et al.* 2018). Leaf samples (0.5 g) were incubated in 25 mL gas-tight vials sealed with rubber serum caps under darkness at 25 ^{o}C for 1 h. 2.5 mL from the air of the tubes was injected into the instrument. Flow rates were 35 mL min^{-1} for He, 30 mL min^{-1} for H_2, and 300 mL min^{-1} for air. The oven, injector, and detector temperatures were 100, 120, and 200 °C, respectively. A set of ET standards was applied to calculate the ET concentration emitted by the leaves.

2.3. Quantification of free polyamine contents

Free PAs were determined from 200 mg leaf fresh weight (FW) (Takács *et al.* 2016). Samples were homogenized in 5 % (v/v) cold perchloric acid and then were kept on ice for 20 min. After centrifugation (10.000 g for 20 min at 4 ^{o}C) 0.4 mL of 2 M NaOH was added to 1 mL of plant extract. The mixture was then vortexed and after the addition of 10 μL benzoyl chloride, it was vortexed again and stored at 25 ^{o}C for 30 min. The benzoylated PA derivates were removed from the aqueous phase by 1.2 mL diethyl ether. An aliquot (0.75 mL) of the organic solvent phase was evaporated to dryness and the residue was resuspended in 200 μL of acetonitrile. Samples were injected onto a reverse-phase column (4.6 mm x 250 mm, 5 μm, Apex octadecyl) at 25 ^{o}C. Analyses were performed at 254 nm by a JASCO high-performance liquid chromatography (HPLC) system coupled to an UV detector (JASCO HPLC system, Japan). The mobile phase consisted of a water/acetonitrile, 55/45 (v/v) mixture applied at a flow rate of 1.0 mL min^{-1}. For PA determination, standards of Put, Spd, and Spm (Sigma-Aldrich, St. Louis MO, USA) were used at 1 mM concentration.

2.4. RNA extraction and Quantitative Real-Time PCR gene analyses

Total RNA was extracted from 100 mg of leaf tissues using the TRIzol method as described in Takács *et al.* (2016). In brief, the samples were incubated in 1 mL TRIzol followed by addition 200 µL chloroform and agitated vigorously for 15 s. Then the tubes were centrifuged for 15 min at 10.000 g at 4 ^{o}C. The upper clear aqueous layer was transferred to a new 1.5 mL tube, and 375 µL chloroform:isoamyl alcohol was added. After 15 seconds of shaking the tubes were centrifuged for 15 min at 10.000 g at 4 ^{o}C. The aqueous layer was transferred to a new 1.5 mL tube and 500 µL of isopropanol was added. After gentle mixing, the tubes were placed on the benches for 10 min at room temperature followed by centrifugation at 10.000 g for 15 min at 4 ^{o}C. The supernatant was discarded and the remaining pellet was washed with 1 mL of 70 % (v/v) ethanol and air-dried for about 10 min. The purified RNA was dissolved in 30 µL of RNase-free water. The RNA concentration and purity were determined with Nanodrop (Eppendorf, Hamburg, Germany) and agarose gel electrophoresis. RNA samples were subjected to DNase I treatment, then the first-strand cDNA was synthesised using reverse transcriptase (Thermo Scientific, Waltham, MA USA) followed the manufacturer's instructions. Gene-specific primers, used for qRT-PCR analyses were mined from Sol Genomics Network (SGN) and National Centre for Biotechnology Information (NCBI) databases. Primers were designed using Primer 3 software (Supplemental Table 1.). Relative transcript accumulation was determined by SYBR Green by quantitative Real-Time PCR (Piko Real-Time qPCR System, Thermo Scientific, Waltham, MA USA). The reaction mixture for PCR analysis contained 10 ng of cDNA template, 400 nM of forward and reverse primers, 5 µL of Maxima SYBR Green qPCR Master Mix (2X) (Thermo Scientific, Waltham, MA USA) and nuclease-free water in a total volume of 10 µL. One cycle of 95 ^{o}C for 7 min is followed by 40 cycles of 95 ^{o}C for 15 s, and 60 ^{o}C for 60 s during the RT-PCR program. After 40 cycles, melting curves were generated by increasing the temperature from 55 to 90 ^{o}C (0.2 C s^{-1}). Data were analysed using PikoReal Software 2.2 (Thermo Scientific, Waltham, MA USA). The fold change value was calculated using the expression $2^{-\Delta\Delta Ct}$ method with the 18S rRNA and elongation factor-1α subunit (*EF1α*) genes of tomato as reference. The obtained results were transformed to $\log_2$ scale.

2.5. Diamine oxidase and polyamine oxidase activity assays

Diamine oxidase (DAO, EC 1.4.3.6) and polyamine oxidase (PAO, EC 1.4.3.4) activities were assayed according to Takács *et al.* (2016). 200 mg of leaf samples were pulverized under liquid nitrogen. 0.6 mL of extraction buffer [100 mM K phosphate buffer (pH 6.6) containing 0.2 M

TRIS (hydroxymethyl)aminomethane (pH 8.0); 10 % glycerol; 0.25 % Triton X-100; 0.5 mM phenylmethylsulfonyl fluoride (PMSF) and 0.01 mM leupeptin] was added to the fine powder of plant tissues. The homogenate was incubated on ice for 20 min, then centrifuged at 7.000 g for 10 min at 4 ^{o}C. The supernatant was applied as the crude enzyme extract. 0.15 mL of this extract, 0.6 mL of 100 mM potassium phosphate buffer (pH 6.6), 50 U of catalase in 50 μL volume, 50 μL of 2-aminobenzaldehyde (0.1 %) and 150 μL of 20 mM Put for DAO or 150 μL of 20 mM Spd for PAO determination were mixed and incubated at 37 ^{o}C for 1.5 h. Then the reaction was stopped with 50 μL of 20 % (w/v) trichloroacetic acid (TCA) and samples were centrifuged (5000 g, 10 min). The formation of Δ^1-pyrroline product was measured by spectrophotometer (KONTRON, Milano, Italy) at 430 nm. The enzyme activity was expressed in nmol Δ^1-pyrroline min^{-1} g^{-1} FW using an extinction coefficient of $1.86x10^3$ mol^{-1} cm^{-1}.

2.6. Statistical analysis

The experiments were repeated 3-4 times. Every investigated parameter had five replicates with the exception of the ET production measurements that had six replicates. Values presented are the averages with standard errors from all experiments. The fold change in gene expression was calculated using Microsoft Excel and was expressed as $\log_2$. Data were analysed using one-way analysis of variance (ANOVA). Statistical analysis was carried out using Sigma Plot 12.0 statistical software (Systat Software Inc., Erkrath, Germany). After ANOVA, Duncan's multiple range test was performed ($P < 0.05$).

3. Results

3.1. Ethylene production

To investigate the role of ET perception in SA-induced PA-ET interaction, the ET production of WT and *Nr* leaves was measured after treatment with 0.1 mM or 1 mM SA under light and dark conditions. Control samples of ET receptor mutant *Nr* leaf exhibited higher ET production than WT under both environmental conditions (Fig. 1A). In WT SA treatments did not affect the ET synthesis under normal photoperiod, however, in *Nr* leaves the maximum of ET emanation was observed in response to 0.1 mM SA. Unexpectedly, ET production decreased slightly compared to the normal photoperiod in the controls of both genotypes under prolonged darkness. Upon 0.1 mM SA treatment in the dark the ET production increased in WT leaves, while it declined to the level of the untreated control in the presence of 1 mM SA (Fig. 1A, B, C). In *Nr* leaves, however ET emanation decreased in response to both SA treatments under

prolonged darkness but it remained higher compared to WT in control samples and in the presence of 1 mM SA (Fig. 1A, C).

3.2. Changes in polyamine concentrations

In control plants Put content was similar, but Spd and Spm levels decreased significantly in *Nr* leaves compared to WT under normal photoperiod. In the dark, however Put concentration was lower in both genotypes and the decline was more pronounced in the mutants (Fig. 2A). In contrast to Put, Spd and Spm levels increased in the leaves of WT while in *Nr*, they remained unaffected compared to light controls under prolonged darkness (Fig. 2D, G).

Put content increased gradually as a function of SA concentration in WT in both environments and reached a much higher value in the dark than in the light at 1 mM SA. In *Nr* leaves the Put levels exhibited near-constant values in the light upon SA treatments, while in the dark they showed a concentration-dependent increase reaching a maximal value after the application of 1 mM SA. However, Put content remained significantly lower in *Nr* plants than in WT upon SA treatments under prolonged darkness (Fig. 2A, B, C).

Spd content slightly increased in the light; but it remained approximately at control level under darkness in WT plants exposed to 0.1 mM or 1 mM SA (Fig. 2D, E, F). Under normal photoperiod, Spd concentration exhibited a maximum in the leaves of *Nr* plants in response to 0.1 mM SA, while it was enhanced in response to SA in a concentration-dependent manner under prolonged darkness (Fig. 2E, F).

1 mM SA induced a significant accumulation of Spm in the WT under normal photoperiod, but under prolonged darkness, the application of both SA concentrations decreased the Spm level of WT leaves compared to untreated controls (Fig. 2G, H, I). In contrast, the Spm content of the mutants was not affected by SA treatments under light and dark conditions and it remained at a very low level compared to WT in all experimental arrangements (Fig. 2G, H, I).

3.3. Changes in the expression levels of polyamine biosynthesis genes

The mRNA levels of the tomato PA biosynthesis genes coding for arginine decarboxylase (*SlADC*), ornithine decarboxylase (*SlODC*) (Fig. 3), S-adenosylmethionine (SAM) decarboxylase (*SlSAMDC*), spermidine synthase (*SlSPDS*) and spermine synthase (*SlSPMS*) (Fig. 4) were determined in WT and *Nr* leaves after 24 hours of 0.1 mM and 1 mM SA treatments under light or dark conditions.

In controls the expression of *SlADC* and *SlODC* was suppressed by dark treatment in both WT and *Nr* leaves, suggesting that the lack of illumination inhibited the *ADC* and *ODC* expression independently of ethylene signalling in tomato (Fig. 3A, D).

In the illuminated leaves, the expression of *SlADC* genes changed only slightly after SA treatments but unexpectedly, the expression of *SlODC* gene was down-regulated by the application of 0.1 mM SA in WT and by 1 mM SA in both genotypes in the light (Fig. 3 E, F). On the other hand, the Put biosynthesis genes were induced (or the inhibition was alleviated) by SA in the dark, in WT especially at 1 mM, while a significant down-regulation could be observed in all treatments in *Nr* leaves compared to the respective WT plants (Fig. 3B, E, C, F).

The changes in the expression of *SlSAMDC*, *SlSPDS* and *SlSPMS* genes encoding enzymes involved in the synthesis of dcSAM, Spd and Spm. are summarized in Fig. 4.

Under the control condition, the expression levels of *SlSAMDC* and *SlSPDS* were higher in the dark than under normal photoperiod, however, the transcript abundance of *SlSPMS* decreased after 24 h of dark treatment in WT. Interestingly, the expression of all genes (*SlSAMDC*, *SlSPDS* and *SlSPMS*) were suppressed by the dark treatment in *Nr* leaves under control condition (Fig. 4A, D, G).

Under normal photoperiod, the expression of *SlSPDS* significantly decreased, but *SlSAMDC* and *SlSPMS* transcripts showed no significant differences compared to the controls in WT leaves in response to 0.1 mM SA. In contrast, in illuminated *Nr* plants, the expression of *SlSPDS* was significantly induced, but those of the *SlSAMDC* and *SlSPMS* were suppressed by 0.1 mM SA treatment (Fig. 4B, E, H). In the dark a very similar expression pattern was observed for *SlSAMDC*, *SlSPDS* and *SlSPMS* genes in WT and *Nr* leaves treated with 0.1 mM SA as in untreated controls (Fig. 4B, E, H).

The expressions of *SlSAMDC*, *SlSPDS* and *SlSPMS* were markedly upregulated by 1 mM SA treatment in illuminated WT leaves, but with the exception of *SlSAMDC*, their expression level showed no significant difference under darkness compared to the respective untreated controls (Fig. 4C, F, I). In illuminated *Nr* leaves, transcription of *SlSAMDC* was induced, while the expression of *SlSPDS* and *SlSPMS* remained at control level at 1mM SA (Fig. 4C, F, I). Additionally, under dark condition, the relative transcript levels of *SlSAMDC* was enhanced in response to 1 mM SA treatment in *Nr* leaves compared to the untreated control while *SlSPMS* remained downregulated (Fig. 4C, F, I).

3.4. Changes in the activity of polyamine catabolic enzymes

To investigate the role of ET perception in SA-induced PA catabolism under light and dark conditions, we compared the activities of the enzymes involved in PAs degradation in WT and *Nr* leaves after 0.1 mM and 1 mM SA treatments. Interestingly, *Nr* leaves exhibited basically higher DAO and PAO activities compared to WT (Fig. 5; Fig. 6). DAO activities in the controls of WT and *Nr* leaves under prolonged darkness showed a similar pattern as in the light, but the increases in *Nr* were more significant. However, PAO activity declined in the dark especially in the mutant leaves compared to the samples exposed to normal photoperiod (Fig. 6, A).

Total DAO activity in illuminated leaves increased as a function of SA concentration, but *Nr* plants responded already to 0.1 mM SA whereas WT leaves only to 1 mM SA with significant increments and its activity showed a similar pattern in WT plants under dark condition (Fig. 5B, C). In contrast, after 24-hour-long treatments, DAO activity in *Nr* leaves declined significantly at 0.1 mM SA while reached a maximum at 1 mM SA under darkness (Fig. 5B, C).

The activity of PAO displayed no significant difference upon exposure to 0.1 mM and 1 mM SA treatments in illuminated WT leaves, however, the activity of the enzyme increased at 1 mM SA in the dark, when compared with respective untreated controls (Fig. 6A, B, C). The leaves of ET receptor mutant *Nr* plants showed much higher PAO activities than those of WT. A significant decrease was found in the enzyme activity after treatment with 1mM SA in the light, but PAO activity increased significantly at both SA concentrations under prolonged darkness (Fig. 6B, C).

3.5. Changes in the expression of polyamine catabolic genes

The expression of *SlDAO1* and *SlDAO2* genes encoding enzymes involved in Put catabolism was suppressed in the dark in the controls of both genotypes (Fig. 7A, D). In the light, the transcript levels of *SlDAO1* and *SlDAO2* decreased in WT as a function of SA concentration, while they were slightly upregulated in *Nr* leaves (Fig. 7B, C, E, F). Under the dark condition, the expression of *SlDAO1* and *SlDAO2* were upregulated by SA in both genotypes when compared to respective untreated controls (Fig. 7B, C, E, F), which means an alleviation of the inhibition.

In the illuminated WT leaves, there was no obvious difference between the transcript levels of *SlPAO1* and *SlPAO2* upon SA treatments. However, both SA treatments had pronounced effects on the expression of *SlPAO1* in *Nr* leaves under light condition, which was

significantly up-regulated (Fig. 8B, C, E, F). Transcript accumulation of *SlPAO1* and *SlPAO2* was generally reduced in the dark in both WT and *Nr* leaves, which was more pronounced in the case of *SlPAO2* (Fig. 8A, B, C, D, E, F). However, the SA exposure slightly alleviated this inhibition in the transcript levels of *SlPAO1* in the WT (Fig. 8A, B, C). However, the transcript levels of *SlPAO2* declined after both SA treatments and this down-regulation was significantly higher in *Nr* than in WT leaves in the dark (Fig. 8D, E, F).

4. Discussion

To better understand the role of ET and PAs in the SA-induced defence mechanism under light and dark condition, we analysed the PA metabolism in the leaves of WT and ET receptor mutant, *Never ripe* tomato plants. In vegetative, non-senescing organs of WT plants, the ET biosynthesis is regulated by a feedback inhibition of ACC synthases by ET, which cannot be observed in *Nr* mutants, which have disrupted ET perception. In accordance with previous results, higher ET production could be detected in untreated controls of *Nr* than in WT, which was reduced slightly but not significantly under prolonged darkness. A similar result was also supported by Lanahan *et al.* (1994), who found that biosynthesis of ET was not impaired in *Nr* mutants.

In the present study, it was found that none of the SA treatments affected ET production under normal photoperiod, while under prolonged darkness ET emanation showed a maximum at 0.1 mM SA in WT plants. These results suggest that the ET biosynthesis in the WT is more sensitive to SA in the dark than under normal photoperiod. Unexpectedly in *Nr* plants, the ET production declined as a function of increasing SA concentrations under both circumstances.

An interesting relationship has been revealed between ET production and PA accumulation in controls of WT plants and *Nr* mutants. While Put content was the same in the controls of the two genotypes, Spd and especially Spm accumulation was significantly reduced in *Nr* compared to WT in both two environments. Since ET production is much intensive in the tissues of *Nr*, this phenomenon raises the possibility of an enhanced shunt of SAM towards the ET biosynthesis. The other important observation is that Spm content increased in the dark in control WT samples, while it remained at a very low level in *Nr* leaf tissues in both environments suggesting that Spm accumulation is minimal in the absence of functional ET signalling.

However, information about the mechanism of SA-induced changes in PA metabolism and the role of ET in this process has been limited. The present study has demonstrated that

exogenous treatment with 0.1 mM SA concentration for 24 h had almost no impact on PA content in WT under normal photoperiod, and there are smaller changes (increased Put and reduced Spm content) in the dark. Similar results were found by Gharbi *et al.* (2016) in the Ailsa Craig tomato cultivar, where the Put, Spd and Spm concentrations of shoot remained unaffected by the application of 0.01 mM SA under normal photoperiod.

Significant increases in PA accumulation could be observed, however, after treatment with 1 mM SA, which resulted in necrotic spots later in leaf tissues.

One of the most important observations of these experiments is that an intensive Put accumulation occurred in WT in both environments, first of all in the dark at the higher SA concentration, which cannot be detected in *Nr* plants. However, in WT plants, Spd and especially Spm accumulated significantly as a function of increasing SA concentrations in the light, but only smaller changes were found in dark samples.

The homeostasis of PA biosynthesis and catabolism is a key factor in PAs-regulated stress tolerance. Since the common substrate for ET, Spd and Spm biosynthesis is SAM, the control of PA metabolism by ET by the promotion or the inhibition of the expression or activities of PA biosynthetic enzymes was suggested (Harpaz-Saad *et al*. 2012). The accumulation of transcripts related to PA biosynthesis also depends on the illumination of tissues. These experiments revealed some general rules about the primary role of ET signalling in certain steps and that of light in other reactions in SA-induced PA metabolism.

In the control samples the expression of *SlADC* and *SlODC*, the enzymes participating in the synthesis of Put, was reduced very significantly in the dark in WT and *Nr* plants suggesting that the effect of dark treatment has not been influenced by the block in ET signalling in the absence of SA. Interestingly, *SlSAMDC* and *SlSPDS* in the WT were up-regulated in the dark in contrast to the expression of the same genes in *Nr* and *SlSPMS* genes, which were down-regulated.

The expression of the two genes in Put synthesis, *SlADC* and *SlODC* was only slightly sensitive to SA with small up and down changes in the light, but as a function of SA concentration, much characteristic induction occurred in the dark, which means significant up-regulation in the WT, and an alleviation of the inhibition in the *Nr* mutants. This means that the enzymes of Put biosynthesis responded to exogenous SA in a light- and ET-dependent manner. The latter means that in the absence of active ET signalling the expression of these genes remained inhibited in the presence of SA. Since the changes in Put level correlated well with

452 the expression pattern of *SlADC* (and less strictly with *SlODC*), it can be concluded that Put
453 accumulation is controlled efficiently by the synthesis in these tissues.

454 PAs with longer carbon skeleton are synthesized from Put by incorporating the
455 aminopropyl group generated by the activity of SAMDC from SAM (Tiburcio *et al.* 2014). The
456 expression of this enzyme was highly dependent on SA concentration and was induced very
457 effectively in the presence of the 1 mM SA. In WT leaves the mRNA levels of *SlSAMDC*
458 increased very significantly by HR-inducing SA concentration compared to untreated controls
459 in WT and *Nr* genotypes, in latter cases this meant a gradual alleviation of the inhibition of
460 gene expression in the dark. Similar results have been found during exogenous SA application
461 when *SAMDC* gene expression increased in illuminated tomato shoot (Gharbi *et al.* 2016). The
462 expression of *SlSPDS* gene exhibited a similar pattern as *SAMDC* in general, but the changes
463 were not so pronounced. More characteristic changes occurred in *SlSPMS* expression upon SA
464 treatments. High Spm content in the illuminated leaves of WT developed in parallel with the
465 increased expression of *SlSAMDC* and *SlSPMS*, which supported that this process depended on
466 the light. In *Nr*, however, Spm content and the expression of *SlSPMS* gene were very low
467 independently of the light conditions and SA treatments. This suggests that Spm synthesis needs
468 functional ET perception. It can be concluded, that exogenous SA, especially at a cell death-
469 inducing 1 mM concentration, may control the expression of the biosynthetic enzymes of higher
470 PAs but the final effect depends on the intact ET signalling and/or on the light conditions.
471 Moreover, the changes in the expression of PA biosynthesis genes correlated well with the free
472 PA accumulation found in the same tissues.

473 The levels of PAs in plant tissues are also determined by the activity of the degrading
474 enzymes. DAO may oxidise Put and Spd and PAO Spd and Spm yielding H_2O_2, thus they affect
475 not only free PA levels but H_2O_2 homeostasis, too (Moschou *et al.* 2008a). In this study, *Nr* leaf
476 exhibited generally higher DAO and PAO activities compared with WT even under control
477 conditions. The activity of PAO was much sensitive to the illumination and exhibited a
478 decreased activity in the dark especially in *Nr* controls. DAO activity increased as a function of
479 SA concentration, and in *Nr* leaves it responded already to the 0.1 mM SA in the light and to 1
480 mM SA in prolonged darkness. PAO activity of WT plants remained constant, but in *Nr* leaves
481 exhibited a gradual decline with increasing SA concentration under normal photoperiod, but in
482 the dark, its activity reached maximal values both in WT and *Nr* plants at 1 mM SA. In our
483 experiments, total activity DAO and PAO activities show only a weak correlation with Put and
484 Spm content.

Since the oxidation of PAs contribute to oxidative stress in plant cells, and at the same time PAs may function as antioxidants, PA metabolism is crucial for successful stress acclimation. Several enzymes can produce ROS in the cell wall of plants, such as PM-localized PAO, NADPH oxidase, cell wall- localized peroxidases and amine oxidases, which determine stress acclimation (Moschou *et al.* 2008b). In our previous study, it was found that H_2O_2 levels are higher in *Nr* leaves than in WT lines under control, 0.1 mM and 1 mM SA treatments both under light and dark conditions (Takács *et al.* 2018). It was also found by other authors that Put elicited ROS-dependent SA pathways during the activation of plant defences in *Arabidopsis* (Liu *et al*. 2020), thus SA-induced PAs may activate SA signalling.

In contrast to these results, it was earlier observed that exogenous PA treatments decreased the levels of H_2O_2 by activating enzymatic and non-enzymatic antioxidants (Mellidou *et al.* 2016). This is consistent with previous studies reporting that transgenic tobacco plants showed decreased PA content and increased inter-/intracellular levels of ROS and the expression of antioxidant genes, which are nevertheless insufficient to scavenge these ROS efficiently (Moschou *et al.* 2008b).

However, there is no consensus about the role of ET in the control of PA degradation. PAO activity was strongly induced by ET in the olive fruit abscission zone (Gil-Amado and Gomez-Jimenez 2012), but Cona *et al.* (2003) found that exogenously supplied ET did not affect PAO activity either after light exposure or in the dark. Depending on the amount of H_2O_2, the defence responses or cell death program are initiated (Moschou *et al.* 2008a) and the oxidation of PAs by DAO and PAO can promote a second phase in the oxidative burst and may induce cell death in biotic stress (Yoda *et al.* 2006). Our results demonstrate that ET can control the DAO and PAO activity in ET receptor mutant *Never ripe*, which in the absence of the functional ET signalling resulted in more effective PA degradation and could modulate the PA (and thus H_2O_2) content under SA treatments, which depended on the presence or absence of light.

Despite the fact that the effect of light on the expression of PAO genes has already been shown (Cervelli *et al.* 2000), no data are available concerning the relationship between the expression of PA catabolic genes during SA treatments in the illuminated and dark-treated leaves in ET receptor mutants. The present study demonstrated that the genes of *SlDAO1*, *SlDOA2, SlPAO1* and *SlPAO2* were down-regulated in control plants under prolonged darkness in both genotypes suggesting that their basal regulation needs normal photoperiod. The expression of *SlDAO1* and *SlDAO2* exhibited a different pattern with increasing SA

concentrations in all samples, since *SlDAO2* showed smaller changes, while the expression of *SlDAO1* was down-regulated in WT in the light by SA, while it was induced in other samples.

Transcript levels of *SlPAO1* and *SlPAO2* did not notably change in illuminated WT leaves during the different SA treatments. However, in *Nr* leaves, the expression of *SlPAO1*, but not that of *SlPAO2*, showed significant induction in response to SA under normal photoperiod. In the dark controls, *SlPAO2* was down-regulated both in WT and *Nr* leaves. Upon SA treatments the expression of *SlPAO2* was almost constant under normal photoperiod but it was inhibited in the dark, which was more pronounced in *Nr*. As it was expected, the changes in the total DAO and PAO activities did not follow the changes in the expression of PA catabolic genes. It is well-known that the enzyme activities do not necessarily correlate with the expression of the coding genes because translational or post-translational regulation of enzyme proteins may determine the extractable activity. Moreover, the enzyme extract contains several isoenzymes from different cell compartments that may show different expression level and besides terminal oxidation, several PAO isoenzymes participate in the back-conversion of Spm or Spd to Put, too. It can be ascertained, however, that *Nr* mutants displayed higher PA degradation capacity almost in all experimental conditions.

5. Conclusions

Summarizing the results, we found that the SA-induced defence reactions, including PA synthesis and metabolism, were highly dependent on the concentration of SA. In our previous work, we found that treating tomato with 0.1 mM SA resulted in 5-6 $\mu g\ g^{-1}$ and with 1mM concentration, ~60 $\mu g\ g^{-1}$ free SA content in the leaf samples, which corresponded to SA levels in the systemic leaves or in the infected tissues during the HR, respectively (Takács *et al*. 2018). It was also found that PA metabolism was different if SA was applied to illuminated plants or to plants in dark environments. It was also of interest whether the inhibition of ET perception and signalling in *Nr* could overwrite or modify the effect of SA in these plants. Although ET production of *Nr* mutants remained higher than that of WT in almost all experimental conditions, PA content displayed a negative correlation with ET emanation only in the case of 1 mM SA application (Put, Spm) and in certain control leaves (Spd, Spm). One day after SA exposure the accumulation of PAs was induced by 1 mM but not by 0.1 mM SA and it was more pronounced in WT than in *Nr* leaves. Light-dependency proved to be the most obvious in Spm accumulation in WT upon 1 mM SA treatment, which was accompanied by increased expression of S-adenosylmethionine decarboxylase (*SlSAMDC*) and Spm synthase (*SlSPMS*).

However, 1 mM SA enhanced the Put content and the expression of Put biosynthetic enzymes, arginine and ornithine decarboxylase (*SlADC* and *SlODC,* respectively) under darkness. The most important finding of these experiments is that Spm accumulation and the expression of *SlSPMS* gene needs an intact ET signalling pathway independently of the light conditions and SA treatments since in all of the samples *Nr* mutants were defective in Spm synthesis and were not able to accumulate this PA. The expression patterns of PA catabolic genes differed during SA treatments in WT and *Nr* leaves under light and dark conditions suggesting that the ET perception controls PA catabolism, too. The activities of diamine and polyamine oxidases were generally higher in *Nr* leaves compared to the WT, which suggests that the terminal oxidation of PAs plays a central role in the maintenance of the free PA level of tissues.

Author contributions

Conceptualization P.P. and I.T.; investigation, Z.T. and P.P.; writing—review and editing, Z.T., P.P. and I.T.

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Conflict of Interest

No conflict of interest is declared.

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Fig. 1 Changes in the ethylene emission from wild type (WT) and ethylene receptor mutant *Never ripe* (*Nr*) leaves under control condition (A) or after 24-h 0.1 mM SA (B) and 1 mM SA (C) treatments under normal light or dark conditions. Means ± SE, n=5. Different letters show significant differences at $P < 0.05$.

Fig. 2 Changes in free polyamine (Put, Spd, Spm) contents in wild type (WT) and ethylene receptor mutant *Never ripe* (*Nr*) leaves under control condition (A, D, G) or after 24-h 0.1 mM SA (B, E, H) or 1 mM SA (C, F, I) treatments under normal light or dark conditions. Means ± SE, n=5. Different letters show significant differences at $P < 0.05$.

Fig. 3 Log_2 fold change of expression of *SlADC* and *SlODC* genes, encoding enzymes involved in biosynthesis of Put in wild type (WT) and ethylene receptor mutant *Never ripe* (*Nr*) leaves under control condition (A, D) or after 24-h 0.1 mM SA (B, E) or 1 mM SA (C, F) treatments under normal light or dark conditions. Means ± SE, n=5. Different letters show significant differences at $P < 0.05$.

Fig. 4 Log_2 fold change of expression of *SlSAMDC*, *SlSPDS* and *SlSPMS* genes, encoding enzymes involved in dcSAM, Spd and Spm biosynthesis in wild type (WT) and ethylene receptor mutant *Never ripe* (*Nr*) leaves under control condition (A, D, G) or after 24-h 0.1 mM SA (B, E, H) or 1 mM SA (C, F, I) treatments under normal light or dark conditions. Means ± SE, n=5. Different letters show significant differences at $P < 0.05$.

Fig. 5 Changes in the activity of DAO in wild type (WT) and ethylene receptor mutant *Never ripe* (*Nr*) leaves under control condition (A) or after 24-h exposure to 0.1 mM SA (B) or 1 mM SA (C) under normal light or dark conditions. Means ± SE, n=5. Different letters show significant differences at $P < 0.05$.

Fig. 6 Changes in the activity of PAO in wild type (WT) and ethylene receptor mutant *Never ripe* (*Nr*) leaves under control conditions (A) and after 24-h 0.1 mM SA (B) or 1 mM SA treatments (C) under normal light or dark conditions. Means ± SE, n=5. Different letters show significant differences at $P < 0.05$.

Fig. 7 Log_2 fold change of expression of *SlDAO1* and *SlDAO2* genes, encoding enzymes involved in catabolism of free Put in wild type (WT) and ethylene receptor mutant *Never ripe* (*Nr*) leaves under control condition (A, D) or after 24-h exposure to 0.1 mM SA (B, E) or 1 mM SA (C, F) under normal light or dark conditions. Means ± SE, n=5. Different letters show significant differences at $P < 0.05$.

Fig. 8 Log_2 fold change of expression of *SlPAO1* and *SlPAO2* genes, encoding enzymes involved in catabolism of free Spd and Spm in wild type (WT) and ethylene receptor mutant *Never ripe* (*Nr*) leaves under control conditions (A, D) or after 24-h exposure to 0.1 mM SA (B, E) or 1 mM SA (C, F) for 24 hours under normal light or dark conditions. Means ± SE, n=5. Different letters show significant differences at $P < 0.05$.