FINAL REPORT

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COMPARISON OF SALICYLIC ACID-INDUCED PROGRAMMED CELL DEATH IN THE PRESENCE OF LIGHT OR IN DARKNESS IN TOMATO PLANTS: THE ROLE OF ETHYLENE AND REACTIVE OXYGEN- AND NITROGEN SPECIES

Keywords

Ethylene, nitric oxide, polyamines, programmed cell death, reactive oxygen species, salicylic acid

Introduction

Light is one of the most important environmental factors, which is required for optimal growth and development or stress responses of plants (Chen et al. 2004; Kangasjärvi et al. 2012). Hence, the absence of light (e.g. night or prolonged darkness) can alter the light-dependent activation of plant developmental or defence responses and induce new signaling and regulation pathways modulated by various signaling molecules (Ballaré 2014).

Salicylic acid (SA) is a natural phenolic compound, which accumulates under abiotic and biotic stress and controls physiological and biochemical functions in plants (Hayat et al. 2010). However, the effect of SA is dependent on the concentration, duration of the SA treatments, on plant genotype and developmental stage (Rivas-San Vicente and Plasencia 2011). Moreover, the SA-induced physiological responses are also light-dependent (Mühlenbock et al. 2008). SA is a key molecule in the induction of both hypersensitive response (HR) and systemic acquired resistance (SAR) (Alvarez 2000). It was shown that SA-induced pathogenesis-related 1 (PR-1) gene expression and bacterial pathogen-induced SA accumulation were light-dependent and both were reduced in darkness (Genoud et al. 2002; Zeier et al. 2004). In addition, reactive oxygen species (ROS), nitric oxide (NO) and ethylene are key component of fine-tuning of these processes (Karpinsky et al. 2003).

SA can generate ROS by PM-localized NADPH oxidases in the location of the infection (Kawano et al. 2004), moreover, SA can inhibit the catalase enzyme (CAT) in the peroxisome. It induces the superoxide dismutase (SOD) thus increases the production of H_2O_2 (Rao et al. 1997; Horváth et al. 2002). ROS, such as H_2O_2 and superoxide radicals in high concentrations are essential mediators of plant PCD, because they damage to the cellular components, such as proteins, lipids and DNA (De Pinto et al. 2012). NO can interplay with

ROS in a variety of ways and it is a crucial partner in determining cell fate or in signaling response in a number of physiological and stress-related conditions such as SA- or ethylene-induced signaling (Ederli et al 2006; Bastianelli et al. 2010; Gémes et al. 2011). SA can inhibit the ethylene synthesis by blocking the conversion of 1-aminocyclopropane-1-carboxylic acid (ACC) to ethylene (Leslie and Romani 1986). In contrast, Rao et al. (2002) found that SA is required to produce ethylene in response to O_3. It was also found that ethylene production was associated with SA treatments in tomato (Gémes et al. 2011). Moreover, it was observed that the conversion of ACC to ethylene was inhibited in the light but it was promoted in the dark (Kao and Yang 1982). SA and ET can induce the accumulation and catabolism of polyamines (PA; Putrescine: Put; Spermidine: Spd, Spermine: Spm), which also play role in several important cellular processes (Jiménez-Bremont et al. 2014).

It can be concluded that the regulation of defence mechanisms and cell death mediated by SA in plant tissues seems to be different in light and dark conditions.

General Aims

The main question of this project was how the light or darkness affects the salicylic acid induced programmed cell death and what are the roles of plant growth regulator polyamines, plant hormone ethylene and reactive oxygen- and nitrogen species in this process. We also wanted to reveal, how ER-stress and the dysfunction of mitochondria affects; how SA, polyamines and ethylene are involved in this process under light and dark conditions.

Results

The current knowledge about the physiological and molecular aspects of dark-modulated effects, as well as production of ROS and NO in different plant species were summarized in a review article and book chapters and discussed their roles in different developmental processes (seedling growth and development or senescence) and during environmental stresses (high and low temperature and biotic stress) in the absence of light.

Dissemination of the results

Péter Poór, Attila Ördög, Zalán Czékus, Péter Borbély, Zoltán Takács, Judit Kovács, Irma Tari (2018) Regulation of the key antioxidant enzymes by developmental processes and

environmental stresses in the dark. *Biologia Plantarum* DOI: https://doi.org/10.1007/s10535-018-0782-7.

Péter Poór, Zalán Czékus, Attila Ördög (2018) Role of nitric oxide in physiological and stress responses of plants under darkness. In: Reactive Oxygen, Nitrogen and Sulfur Species in Plants: Production, Metabolism, Signaling and Defense Mechanisms, ISBN 978-1-1194-6869-1 submitted book chapter (31-Jan-2018)

Péter Poór, Gábor Laskay, Irma Tari (2015): Role of Nitric Oxide in Salt Stress-induced Programmed Cell Death and Defense Mechanisms. In: Khan M N, Mobin M, Mohammad F, Corpas F J (szerk.) Nitric Oxide Action in Abiotic Stress Responses in Plants. Cham (Németország): Springer International Publishing, 2015. pp. 193-219.

Methods

Wild type (*Solanum lycopersicum* Mill. L. cvar. Ailsa Craig) and ethylene receptor mutant *Never ripe* (*Nr*) tomato plants were grown in hydroponics, under controlled condition in the greenhouse (Poór et al. 2011). Tomatoes were treated with 0.1 mM or 1 mM salicylic acid (SA) supplied in the nutrient solution. Half of the plants remained for 24 hours under the growing light/dark cycle (light samples) or half of them were put into prolonged darkness (dark samples) at 25 ^{o}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. Viability of leaf tissues was determined based on the measurements of electrolyte leakage (EL) and lipid peroxidation (Poór et al. 2013; Horváth et al. 2015). Changes in H_2O_2 contents were measured using spectrophotometer by the method of Horváth et al. (2015). Histochemical detection of superoxide and H_2O_2 production was carried out according to Wohlgemuth et al. (2002). Native PAGE analysis of NADPH oxidase activity was performed as described by Carter et al. (2007). Activities of superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and guaiacol-dependent peroxidase (POD) were determined by Horváth et al. (2015). NO production of tomato leaves was visualized using 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate (DAF-FM DA) based on Gémes et al. (2011). Measurement of ethylene production with a Hewlett-Packard 5890 Series II gas chromatograph was carried out by Poór et al. (2013). Free PA contents were determined with HPLC as described by Szepesi

et al. (2009). Determination of terminal diamine- (DAO) and polyamine oxidase (PAO) activities were estimated spectrophotometrically with minor modifications of the method described by Quinet et al. (2010). Quantitative real-time reverse transcription-PCR (qRT-PCR; Piko Real-Time qPCR System, Thermo Scientific) was used to detect the expression pattern of "anti-apoptotic" Bax Inhibitor-1 (BI-1), cysteine proteases and protease inhibitors, as well as ER stress marker genes mined from Sol Genomics Network (SGN; http://solgenomics.net/) and National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/) databases (Gallé et al. 2013). Immuno-blotting analysis was carried out after protein separation with SDS-PAGE based on Chen et al. (2009). Isolation of tomato leaf mitochondria was carried out as previously described by Camacho-Pereira et al. (2009). Transmission electron microscopy and post-embedding immunohistochemistry was applied based on Talapka et al. (2016). Azocasein was used as a non-specific substrate to measure the total proteolytic activity (Pereira et al. 2010). Chlorophyll *a* fluorescence and signal from oxidized P700 were detected with Dual-PAM-100 instrument (Heinz-Walz, Effeltrich, Germany) (Klughammer and Schreiber, 1994). Changes in mitochordrial electron transport were determined with Clark-type oxygen electrode (Hansatech Oxytherm, Hansatech UK) in isolated mitochondria by the method of Camacho-Pereira et al. (2009).

1st year

Aims

Production of reactive oxygen- (ROS) and nitrogen species (RNS), ethylene emission, polyamine contents induced by salicylic acid (SA) treatments in dark and light conditions. Determination of tissue viability after various treatments. In parallel, measurement of the expression of "anti-apoptotic" Bax Inhibitor-1 (BI-1) and cysteine proteases, protease inhibitors by qRT-PCR and detection of protein levels by Western Blot. Determination of mitochondrial stress leading to the initiation of cell death. Preparation of functional mitochondrial fraction, cytochrome *c* (cyt *c*) release from mitochondria, measurement of protease and diamine oxidase activities as a function of time. Detection of the source of ROS and RNS in dark and light conditions: determination of photosynthetic and mitochondrial electron transport chain, activity of plasma membrane localized NADPH oxidase and polyamine oxidases.

Results

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 signaling 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. Moreover, 1 mM SA application induced NADPH oxidase activity leading to increased superoxide production in the first hours of SA treatments in the light. The activity of NADPH oxidase and expression *SlRBOH1* gene encoding a NADPH oxidase subunit was much lower in the dark. In addition, 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. Interestingly, changes in APX activities were the most light-dependent and were reduced under the dark condition. Analysis of the expression levels of the different genes coding for antioxidant enzymes (SOD: *SlCuZnSOD*, *SlFeSOD*, *SlMnSOD*; CAT: *SlCAT1*, *SlCAT2*, *SlCAT3*; APX: *SlAPX1*, *SlAPX2*) showed also significant differences under light or dark conditions. 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 of tomato.

Besides ROS, 1 mM SA induced accumulation of reactive nitrogen species (RNS), such as nitric oxide (NO) and peroxynitrite, which were also lower in the dark compared to the SA treated plants in the light.

Both SA concentration induced ethylene emission in the first 6 hours of treatments which was significantly higher in the dark. However, ethylene emission was blocked by SA after 12 and 24 hours.

Exogenously SA treatments and darkness significantly promoted the free polyamine (Put, Spd and Spm) accumulation. The highest levels of Put and Spm were observed in the leaves treated with 1 mM SA in the dark, which suggested that polyamines play an important role in plant defence mechanisms under prolonged darkness.

To detect the light-dependent effects of SA treatments on PA catabolism, changes in diamine oxidase (DAO) and polyamine oxidase (PAO) activity were also investigated. 1 mM SA induced significantly higher DAO activity in the dark after 1 and 24 h. Similar to the DAO enzyme activity, the expression of DAO coding two selected tomato genes, *SlDAO1* and *SlDAO2* showed significantly higher expression after SA treatments. Moreover, 1 mM SA increased significantly the PAO activity after 24 h under light and it was also enhanced in the dark, but reduced after 3, 6 and 12 h. The expression pattern of the two selected PAO genes (*SlPAO1* and *SlPAO2*) showed similar tendencies to the enzyme activity.

To detect the effects of SA on the viability of leaf tissues, membrane integrity was measured by electrolyte leakage (EL) during the experiments. Exogenous application of 0.1 mM SA did not induce the loss of membrane integrity of leaf cells, but it was increased by the treatment with 1 mM SA after 24 h, which was significantly higher in light than in dark condition. Moreover, malondialdehyde (MDA) content, the product of lipid peroxidation was significantly increased in 1 mM SA treated leaves under light condition but remained lower in the dark.

In parallel, the expression levels of specific genes involved in cell death program and proteolysis were analysed by qRT-PCR. 1 mM SA induced the expression of "proapoptotic" *SlCYP1* (papain-like cysteine protease), *SlVPE1* (vacuolar processing enzyme) and *SlMCA1* (metacaspase) genes after 1 h in the leaves. However, the protein levels slightly decreased in the 1 mM SA treated leaves but proteolytic activity was induced after 24 h. The sublethal, 0.1 mM SA induced the expression of "anti-apoptotic" *SlPI2* (Protease Inhibitor-2 protein) and *SlLTC* (cystatin) genes after 6 hours in the leaves. *SlBI-1* gene was also up-regulated in 1 mM SA treated leaves after 24 h, which was significant higher in illuminated plants than plants treated in the dark.

Production of ROS by mitochondrial electron transport chain, cytochrome *c* (cyt *c*) release from mitochondria, and loss of mitochondrial integrity take place before cell death execution in plant tissues. Hexokinases (HXKs) are the first enzymes in the glycolysis, which play regulatory function in the sugar metabolism and sugar sensing. In addition, the mitochondria-associated hexokinases have a role in the control of PCD in plants. The mitochondria-associated hexokinases inhibit the opening of the voltage dependent anion channels (VDACs) and thus inhibit cyt *c* release from the intermembrane space of mitochondria to the cytosol. The upstream promoter sequences of hexokinase genes of tomato (*SlHXK*) were analyzed *in silico* to identify their *cis*-regulatory elements (CREs). The most

abundant CRE was *GT1CONSENSUS* with 45 duplications, which plays a significant role in the regulation of many light-dependent genes. 24-h-long dark period reduced the expression of the mitochondrial *SlHXK1* and chloroplastic *SlHXK4* in the young and mature leaves, but induced the expression of all *SlHXKs* especially that of *SlHXK3* in the old leaves. In contrast to *HXK* expression, HXK activity decreased at all leaf positions, however smallest changes were observed in young, sink leaves. Images prepared by transmission electron microscopy (TEM) showed swelling and disorganisation of mitochondrial cristae, disintegration and vacuolization of mitochondria after SA exposure. Using WB and post-embedding immunohistochemistry, cyt *c* release from mitochondria was also detected. Both SA treatments decreased the activity and transcript levels of HXKs, which could contribute to the higher mitochondrial ROS production but not to NO generation in SA-treated leaf mitochondria.

Moreover, SA has light-dependent effects not only the mitochondrial- but also the photosynthetic electron transport chain, which was measured by DUAL-PAM. Treatment with 1 mM of SA caused significant decrease in the maximum quantum yield of PSII (Fv/Fm) independently from the presence or absence of light, but 0.1 mM SA decrease significantly Fv/Fm only in the dark. Similar tendencies were observed in case of the effective quantum yield of PSII [Y(II)] and also PSI [Y(I)], which decreased after all SA treatments in the light. Decline in photosynthetic activity and damage of chloroplasts (investigated by using TEM) could contribute to higher ROS production after 1 mM SA in the light.

Dissemination of the results

Planned and published articles in international and peer-reviewed journals:

(Péter Poór, Péter Borbély, Nikolett Bódi, Mária Bagyánszki, Irma Tari: Effects of salicylic acid on photosynthetic activity and chloroplast morphology under darkness. *Manuscript*)

Péter Poór, Zoltán Takács, Gábor Patyi, Péter Borbély, Ottó Bencsik, András Szekeres, Irma Tari: Dark-induced changes in the activity and the expression of tomato hexokinase genes depend on the leaf age. *Submitted article to Journal of Plant Growth Regulation* (17-JAN-2018)

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2nd year

Aims

Investigation of SA effect in ethylene receptor mutant tomato. Determination of ROS and RNS production, polyamine and ethylene levels induced by SA treatments as well as cell viability in ethylene insensitive *Never ripe* tomato mutant in dark and light conditions. Measurement the polyamine oxidase (PAO) and diamine oxidase (DAO) activity and gene expression induced by SA treatments in parallel with ROS and RNS production in ethylene insensitive mutant in control condition and after inhibition of PAO/DAO.

Results

Since the SA-mediated signaling pathways and the formation of ROS and RNS are light-dependent, the time- and concentration-specific induction of defence signaling was investigated in leaves of wild type (WT) and ethylene insensitive *Never ripe* (*Nr*) tomato plants. Our work focused on the ethylene-dependent effects of sublethal (0.1 mM) and lethal (1mM) SA treatments under light and dark conditions on ROS, RNS and PA metabolism

To detect the effects of SA on membrane integrity and cell viability of leaf tissues, EL was firstly measured as a function of time. Exogenous application of 0.1 mM SA did not lead to the loss of membrane integrity, but 1 mM SA induced an increase in EL after 24 h, which was significantly higher under light than in the dark, however it was significantly lower in the leaves of *Nr* plants in the light, but higher in the dark. Another important injury indicator of plant cells under stress condition is the degree of lipid peroxidation, which was estimated as the amount of the thiobarbituric acid-reactive substances, mainly MDA. Sublethal SA concentration had no impact on leaf MDA content, but the product of lipid peroxidation was significantly increased in 1 mM SA treated WT leaves. This change was significantly higher in the illuminated leaves than in those exposed to darkness. In contrast, lipid peroxidation was lower in *Nr* leaves after lethal SA treatment. These results suggest that the stability and integrity of plasma membrane was significantly reduced in illuminated WT leaves after the treatment with 1 mM SA and it was dependent on the active ethylene signaling.

Ethylene production was decreased by both SA concentrations in the leaves of WT under both environmental conditions. However, ethylene levels were basically higher in *Nr* than in WT under same condition. Interestingly, the expression of *SlACS6*, the 1-aminocyclopropane-1-carboxylate (ACC)-synthase coding gene which takes part in ethylene biosynthesis, decreased significantly after SA treatments in WT plants, but increased slightly

in *Nr* leaves. In contrast, the expression of ACC-oxidase coding genes, *SlACO1* and *SlACO4* increased in the light but decreased under the dark condition in both tomato genotypes.

ROS play key role in SA- and ethylene-mediated signaling, thus H_2O_2 content was determined in the leaves of WT and *Nr* plants after SA treatments. Upon control condition a slightly increased H_2O_2 content was observed in the light compared to the dark. Both SA treatments increased H_2O_2 content in concentration- and time-dependent manner in WT plants, but it was significantly lower in the dark. However, H_2O_2 production was lower after 1 mM SA in the leaves of *Nr* plants compared to the WT plants. Moreover, 0.1 mM SA induced significantly higher H_2O_2 production compared to 1 mM SA treatments after 24 hours in the illuminated leaves of *Nr* plants. Interestingly, H_2O_2 content was significantly higher in the dark after 1 mM SA treatment in *Nr* leaves. In parallel, SA treatments induced SOD and decreased catalase CAT activities, thus increased the production of H_2O_2 in WT, while the mutants maintained enhanced CAT enzyme activity in the dark. SA increased also APX and POD activities, but APX was the most light-dependent because it showed higher activity in the light. Analysis of the expression levels of the different genes coding for antioxidant enzymes showed significant differences between the WT and *Nr* plants. Relative transcript levels of *SlMnSOD* and *SlCuZnSOD* were significantly higher in WT exposed to 1 mM SA. The expression of *SlCuZnSOD* was also high in *Nr* leaves under darkness SA treatments inhibited the expression levels of *SlCAT1* and induced *SlCAT3* in WT but *CAT* expression did not change in *Nr* plants. Moreover, 1 mM SA elevated also the transcript levels of *SlAPX*1 and *SlAPX2* in WT in the light but transcript abundance did not increase in *Nr* and under darkness.

SA treatments induced not only H_2O_2 accumulation but also the production of NO in a light dependent manner. NO production exhibited a small decline during the day in the control leaves and in the WT leaves treated with 0.1 mM SA in both environments. In contrast, 1 mM SA induced significant NO production after the 6^{th} h compared to the control in illuminated WT leaves, but NO level declined under the dark. In contrast to WT plants, NO levels were basically higher in *Nr* leaves which increased significantly after SA treatments in the light.

To examine the importance of light and dark conditions in PA metabolism, the free PA contents were measured in the leaves of WT and *Nr* tomato plants after the different SA treatments. Under the light, the accumulation of PAs showed diurnal rhythm in the control leaves leading to increased PA level in the late afternoon. In contrast to 0.1 mM SA treatment,

1 mM SA caused pronounced increase in free Put, which was markedly higher under the dark condition after 24 h than under the light in WT plants, but it was significantly lower in *Nr* leaves. However, 1 mM SA increased also Spm levels, but only in the illuminated WT leaves. Spm content did not change in *Nr* leaves under both environmental conditions. Interestingly, not only Spm contents, but also Spd were basically lower in *Nr* leaves. Expression of PA biosynthetic genes showed similar tendencies. 1 mM SA caused significantly higher expression of *SlADC* and *SlODC* genes after 24 h, respectively under the dark condition in WT leaves, which take part in the biosynthesis of PAs, but it decreased the expression of both genes in *Nr* leaves under both environmental conditions. Expression level of *SlSPMS* was significantly higher after 24 h in the illuminated WT plants but lower in *Nr* leaves, which confirmed the lower Spm content in the ethylene insensitive *Nr* plants.

To detect the light- and ethylene dependency of SA-induced PA catabolism, activity of DAO and PAO were investigated. DAO and PAO activity was remained at constant level in the presence of 0.1 mM SA in illuminated WT samples, but 1 mM SA induced DAO activity after 24 h. In contrast to this result, higher DAO and PAO activities were found in *Nr* plants in the light. Moreover, 1 mM SA decreased PAO activity in the light but not in the dark in *Nr* leaves. Similar tendencies can be found in the expression levels of *SlPAO1*, the gene encoding PAO. Expression of *SlDAO2*, which plays role in DAO synthesis was significantly higher in *Nr* leaves under darkness. Inhibition of polyamine oxidase by a spermine analogue, MDL-72,527 resulted lower H_2O_2 production and polyamine oxidase activity after 1 mM SA treatment in the light but not in the dark. Moreover, ethylene production and Spd and Spm levels were also higher in the presence of MDL after SA treatments in the light.

Dissemination of the results

Planned and published articles in international and peer-reviewed journals:

(Zoltán Takács, Péter Poór, Irma Tari: Interaction between polyamines and ethylene in *Never ripe* tomato in response to different salicylic acid during normal photoperiod and prolonged darkness. *Manuscript*)

Zoltán Takács, Péter Poór, Péter Borbély, Zalán Czékus, Gabriella Szalai, Irma Tari: H_2O_2 homeostasis in wild-type and ethylene-insensitive *Never ripe* tomato in response to salicylic

acid treatment in normal photoperiod and in prolonged darkness. *Submitted to Plant Physiology and Biochemistry after revision* (22-JAN-2018)

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Péter Poór, Zoltán Takács, Péter Borbély, Zalán Czékus, Gábor Patyi, Irma Tari: Involvement of ethylene in hydrogen-peroxide metabolism in the leaves of salicylic-acid treated tomato. In: Albrechtova J, Santrucek J (szerk.) Plant Biology Europe EPSO/FESPB 2016 Congress: Abstracts. Konferencia helye, ideje: Prague, Csehország, 2016.06.26-2016.06.30. Prague: p. 198.

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Poór Péter, Takács Zoltán, Borbély Péter, Czékus Zalán, Ördög Attila, Szalai Gabriella, Tari Irma: Szalicilsav kezelést követő etilénfüggő változások paradicsom növények H2O2 homeosztázisában fényben és sötétben. In: Györgyey János (szerk.) A Magyar Növénybiológiai Társaság XII. Kongresszusa. 72 p. Konferencia helye, ideje: Szeged, Magyarország, 2017.08.30-2017.09.01. Szeged: Magyar Növénybiológiai Társaság, 2017. p. 11. (ISBN:978-963-12-9736-2)

Péter Poór, Zoltán Takács, Péter Borbély, Zalán Czékus, Irma Tari: Ethylene dependent changes in hydrogen-peroxide homeostasis after salicylic acid treatment in tomato. In: Andreas Bachmair, Alisher Touraev (szerk.) International Conference Plant Molecular

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Aims

Detection of ER stress induced by tunicamycin and azetidin-2-carboxylic acid, the effect of ER stress inducers on PCD induction by mitochondria: cyt c release, ROS and RNS production in the presence or absence of SA. Determination of oxidized and misfolded proteins. Effect of ER stress inhibitors, Ca^{2+} signaling inhibitors and the inhibition of mitochondrial permeability transition pore (PTP) on the BI-1 expression and protein level in the leaves under SA treatments in light and dark conditions.

Results

SA can induce the accumulation of unfolded proteins in the ER, which is sensed as ER stress signal. Chemicals, such as the *N*-glycosylation inhibitor tunicamycin (Tm) or azetidin-2-carboxylic acid (Aze) is used to induce and examine ER stress. Generation of ROS and cell death-promoting Ca^{2+} signaling at the ER and mitochondria can involve in ER-mediated cell death. Effects of applied proline analog Aze in tomato plants were not significant within 24 hours. Significant ethylene, ROS and RNS production, as well as cell death induction based on EL could be not detected. In contrast, 5 μg ml^{-1} Tm significantly elevated EL from the leaves of WT but not in *Nr* plant in the light, but Tm was not able to induce cell death and cyt *c* release within 24. In the presence of 0.1 mM SA, Tm did not change significantly the viability of leaves in both genotypes, but Tm reduced the high EL caused by 1 mM SA in the light. Interestingly, EL was significantly lower in the dark. Unfortunately, application of Ca^{2+} signaling inhibitor was not specific and ineffective through the root system. Instead, other ER stress inhibitor, the small molecular chaperone 4-phenylbutyrate (PBA) was applied beside SA treatments. Basically, PBA did not reduce the EL, but the effect of 1 mM SA on EL was moderated by application of 1 mM PBA.

Tm elevated H_2O_2 contents in control and SA treated leaves, which was significantly lower in *Nr* leaves after 1 mM SA. Interestingly, H_2O_2 levels were higher after Tm treatment in the control leaves of WT under darkness. At the same time, application of PBA decreased H_2O_2 levels in case of all treatments in the WT plants but not in *Nr* leaves. Lipid peroxidation, based on MDA content was significantly higher only in 1 mM SA treated illuminated WT

leaves and did not change in the presence of Tm or PBA in these plants. In contrast, oxidized protein levels based on protein carbonylation were significantly elevated by 1 mM SA in the absence and presence of TM in the light. Moreover, the oxidized protein levels were basically higher in *Nr* leaves after the same treatments. SA and Tm induced oxidative stress and they caused the significantly higher proteolytic activity in WT and *Nr* leaves. Tm induced itself the significantly higher proteolytic activity in WT plants, but it was higher in *Nr* leaves and under darkness.

NO production was increased by SA treatments in the light. However, Tm also induced the NO production in leaf tissues but the effects of PBA on NO production were more significant. PBA induced NO release in the leaves of both tomato genotypes could contribute to the defence responses of the plants.

Ethylene production was inhibited by both SA treatments in WT leaves under both environmental conditions. In contrast, it was basically higher in *Nr* plants. Tm increased significantly the ethylene emission of the illuminated leaves of both genotypes, but this tendency was significantly higher in *Nr* plants, especially in the presence of SA. Ethylene production was significantly higher in *Nr* leaves after Tm and SA treatments in the light but it was lower in the dark in case of both genotypes. Interestingly, treatment with PBA significantly decreased the ethylene production in *Nr* leaves.

Expression of specific marker genes were also analyzed to understand the role of SA in ER stress processes. Interestingly, not only SA but also Tm induced the relative transcript levels of *PR1*, which plays important role in SA signaling pathway. The expression of *PR1* was significantly higher in *Nr* leaves after Tm treatment. The relative transcript amount of ER stress marker *BiP* was also elevated by the treatments with Tm in WT plants and it was significantly higher in *Nr* leaves. Moreover, expression of *BiP* was also significantly induced by both SA treatments in both genotypes. In contrast, treatment with PBA decreased *BiP* expression after SA treatments, especially in illuminated *Nr* leaves. Expression of two ER signaling components, *IRE1*α and *IRE1*β was also investigated. Transcript levels of both *IRE1* genes were elevated by Tm and by 0.1 mM SA treatments, especially in illuminated *Nr* leaves. Interestingly, the expression of both *IRE1* genes was significantly lower under darkness.

BI-1 can involve in restriction and regulation of ER-stress in plants. Interestingly, Tm significantly induced the relative expression of *BI-1*. Moreover, PBA and both SA treatments also elevated the relative transcript levels of *BI-1*, but this tendency was significantly lower

compared to the treatment with Tm. In addition, relative transcript levels of *BI-1* were significantly lower in illuminated *Nr* leaves after SA exposure and under darkness in both tomato genotypes.

It can be concluding that the active ethylene signaling can contribute to ER stress and defence mechanism of leaf cells regulated by H_2O_2 and NO after SA exposure in the light.

Dissemination of the results

Planned manuscripts in international and peer-reviewed journals:

(Zalán Czékus, Orsolya Csíkos, Attila Ördög, Péter Poór: Investigation of salicylic acid induced ER stress in wild-type and ethylene-insensitive *Never ripe* tomato.)

(Péter Poór, Zalán Czékus, Orsolya Csíkos, Attila Ördög: The role of ethylene in tunicamycin induced ER stress under darkness.)

International- and national conference issues:

Czékus Z, Csíkos O, Takács Z, Ördög A, Bódi N, Bagyánszki M, Tari I, Poór P: Szalicilsav okozta ER stressz hatásának vizsgálata paradicsomban. In: Györgyey János (szerk.) A Magyar Növénybiológiai Társaság XII. Kongresszusa. 72 p. Konferencia helye, ideje: Szeged, Magyarország, 2017.08.30-2017.09.01. Szeged: Magyar Növénybiológiai Társaság, 2017. p. 44. (ISBN:978-963-12-9736-2)

Czékus Zalán, Csíkos Orsolya, Ördög Attila, Poór Péter: Szalicilsav indukálta ER stressz vizsgálata paradicsomban. In: Mézes Miklós (szerk.) Magyar Szabadgyök-Kutató Társaság IX. Kongresszusa és az MTA ÉKB Mikroelem Munkabizottságának Tudományos Ülése. Konferencia helye, ideje: Gödöllő, Magyarország, 2017.08.25-2017.08.26. Gödöllő: SzIE, 2017. p. 26. (ISBN:978-963-269-666-9)

Czékus Zalán, Csíkos Orsolya, Takács Zoltán, Ördög Attila, Bódi Nikolett, Bagyánszki Mária, Tari Irma, Poór Péter: Szalicilsav indukálta ER stressz vizsgálata paradicsom növényekben. In: Kristóf Zoltán, Solymosi Katalin (szerk.) XV. Magyar Növényanatómiai Szimpózium. Konferencia helye, ideje: Budapest, Magyarország, 2017.09.07 Budapest: ELTE TTK Biológiai Intézet, 2017. p. 28. (ISBN:978-963-12-9834-5)

Significance of the research

To best of our knowledge, this was the first occasion when the effects of light and darkness on ROS and RNS production and their source were investigated after treatment with lethal concentration of SA in parallel with the role of ethylene and specific proteases, protease inhibitors and "anti-apoptotic" proteins. The role of polyamines in this process has not been fully elucidated yet, and it is of interest how polyamine catabolism contributes to cell death induction and how it is connected to mitochondrial dysfunctions. We were also interested in how ER stress, the accumulation of unfolded proteins in the ER lumen and disintegration of mitochondria in SA-treated tissues affect the cell viability. This may contribute to understand the complexity of SA-induced PCD and to reveal what are the main features of the "apoptotic-like" plant cell death in the light and in the dark. This research will partly answer the question, why plant responses are different against some microorganisms in the light and dark conditions.

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