FINAL REPORT

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FINE-TUNING OF PLANT DEFENCE IN THE DARK: THE ROLE OF SALICYLIC ACID, JASMONIC ACID AND ETHYLENE

Keywords

Ethylene, jasmonic acid, nitric oxide, reactive oxygen species, salicylic acid, stomata

Introduction

Light is the most important energy source for biomass production. It is required for optimal growth and developmental processes in plant kingdom. Light may also control the plant defence mechanisms, while excess of light could lead to oxidative stress, which may contribute to the initiation of cell death in tissues (Karpinski et al. 2003; Kangasjärvi et al. 2012). Plants are able to sense the amount of photons, the intensity and quality of light as well as the changes in light/dark cycles. Hence, the absence of light (i.e. prolonged darkness or night) can alter the light-dependent activation of plant defence responses and can turn up new signalling and regulation pathways (Ballaré 2014). Thus, the regulation of defence mechanisms and cell death in plant tissues seems to be different in light and dark conditions. It has been found that light is necessary for the development of resistance responses to *Pseudomonas solanacearum* in tobacco, to *Xanthomonas oryzaein* in rice, to *Pseudomonas syringae* and to *Peronospora parasitica* in *Arabidopsis* (Roberts and Paul 2006). It was also found in many cases that hypersensitive cell death response (HR) was suppressed or delayed after pathogen infection in the dark (Chandra-Shekara et al. 2006; Grimmer et al. 2012), but the role of plant hormones and hormone crosstalk-induced signalling in this process is still unclear.

Hormone signalling crosstalk plays a major role in plant defence against a wide range of biotic stressors. The antagonistic and cooperative interactions between SA, JA and ET can determine the plant fate after the infection (Derksen et al. 2013). SA plays an important role in many plant-pathogen interactions by inducing localized death of infected cells, the hypersensitive reaction (HR) and the systemic acquired resistance (SAR) in long-distance tissues (Glazebrook 2005). SA is typically involved in defence against biotrophs and in the infection during the biotrophic stage of hemibiotrophs (Spoel et al. 2007). However, the effect of SA is dependent on the concentration, duration of treatment and on the plant genotype or developmental stage (Rivas-San Vicente and Plasencia 2011). It has long been recognized that local and systemic SA accumulation following the pathogen infection induces the expression of number of marker genes such as PR genes. SA-responsive PR genes are among others PR-1, PR-2 (β-1,3-glucanase), PR-3 (chitinase) and PR-5 (thaumatin-like protein). Some of these PRs, possess antimicrobial activity and are related with pathogen-induced expression, hence participating in defence (Durrant and Dong 2004). The other important defence regulator phytohormone is JA, which is generally effective against necrotrophic pathogens or wounding. JA pathway-related defence genes are induced after the degradation of Jasmonate Zim-domain (JAZ) proteins (Pieterse et al. 2012). Interestingly, many protease inhibitors were blocked in jasmonic acid-insensitive1-1 (jai1-1) tomato mutant (Li et al. 2004). ET plays an important part in fine-tuning of JA mediated signalling (Vos et al. 2013). The biosynthesis of ET by 1-aminocyclopropane-1-carboxylic acid (ACC) synthase (ACS) and ACC oxidase (ACO) is regulated by several developmental and environmental factors, such as the presence of light and it could be also inhibited by SA (Zhang et al. 2016). ET receptors are transmembrane proteins bound to endoplasmic reticulum (ER) membranes and have structural similarity to bacterial two-component histidine kinases. In tomato there are eight ET receptors, SlETR1, -2, to -7, and Never ripe (NR) and five of them were shown to bind ET with high affinity (Kamiyoshihara et al. 2012). Generally, the antagonistic effects of SA on the JA/ET pathways are the most studied steps of regulation. However, ET can interact both positively and negatively with SA signalling, depending on the plant-pathogen interactions (Broekgaarden et al. 2015). Moreover, the amount and timing of phytohormone production under stress or diverse environmental conditions, such as in light or darkness could lead to reprogramming of defence or other cellular processes, such as cell death (Pieterse et al. 2012). The physiological processes regulated by phytohormone interactions remained mostly unclear, especially in the case of dark. Light condition has been shown to strongly influence hormone-regulated defence responses (Roberts and Paul 2006). Plants infected under dark condition showed reduced lesion formation and HR in response to non-host and avirulent pathogens (Zeier et al. 2004). Phenylalanine ammonia-lyase (PAL) activity, which catalyzes the first step in the phenylpropanoid pathway and plays dominant role in SA biosynthesis, was decreased in the dark. Moreover, SA remained at basal level, JA increased significantly in the dark after *Pseudomonas* infection, while in the presence of light the opposite results were observed (Zeier et al. 2004). It is well known that prolonged darkness induces the accumulation of ET, breakdown of chlorophyll and carbohydrates or recycling of chloroplast proteins (Lim et al. 2007). The sensing of ET by the ET receptors is also mediated by light and dark conditions (Wilson et al. 2007).

Oxidative- and nitrosative burst contributes to the pathogen-triggered plant immunity and HR mediated by phytohormones. Thus to the regulation of the rapid production and accumulation of ROS and RNS by plant hormones, which is essential in downstream signalling. Many plant hormones, such as SA and ET can generate ROS but can also contribute to maintain cellular redox homeostasis through the regulation of antioxidant enzymes activities under stress (Xia et al. 2015). However, the spatial and temporal regulation of production and the decomposition of ROS, furthermore the specificity of hormone crosstalks remained poorly documented in the dark. The gaseous free radical nitric oxide (NO) can also interplay with ROS in a variety of ways and it is a crucial partner in determining the cell fate or in the signalling response in a number of physiological and stress-related conditions such as SA-, JA- or ET-induced signalling (Kocsy et al. 2013).

Stomata are important entry sites for foliar bacterial and fungal plant pathogens, which can play an active role in limiting pathogen invasion as part of the plant innate immune system (Melotto et al. 2008). Regulation of stomatal function is also controlled by hormone interactions. ABA is one of the key players inducing stomatal closure, but among others SA, JA and ET also contribute to stomatal aperture regulation (Acharya and Assman 2009). It is well known that H_2O_2 and NO are essential signal molecules in the ABA- (Bright et al. 2006), SA-(Poór and Tari 2012), ET- (He et al. 2011) and JA-induced (Hossain et al. 2011) stomatal closure and play crucial role in dark/light regulated stomatal movement (She et al. 2004). Moreover, chitosan (Srivastava et al. 2009) or flagellin (Deger et al. 2015) also induced stomatal closure by elevating ROS and NO levels in the guard cell as plant defence response to prevent the penetration of pathogenes. Interestingly, chitosan inhibited the light-induced stomatal opening (Srivastava et al. 2009) and reduced the photosynthetic electron transport of guard cells (Ördög et al. 2011). Chloroplast and photosynthesis in guard cells also contributes to stomatal function which can be regulated by ROS and NO (Ördög et al. 2013), but the role of guard cell photosynthesis under biotic stress remained still unclear. Delayed stomatal response was also found after pathogen infection in the dark, which was associated with delayed HR (Grimmer et al. 2012) suggesting the significant role of light and alternative signalling ways in the dark after pathogen attack.

Toxins produced by bacteria or fungi can be stimulate plant defence responses or cell death in plants. Fumonisin B1 (FB1), a programmed cell death–eliciting mycotoxin produced by the *Fusarium moniliforme* stimulated ROS production and the degradation of chloroplastic proteins and up-regulates PAL-mediated SA synthesis, which was light-dependent (Xing et al. 2013). Moreover, coronatine (COR) produced by a plasmid-encoded operon of genes in several strains of *Pseudomonas syringae* can suppress SA accumulation and can inhibit stomatal closure or induce stomatal re-opening which can facilitate bacterial infection at night (Panchal et al. 2016). Both toxins can induce phytotoxic effects on tomato plants which can be significant from agricultural aspect. Thus, investigation of stomatal movement, ROS/RNS ratio and hormone signalling in the dark is very important to understand plant defence responses under different light condition.

General Aims

Wild type, ET insensitive *Never ripe* (*Nr*) and JA signalling defective *Jasmonic acid*–The main question of this project is how the light or darkness affects microbial-associated molecular pattern (MAMPs, such as flagellin and chitosan) and toxin (coronatine, fumonisin B_1) induced defence or cell death in tomato plants. What is the difference between the physiological responses of tomato plants under the two environmental conditions?

Materials and methods

Wild type, ET insensitive *Never ripe* (*Nr*) and JA signalling defective *Jasmonic acid–insensitive 1* (*jai1-1*) tomato plants (*Solanum lycopersicum* Mill. L. cvar. Ailsa Craig and Castlemart) were grown controlled condition in the greenhouse. Plants were treated foliar with flagellin and chitosan or toxins (coronatine, fumonisin B_1) under light or dark conditions (Ördög et al. 2014; Takács et al. 2016). The width of stomatal apertures was measured on epidermal strips with microscope (Zeiss Axiovert) and by measuring stomatal conductance (Li-COR). Chlorophyll *a* fluorescence was monitored in individual guard cells with Microscopy-PAM chlorophyll fluorimeter (Waltz GmbH) and in intact leaves with Dual PAM-100 (Waltz GmbH). CO_2 assimilation and transpiration rate were monitored by LI-6400 Portable Photosynthesis and Fluorescence System (Li-COR) (Poór et al. 2012; Ördög et al. 2014). Levels of ROS and RNS in the leaf tissues and stomata were detected by spectrophotometric methods or by fluorescent staining using microscopes (Ördög et al. 2014; Poór et al. 2015). JA and SA were quantified fluorimetrically with HPLC (Pál et al. 2005). ET production will be measured by gas chromatography (Hewlett Packard) (Poór et al. 2013). Expression of hormone regulated defence marker genes (e.g. ERFs, PRs, Bax inhibitor-1, protease inhibitors) were determined by qRT-PCR (Analytik Jena) (Horváth et al. 2015). Enzyme activities and proteins were analyzed by native-PAGE, WB (Cleaver Scientific) and by spectrophotometer (KONTRON) (Kurepa et al. 2008). Localisation and quantification of specific stress proteins (e.g. PRs) were determined by post-embedding immunohistochemistry using electron microscope (Philips) (Talapka et al. 2016). Secondary metabolites were measured by UHPLC (Akram et al. 2008). The viability of tissues was determined by conductivity meter (Mettler-Toledo) and by staining (Poór et al. 2013).

1st year

Aims

Studying the effects of MAMPs (flagellin, chitosan) on the stomatal regulation of tomato plants and on the role of photosynthesis under light and dark conditions. Detecting the light-dependent changes in ROS and RNS levels induced by MAMPs in stomata and leaves. Sampling for hormonal-, gene expression- and protein analysis. Implementation of RNA extraction, genomic DNA digestions and cDNA synthesis. Design of experiments for optimization of SA treatments.

Results

Previous studies showed conflicting results regarding the ability of stomatal closure upon fungal and bacterial pathogens. Several factors can play role in these result, among others the pathogen- and host-derived compounds, the link between the infected leaf and whole-plant physiology or the daytime- and/or light-dependent activation of plant defence responses (Grimmer et al. 2012). Application of the elicitor chitosan (CHT; the deacylated derivative of the fungal cell wall component chitin) can mimic the attack of fungal pathogens and flagellin 22 (flg22; a peptide corresponding to the most conserved domain of bacterial flagellin) and provide easy tool to detect the effect on stomata and plant defence system (Ördög et al. 2014). To study the daytime- and light-dependent effects of CHT and flg22, we treated one developed leaf level of intact tomato plants at several daytimes, i.e. in the late afternoon (17:00 p.m.), in the evening (21:00 p.m.), at dawn (5:00 a.m.) and in the morning (8:00 a.m.) and measured the plant defence responses at several daytimes (6:00 a.m.; 9:00 a.m.; 15:00 p.m.) after each treatment (except after the treatment in the morning). To examine whether light regulation plays role in CHT- and flg22-induced defence reactions, artificial darkening experiments were set in the morning (from 8:00 a.m.) and the effect of elicitors was similarly detected during the day at 9:00 a.m. and at 15:00 p.m. The applied day/night cycle consisted of 12 h light (200 μ mol m^{-2} s^{-1} photon flux density) starting from 6:00 a.m. until 18:00 p.m., and 12 h dark period during the remaining daytime. To detect the role of the first line of defence, changes in the stomatal movement upon elicitor treatments, abaxial tomato leaves of the 6th developed leaf levels were treated with a squirrel hair brush.

In tomato leaves, stomata started to open at dawn and reached the maxima of stomatal pore size around 12 h and then started to close during the afternoon. Interestingly, CHT not only inhibited the light-induced stomatal opening at dawn, at 6:00 am, but it also induced the stomatal closure on the abaxial epidermis of intact plants in the morning at 8:00 am both in the light and dark, which changes were independent from the daytime of the application. Flg22 also caused stomatal closure at these daytimes, but the intensity of stomatal closure depended on the daytime of the treatments. In contract to flg22, CHT did not affect stomatal movement in the late afternoon, where stomata started to close and accumulated photoassimilates. At the same time, CHT treatments did not induce significant stomatal responses in the distal leaves from CHT-treated leaves in the first light phase of the experiment.

Because stomatal movements regulated by ROS and NO, which are mediated by several signalling pathways (Melotto et al. 2008) and are linked with light- and circadian rhythm (Kangasjärvi et al. 2012), the possible daytime effects on elicitor-induced stomatal closure were also examined on the production of ROS and RNS in the guard cell pairs. In all cases, CHT and flg22 induced significant ROS generation in the guard cell pairs of the abaxial epidermis of tomato leaves in the first part of the light phase of the day independently of the daytime of elicitor treatments. However, ROS burst was significantly higher at 9:00 am compared to 6:00 a.m., especially in the case of the evening (21:00 p.m.) treated leaves. Interestingly, CHT promoted significant NO generation only in the afternoon (15:00 p.m.) and upon artificial dark treatment combined with CHT, which also resulted in ROS production in the guard cell pairs.

Pathogens and elicitors, like CHT and flg22, not only induce high ROS and RNS production especially in chloroplasts but also have essential effects on photosynthetic activity (Srivastava et al. 2009; Ördög et al. 2011). Inhibition and/or degradation of the photosynthetic electron transport can result in a decrease in ATP production and ROS/RNS generation leading to initiation of stomatal closure (Lawson, 2009). Chlorophyll *a* fluorescence parameters demonstrate well the effects of different stressors and elicitor effects on PSII activity in stomata and mesophyll cells, respectively (Poór and Tari, 2012). Therefore, the daytime- and light-dependence of the Photosystem II (PSII) activity was investigated after elicitor treatments in order to ascertain the possible effects of CHT and flg22 mediated by light- and circadian rhythm on PSII activity. The maximal quantum yield of PSII photochemistry (Fv/Fm) decreased only slightly upon both elicitor treatments. In contrast, the actual quantum yield of PSII electron transport in the light adapted-state (Φ_{PSII}) and the photochemical quenching coefficient (qP) decreased upon CHT treatments at dawn independently from the daytime of CHT treatments. In contrast, flg22 decreased both parameter in the late light phase. Interestingly, when leaves were treated with CHT in the morning at 8:00 a.m., when stomata were already opened, Φ_{PSII} and qP did not change significantly in the guard cells. Moreover, CHT and flg22 also did not influence significantly the non-photochemical quenching (NPQ) parameter, which indicates the ability of chloroplasts to dissipate excess excitation energy as heat. As observed in the case of stomatal movement, there were not measured significant changes in chlorophyll *a* fluorescence parameters in the distal leaves from the elicitor-treated ones in this first light period after the fungal elicitor application.

To assess whether the foliar application of CHT and flg22 with a brush on intact plants results in changes and when in the chlorophyll *a* fluorescence parameters of mesophyll cells, photosynthetic activity measurements were carried out in the leaves after the different daytime applied elicitor treatments. Unexpected results were detected in the CHT-treated leaves, where Φ_{PSII} also significantly decreased already at dawn independently from the daytime of CHT treatments like it was observed in the guard cells. These suggest that CHT perception on the epidermis, which means the first line of plant defence system, induce significant changes in the photosynthetic activity and thus signalling and metabolism of mesophyll in the earliest phase of the light period of the day inducing defence in leaves and plant.

Optimal timing of phytohormone-mediated defence signalling and SAR development is crucial upon pathogen infection in plants (Karapetyan and Dong, 2017). It was next assessed which defence hormone-responsive gene expressed upon CHT and flg22 treatments and whether the defence hormones inducible genes show any daytime- light- or organ-specific pattern after elicitor treatments (effects of flg22 is under investigation), which had previously been shown in case of bacterial pathogen *Pseudomonas syringae* in *Arabidopsis* plants (Griebel and Zeier, 2008). The most significant expression was observed in the case of SA-induced *PR1* marker gene. Early *PR1* expression was induced already at dawn (6:00 a.m.) in the CHT-treated leaves which raised the maxima in the early light phase of the day upon CHT treatment in the afternoon (17:00 p.m.) and evening (21:00 p.m.). The highest value was observed upon the CHT application in the early night, at 21:00 p.m. in the dark phase. The most important results were observed in the morning in the early light phase, where CHT induced *PR1* expression within 1 hour in the light, but the simultaneously dark was able to inhibit this increase in *PR1* transcript levels suggesting the potential light-dependent regulation of CHT-induced defence responses in tomato plants. Interestingly, the expression of Ethylene Response Factor 1 (*ERF1*), which can be induced rapidly by ethylene and JA as well as synergistically by both hormones (Müller and Munné-Bosch, 2015), did not change significantly after the CHT treatments in the examined daytimes of the first light phase. However, CHT induced the expression of *ERF1* after 1 hour of the elicitor treatments, which suggest the role of ET and JA in the first hours after recognition of CHT and the dominant role of SA based on *PR1* expression upon CHT in tomato plants. In contrast, *ERF1* transcript levels significantly increased under darkness, where *PR1* expression decreased in parallel suggesting the crucial role of the presence of light in the regulation of plant responses to CHT.

Dissemination of the results

Published articles in international and peer-reviewed journals:

- Gallé, Á., Czékus, Z., Bela, K., Horváth, E., Ördög, A., Csiszár, J., & Poór, P. (2019). Plant glutathione transferases and light. *Frontiers in plant science, 9*, 1944.

International- and national conference issues:

- Czékus Zalán, Kukri András, Ördög Attila, Poór Péter: A kitozán által kiváltott immunválasz fényregulációja és napszak függése paradicsomban: a zárósejtek fotoszintézisének szerepe, Garab, Győző; Janda, Tibor; Darkó, Éva; Pál, Magda; Pusztai, Magdolna; Solymosi, Katalin; Zsiros, Ottó (szerk.) Hazai Fotoszintézis-kutatók Találkozója: Program és Össze, 2018
- Péter Poór, Zalán Czékus, Attila Ördög: Chitosan elicited immune response reduced photosynthetic electron transport in the guard cells of tomato plants under different light conditions, Frank, Takken; Alisher, Touraev (szerk.): Plant Biotic Stresses & Resistance Mechanisms III, 2018

2nd year

Aims

Our goal was to reveal the changes in endogenous hormone levels (SA, JA, ET), in expression of specific genes (hormone biosynthesis and response genes, defence marker genes), in protein levels as well as in production of secondary metabolites following the MAMP (flagellin, chitosan) triggered immune response of tomato under light and dark conditions. Contribution of hormonal signalling and hormone-mediated ROS and RNS in this process will be investigated and compared in the light and dark in ET (*Never ripe*) and JA signalling mutants (*jai1-1)* of tomato plants. We wanted to investigate, if the effect of SA on JA/ET signalling is antagonistic or cooperative, and to reveal the possible differences between treatments in the dark and light. Starting and optimizing the experiments for appropriate toxin treatments.

Results

We treated leaves on the 6th leaf levels (counted from the top) of intact tomato plants at several daytimes, i.e. in the late afternoon (5:00 p.m.), in the evening (9:00 p.m.), at dawn (4:00 a.m.) and in the morning (8:00 a.m.) and we measured the plant defence responses at different time points (5:00 a.m.; 9:00 a.m.; 3:00 p.m.) after each treatment (except after the treatment in the morning). To examine whether light regulation plays role in elicitor-induced defence reactions, artificial darkening experiments were set up in the morning (from 8:00 a.m.) and the effect of elicitor was similarly detected during the day at 9:00 a.m. and at 3:00 p.m. The applied day/night cycle consisted of 12 h light (200 µmol m^{-2} s^{-1} photon flux density) starting from 6:00 a.m. until 18:00 p.m. and 12 h dark period during the remaining daytime. To detect the role of the first line of defence, the elicitor-treated 6th leaf levels were used for the experiments. To examine the systemic responses of tomato plants, the distal 7th leaf levels above the elicitor-treated ones were also analysed.

Based on the results of the first year, we focused on the elicitor treatments in the late afternoon (5:00 p.m.; light) and in the evening (9:00 p.m.; dark) in the hormone mutant plants in this year. In addition, experiments were carried out in the morning (8:00 a.m.) under light and dark conditions, respectively. Contribution of hormonal signalling and hormone-mediated ROS and RNS in this process have been investigated and compared in *Nr* and *jai1-1* tomato plants.

Significant stomatal closure was induced after flg22 treatment in WT plants at 5:00 p.m. but stomata did not close in the evening at 9:00 p.m. Moreover, stomatal aperture did not change in *Nr* leaves suggesting the potential signalling role of ET in flg22-induced defence reactions. Similar changes were observed in the case of CHT treatments. CHT application did not induce significant stomatal closure in *Nr* plants. Not only ET but JA also plays a role in stomatal closure after the treatments with both elicitors. Similarly to *Nr* plants, flg22 and CHT did not cause significant stomatal closure in *jai1-1* plants. In addition, the diameter of stomatal pores was smaller in *jai1-1*. Stomatal closure was also not observed upon none of the treatments in the morning at 8 a.m. in the mutant plants. Surprisingly, the systemic response was detected after both treatments in the upper leaves of the flg22- or CHT-treated ones of WT plants in the morning but it was not observed in *Nr* and *jai1-1* plants.

Flg22 induced ET emission and JA and SA accumulation in the light phase but ET emission was not significant treated in the dark. Similarly to ET, SA content also did not change significantly at 9:00 p.m., suggesting the light dependence of hormone signalling pathways upon flg22. CHT also induced significantly high ET emission in the light phase of the day. Interestingly, ET production did not change in distal leaves upon none of the elicitor treatments. Based on gene expression analysis, elicitor treatments induced the expression of ACC synthase-coding tomato genes only at the light phase treated leaves.

Accumulation of superoxide, H_2O_2 and NO was significantly different after flg22 and CHT treatments in the late dark phase and in the early night. Namely, ROS and NO levels were significantly lower in case of treatments at night. Interestingly, NADPH oxidase activity upon flg22 treatment was slightly higher in the dark period of the day but it remained lower in *Nr* mutants. In *jai1-1* leaves, H_2O_2 levels did not change significantly after flg22 treatment in both daytimes, while it slightly decreased upon CHT application. Interestingly, superoxide dismutase activity also decreased after flg22 treatment dependently on active ET signalling.
Investigation of hormone response genes, *PR1*, *ERF1* and various defensins (*DEF5-9*) showed significant differences in several daytimes upon flg22 and CHT treatments. *PR1* expression was significantly higher after 1 hour later upon elicitor treatments at 5:00 p.m. but did not change at 21:00 p.m. In contrast, *ERF1* increased significantly in the dark. The most characteristic changes in *DEF* genes were observed upon CHT treatments. CHT-induced accumulation of *DEF* transcript levels was decreased by dark. *PR1* expression was basically higher in *Nr* plants and did not change upon flg22. In contrast to *Nr* leaves, *PR1* and *ERF1* expression were elevated by flg22 in *jai1-1* plants. CHT treatment also induced *PR1* expression in *jai1-1* leaves but *ERF*1 did not change significantly in these plants. Analysis of PRs, especially PR3 protein showed significant changes especially upon CHT treatment in WT leaves compared to the mutants.

Based on our results we can conclude that early biotic signalling in intact leaves is an ET/JA- and light-dependent process, which has great importance on the guard cell-mediated plant defence responses.

Dissemination of the results

Published articles in international and peer-reviewed journals:

- Kolbert Zsuzsanna; Feigl Gábor; Freschi Luciano ; Poór Péter: Gasotransmitters in Action: Nitric Oxide-Ethylene Crosstalk during Plant Growth and Abiotic Stress Responses, Antioxidants 2019, 8(6), 167; https://doi.org/10.3390/antiox8060167, 2019

- Péter Poór; Zalán Czékus; Irma Tari; Attila Ördög: The Multifaceted Roles of Plant Hormone Salicylic Acid in Endoplasmic Reticulum Stress and Unfolded Protein Response, Int. J. Mol. Sci. 2019, 20(23), 5842; https://doi.org/10.3390/ijms20235842, 2019

Published book chapters in international books:

- Péter Poór; Zalán Czékus; Attila, Ördög: Role of Nitric Oxide in Physiological and Stress Responses of Plants Under Darkness, Reactive Oxygen, Nitrogen and Sulfur Species in Plants: Production, Metabolism, Signaling and Defense Mechanisms, 2019

International- and national conference issues:

- Attila Ördög; Zalán Czékus; András Kukri; Angela Girón Lafuente; Marina Zafra Salcedo; Péter Poór: Organ-specific and daytime-dependent effects of exogenous flg22 elicitor treatments on the photosynthetic activity of tomato leaves, 9th Conference of the Polish Society of Experimental Plant Biology ABSTRACT BOOK: New trends in plant reproduction and growth regulation (2019) p. 90, 2019
- Czékus Zalán; Poór Péter; Kukri András; Tari Irma; Ördög Attila: Kitozán indukálta védekezés napszak- és fény-függő hatásának vizsgálata, Poór, Péter; Blázovics, Anna (szerk.) Magyar Szabadgyök-Kutató Társaság X. Kongresszusa: Program és összefoglalók Szeged, Magyarország : Szegedi Tudományegyetem, (2019), 2019
- Gallé Ágnes; Pelsőczi Alina; Czékus Zalán; Csiszár Jolán; Poór Péter: Glutation-transzferáz enzimek fényindukálahtósága búzában, Poór, Péter; Blázovics, Anna (szerk.) Magyar Szabadgyök-Kutató Társaság X. Kongresszusa: Program és összefoglalók Szeged, Magyarország : Szegedi Tudományegyetem, (2019), 2019
- Kukri András; Czékus Zalán; Pollák Boglárka; Csóré Dóra; Ördög Attila; Poór Péter: Flg22 elicitor etilénfüggő hatásainak vizsgálata paradicsom levelében, In: Poór, Péter; Blázovics, Anna (szerk.) Magyar Szabadgyök-Kutató Társaság X. Kongresszusa: Program és összefoglalók Szeged, Magyarország: Szegedi Tudományegyetem, (20, 2019
- Ördög Attila; Czékus Zalán; Kukri András; Poór Péter: Exogén flg22-kezelés napszak- és szerv-függő hatása paradicsom növények fotoszintézisére, In: Poór, Péter; Blázovics, Anna (szerk.) Magyar Szabadgyök-Kutató Társaság X. Kongresszusa: Program és összefoglalók Szeged, Magyarország : Szegedi Tudományegyetem, (20, 2019
- Péter Poór; Zalán Czékus; András Kukri; Angela Girón Lafuente; Marina Zafra Salcedo; Attila Ördög: Activation of defense responses by flg22 elicitor is dependent on the daytime and ethylene in intact tomato leaves, 9th Conference of the Polish Society of Experimental Plant Biology ABSTRACT BOOK: New trends in plant reproduction and growth regulation (2019) p. 89, 2019
- Poór Péter; Czékus Zalán; Farkas Máté; Bakacsy László; Ördög Attila; Gallé Ágnes: Herbicid alkalmazásának napszak függő hatásai az antioxidáns védelmi rendszerre, In: Poór, Péter; Blázovics, Anna (szerk.) Magyar Szabadgyök-Kutató Társaság X. Kongresszusa: Program és összefoglalók Szeged, Magyarország : Szegedi Tudományegyetem, (20, 2019
- Zalán Czékus; András Kukri; Péter Poór; Attila, Ördög: Chitosan-induced plant defence responses are influenced by light and daytime in tomato, 9th Conference of the Polish Society of Experimental Plant Biology ABSTRACT BOOK: New trends in plant reproduction and growth regulation (2019) p. 92, 2019

3rd year

Aims

Further analysis of SA treatments on MAMP induced physiological effects of plants, based on results of the previous year. Studying the effects of toxins (coronatine, fumonisin B_1) on the stomatal regulation of tomato plants and on the role of photosynthesis under light and dark conditions. Detecting the light-dependent changes in ROS/RNS levels and cell viability induced by toxins in stomata and leaves. Starting of the hormonal-, gene expression- and protein analysis. Implementation of RNA extraction, genomic DNA digestions and cDNA synthesis.

Results

Based on the results of the second year, we focused on the role of ET and SA in the fast and probably light-dependent responses of plants. Experiments with CHT were carried out in the morning (8:00 a.m.) under light and dark conditions in ethylene receptor mutant (*Never ripe*; *Nr*) and JA signalling mutant (*jai1-1*) tomato plants. Based on our measurements, significant ET production was observed in wild-type and *Nr* plants upon CHT locally in the light, which was significantly lower under darkness in the wild-type plants. The production of ROS as signalling molecules changed also significantly. CHT induced the generation of superoxide locally and systemically, respectively. This systemic response upon CHT was not observed in *Nr* plants, suggesting the role of ET in the development of rapid defence responses of intact plants. However, levels of hydrogen peroxide, as long-distance signalling molecule did not change at this time point. At the same time, NO generated also after CHT exposure locally and systemically in the wild-type plants as well as in *Nr* but only in the light. These results suggested that ROS/NO signalling is a light-dependent process upon CHT. Besides the detection of oxidative/nitrosative stress, PR proteins have been also investigated in the stress responses of tomato plants. PRs play role in suppressing pathogens via detoxifying virulence factors or degrading cell walls. Among PR proteins, PR-3 chitinase group is specifically responsible for preventing microbial infection, while expression of *PR-1* is strongly correlated with the development of systemic responses. We observed that expression of *PR3* was induced after CHT treatment both locally and in the systemic leaves, which expression was significantly higher in *Nr* leaves and interestingly in the dark. The levels of PR3 protein determined by Western-blot analysis showed similar tendencies but chitinase activity did not change significantly after one hour of the elicitor treatments. The excessive production of antimicrobial proteins may exceed the folding capacity of the endoplasmic reticulum (ER) under biotic stress and cause ER stress that triggers unfolded protein response (UPR) in plants. Thus we investigated the role of UPR marker lumenal binding protein (BiP) in CHT-induced defence mechanisms. Based on the results, expression of tomato *BiP* and protein levels of BiP changed similarly to PR3 which suggest that this part of CHT-triggered defence is not dependent on ethylene and the presence/absence of light but dark can amplify them.

The potential role of ET in systemic responses of tomato plants generated by flg22 was further investigated using ET modulators. Co-application of ET biosynthesis inhibitor aminoethoxyvinylglycine (AVG) with flg22 reduced the ET emission locally and flg22-induced systemic responses such as stomatal closure, moreover, superoxide production and *PR1* expression were significantly lower as compared to the only flg22-treated plants. In addition, the distal, systemic leaves from the flg22-treated ones were exposed to the ET receptor inhibitor silver thiosulfate resulting also lower stomatal closure, superoxide production and *PR1* expression. These results confirmed the role of ET in the development of fast systemic response of tomato plants upon flg22 treatments.

The light-dependent defence responses of plants were further examined using toxins. FB1 is one of the most harmful mycotoxins which poses serious threats to humans, animals and plants, respectively. FB1 induces the perturbation of sphingolipid metabolism, oxidative stress and hypersensitive response in plants while coronatine mimicks the bacterial-induced pathogenesis inhibiting the stomatal closure in plants. The phytohormones such as SA, JA and ET are one among the key regulators in defence responses upon toxins. At the same time, the effects of toxins on the photosynthesis, which can contribute to defence responses of plants, are not known in full details. We found that toxin-induced cell death is more significant in the light as compared in the dark. This result suggested that the active photosynthesis and its inhibition can contribute to higher ROS production leading to faster cell death in the toxin-treated leaves. We detected the sublethal, 1 μM and a cell death-inducing, 10 μM concentrations of FB1 on tomato plants for the first time. In addition, we tested the effects of fusaric acid (FA) in two different concentrations (0.1 and 1 mM), respectively. The maximal quantum yield of photosystem II (F_v/F_m) did not change significantly after the 3-days-long FB1 treatments in the investigated tomato genotypes. However, the effective quantum yields of PSII [Y(II)] and PSI [Y(I)] decreased in case of the higher FB1 concentration, especially in *Nr* leaves suggesting that these plants are more sensitive to the mycotoxin in case of the lack of the active ethylene signalling. At the same time, the non-photochemical energy dissipation [Y(NPQ)] significantly elevated in these plants. Moreover, the quantum yield of non-photochemical energy dissipation in PSI due to donor side limitations Y(ND) followed the same trend as Y(NPQ), while in case of the acceptor side limitations Y(NA) decreased slightly during 10 μM FB1 exposure in *Nr* plants. In addition, the yield of cyclic electron flow (CEF) around PSI also elevated after 10 μM FB1 in these plants. In contrast to FB1, F_v/F_m significantly decreased after the 3-days-long FA treatments in the investigated genotypes but it was lower in *Nr* leaves. In addition, Y(II) and Y(I) also significantly decreased in the case of the higher FA concentration. At the same time, NPQ significantly elevated in these plants. Moreover, Y(ND) followed the same trend as Y(NPQ), while Y(NA) decreased during 1 mM FA exposure in *Nr* plants. The higher concentration of FA elevated also the rate of lipid peroxidation and caused the loss of membrane integrity in both genotypes.

Our results suggest that these toxins have a significant effect on photosynthetic activity in plants and confirmed the ethylene-regulated photoprotective mechanisms in plants exposed to mycotoxin treatments.

Dissemination of the results

Published manuscripts in international and peer-reviewed journals:

- Czékus Zalán; Csíkos Orsolya; Ördög Attila; Tari Irma; Poór Péter: Effects of Jasmonic Acid in ER Stress and Unfolded Protein Response in Tomato Plants, BIOMOLECULES 10: 7 Paper: 1031, 20 p. (2020), 2020
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- Czékus Zalán; Poór Péter; Tari Irma; Ördög Attila: Effects of Light and Daytime on the Regulation of Chitosan-Induced Stomatal Responses and Defence in Tomato Plants, PLANTS-BASEL 9: 1 Paper: 59, 21 p. (2020), 2020
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- Iqbal Nadeem; Czékus Zalán; Poór Péter; Ördög, Attila: Ethylene-dependent effects of fumonisin B1 on the photosynthetic activity of tomato plants, Barna, Boglárka Johanna; Kovács, Petra; Molnár, Dóra; Pató, Viktória Lilla (szerk.) XXIII. Tavaszi Szél Konferencia Absztrakt Kötet : "Mi és a tudomány jövője", 2020
- Nadeem Iqbal; Zalán Czékus; Péter Poór; Attila Ördög: Ethylene-dependent effects of Fusaric acid on the photosynthetic activity of tomato plants, Csiszár, B; Hankó, Cs; Kajos, L F; Kovács, O B; Mező, E; Szabó, R; Szabó-Guth, K (szerk.) IX. INTERDISZCIPLINÁRIS DOKTORANDUSZ KONFERENCIA 2020 ABSZTRAKTKÖTET: 9th INTER, 2020
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4th year

Aims

Our goal was reveal the changes in endogenous hormone levels (SA, JA, ET), in expression of specific genes (hormone biosynthesis and response genes, defence and cell death marker genes), in protein levels as well as in production of secondary metabolites following toxin (coronatine, fumonisin B_1) treatments of tomato under light and dark conditions. Contribution of hormonal signalling and hormone-mediated ROS and RNS in this process will be investigated. The given results will be compared between the light and dark conditions in both the ET (ET receptor mutant *Never ripe*) and JA signalling mutants (*jai1-1)* of tomato plants. We would like to investigate, if the effect of SA on JA/ET signalling is antagonistic or cooperative, and to reveal the possible differences between treatments in the dark and light after the toxin treatments.

Results

Based on the results of the third year, we were focusing on the role of ET and SA in the fast and probably light-dependent responses of plants upon toxin exposure. Firstly, the role of defence-related phytohormones in the regulation of photosynthesis under FB1 or FA exposure was examined. FB1 and FA are harmful mycotoxins produced by several *Fusarium* species, which results in triggered oxidative stress leading to cell death in plants. FB1 perturbs the metabolism of sphingolipids by inhibiting ceramide synthase activity and causes growth and yield reduction in many crops, while FA induces lethal oxidative stress in plants. We found that both toxins induced a significant and rapid ET emission, thus were focusing on the role of ET in plant defence responses upon toxin treatments. Unfortunately, the JA-mediated signalling was not so significant as compared to ET. Thus our investigation focused to the application of ET receptor mutant *Nr* instead of *jai-1* plants. We measured a concentration-dependent ET production upon both mycotoxins in WT and *Nr* leaves. Moreover, FB1 significantly affected the photosynthetic parameters of PSII and PSI and activated photoprotective mechanisms such as nonphotochemical quenching (NPQ) in both genotypes, especially under 10 μM FB1 concentration. Further, the net photosynthetic rate and stomatal conductance were significantly reduced in both genotypes in an FB1 dose-dependent manner. Interestingly, lipid peroxidation and loss of cell viability were also more pronounced in WT as compared to *Nr* leaves confirming the role of ET in cell death induction in the leaves upon toxin treatments. Thus, FB1-induced oxidative stress (higher $O_2^{\cdot-}$ and H_2O_2 levels) affected the working efficiency of PSI and PSII in both tomato genotypes. NO levels were decreased upon toxin treatments suggesting the vital role of NO in the defence responses against FB1. Interestingly, ET-dependent antioxidant enzymatic defense mechanisms were activated by FB1 and manifested in significantly elevated superoxide dismutase, ascorbate peroxidase and glutathione S-transferase activities, especially in *Nr* plants as compared to WT tomato plants confirming the role of ET in the regulation of cell death and defense mechanisms under the mycotoxin exposure.

In the case of FA we found that FA induced the accumulation of ROS ($O_2^{\cdot-}$ and H_2O_2) in a time- and concentration-dependent manner in both tomato genotypes. Lethal FA concentration at the 72nd h followed by the treatment resulted in oxidative burst, activated NADPH oxidase, and triggered elevated antioxidant defence mechanisms even at transcription levels in WT plants. Further, FA treatment enhanced the activities of POD, SOD, APX antioxidants while CAT activity was reduced particularly under 1 mM FA concentration after 72 h. Furthermore, AsA level was decreased and GSH content was recorded to be higher under 1mM FA exposure but there were significant differences in their levels in WT and *Nr* leaves. The decrease in CAT activity was compensated by the increase of APX and POD activities to decompose H_2O_2. The activity of APX was higher but POD was significantly lower in *Nr* as compared to WT leaves. However, the expression levels of *SOD-CuZn*, *CAT2*, and *CAT3* were elevated significantly in both genotypes after 72 h than but not after 24 h following treatment. ET is involved in the induction of cell death by increasing oxidative stress while on the other hand, it plays a crucial role in the activation of key antioxidants reflecting defence response under FA exposure even at the genetic level. Conclusively, ET serves as a potential candidate among phytohormones in mitigating mycotoxin-induced stress by activating several enzymatic and non-enzymatic antioxidant mechanisms to detoxify excess ROS accumulation for the proper functioning and undistributed growth of plants. These findings could assist in future research on mycotoxin-induced alleviation of environmental stresses in economically important plants.

The effects of the bacterial effector coronatine (COR) were also tested which mimics the bacteria-induced pathogenesis inhibiting the stomatal closure in plants. It is well known that COR can overwrite the flg22-induced stomatal closure as the defence response of plants. In addition, we found that flg22-induced stomatal closure is mediated by ET and JA using *Nr* and *jai-1* plants. At the same time, the effects of COR on SA-induced stomatal closure and the light-dependent effects of COR remained unclear. In addition, the function of stomatal photosynthesis and the role of chloroplasts have not been investigated in this process. We found that COR application in the morning at 9:00 a.m. resulted in stomatal opening in 24-hours-long SA pre-treated plants within hours. The effects of COR on the regulation of stomatal movement were light-dependent. Significantly lower ROS and NO production was detected in stomata of COR-sprayed and SA pre-treated leaves contributing to the stomatal opening. The role of chloroplast in this process was investigated by microscopy-PAM technique and significant changes were found in Yield and NPQ influencing stomatal movement. Expression of hormone response genes, *PR1*, *ERF1*, and various defensins (*DEF5-9*) showed significant differences in COR-treated leaves. Analysis of PR3 and DEF proteins on whole leaf and guard cell levels is under process.

Dissemination of the results

<u>Published manuscripts in international and peer-reviewed journals:</u>

- Czékus Zalán; Kukri András; Hamow Kamirán Áron; Szalai Gabriella; Tari Irma; Ördög Attila; Poór Péter: Activation of Local and Systemic Defence Responses by Flg22 Is Dependent on Daytime and Ethylene in Intact Tomato Plants, INTERNATIONAL JOURNAL OF MOLECULAR SCIENCES 22: 15 Paper: 8354 , 21 p. (2021), 2021
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<u>Planned manuscripts in international and peer-reviewed journals:</u>

- Z. CZÉKUS, P. KOPRIVANACZ, A. KUKRI, N. IQBAL, A. ÖRDÖG, P. POÓR: The role of photosynthetic activity in the regulation of flg22-induced local- and systemic defence reaction in tomato. *Under major revision at Photosynthetica*
- Nadeem Iqbal, Zalán Czékus, Cserne Angeli, Tibor Bartók, Péter Poór, Attila Ördög: FB1-induced Oxidative Burst Perturbed Photosynthetic Activity and Affected Antioxidant Enzymatic Response in Tomato Plants in Ethylene-dependent Manner. *Submitted to JPGR*
- Zalán Czékus; Atina Martics; Boglárka Pollák; András Kukri; Tari Irma; Attila Ördög; Péter Poór: Effects of ethylene modulators on the flg22-induced rapid local- and systemic defence responses of tomato plants. *Ready to submit to JPR*
- Ethylene-dependent regulation of oxidative stress in the leaves of fusaric acid-treated tomato plants. *Will be submit to JPP*

Significance of the research

Our research was the first occasion when the effects of light and darkness on MAMP (flagellin, chitosan) and toxin (coronatine, fumonisin B_1) induced defence or cell death reaction of tomato plants and the hormonal crosstalk (SA, JA, ET)-mediated ROS and RNS production was investigated. The role of phytohormones in the presence and absence of light has not been fully elucidated yet, and it is of interest why plant defence and cell death is different under the two environmental conditions and what is the role of SA, JA and ET signalling in this process. Dysfunction in stomatal movement and the photosynthesis caused by flagellin and chitosan can control the basal defence reactions since it determines the production of ROS and RNS. We were also interested in how hormonal crosstalk can mediate the local and systemic defence reaction of plants in the light and darkness. Analysis of specific genes and proteins in wild type and hormone signalling mutants helped to understand the fine-tuning of defence in light and dark conditions mediated by phytohormones. Results of this project could contribute to developing innovative procedures in plant protection against invading pathogens under dark conditions in the future.

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