Role of ethylene and light in chitosan-induced local and systemic defence responses of tomato plants

Zalán Czékus[1,2], Nadeem Iqbal[1,3], Boglárka Pollák[1], Atina Martics[1], Attila Ördög[1], Péter Poór[1,*]

[1]Department of Plant Biology, University of Szeged, H-6726 Szeged, Közép fasor 52., Hungary
[2]Doctoral School of Biology, University of Szeged, Szeged, Hungary
[3]Doctoral School of Environmental Sciences, University of Szeged, Szeged, Hungary
[*]Corresponding author (poorpeti@bio.u-szeged.hu; Tel/Fax: +36 62 54 4307)
E-mail addresses: czekus.z@bio.u-szeged.hu (Z. Czékus), nadeemiqbal814@gmail.com (N. Iqbal), pollak.boglarka11@gmail.com (B. Pollák), martics.athena@gmail.com (A. Martics), aordog@bio.u-szeged.hu (A. Ördög)

Highlights

- CHT induced ET and $O_2^{\cdot-}$ production and stomatal closure locally and systemically
- ET and $O_2^{\cdot-}$ production was inhibited in the absence of light upon CHT treatment
- CHT increased *SlPR3* and PR3 accumulation, but the systemic response is ET-dependent
- CHT-induced systemic UPR is mediated by ET

Abstract

Plant defence responses can be triggered by the application of elicitors for example chitosan (β-1,4-linked glucosamine; CHT). It is well-known that CHT induces rapid, local production of reactive oxygen species (ROS) and nitric oxide (NO) resulting in fast stomatal closure. Systemic defence responses are based primarily on phytohormones such as ethylene (ET) and salicylic acid (SA), moreover on the expression of hormone-mediated defence genes and proteins. At the same time, these responses can be dependent also on external factors, such as light but its role was less-investigated. Based on our result in intact tomato plants (*Solanum lycopersicum* L.), CHT treatment not only induced significant ET emission and stomatal closure locally but also promoted significant production of superoxide which was also detectable in the distal, systemic leaves. However, these changes in ET and superoxide accumulation were detected only in wild type (WT) plants kept in light and were inhibited under darkness as well as in ET receptor *Never ripe* (*Nr*) mutants suggesting pivotal importance of ET and light in inducing resistance both locally and systemically upon CHT. Interestingly, CHT-induced NO production was mostly independent of ET or light. At the same time, expression of *Pathogenesis-related 3* (*PR3*) was increased locally in both genotypes in the light and in WT leaves under darkness. This was also observed in distal leaves of WT plants. The CHT-induced endoplasmic reticulum (ER) stress, as well as unfolded protein response (UPR) were examined for the first time, via analysis of the lumenal binding protein (BiP). Whereas local expression of *BiP* was not dependent on the availability of light or ET, systemically it was mediated by ET.

Keywords

dark; chitosan; nitric oxide; reactive oxygen species; stomata; unfolded protein response

Introduction

Plants are challenged by a large scale of factors limiting their optimal growth, development and yield that can include both abiotic (light, water availability or temperature) and biotic environmental issues. Although plants lack specific immune cells, sophisticated signalling cascade activations of innate immunity enable them to defend themselves against diverse pathogens and pests (Ballaré, 2014). Molecular evolution provides a highly plastic recognition system allowing them to identify various kinds of attackers, which can be recognised by the conserved damage- or pathogen-associated molecular patterns (DAMPs or PAMPs) through cell surface-located pattern-recognition receptors (PRRs). Recognition of PAMPs triggers the so-called PAMP-triggered immunity (PTI), in which stomatal closure plays a pivotal role as the main barrier of pathogen invasion (Melotto et al., 2008; Han, 2019). In response to activation of plant PTI, pathogens have developed complex strategies to elude and hinder plant defence reactions. Production of specific effector proteins can provoke the effector-triggered immunity (ETI) response of plants (Pieterse et al., 2012). Upon activation of ETI, the most elementary defence reactions result in a hypersensitive response (HR) manifested in localized programmed cell death (PCD) at the infection site (Han, 2019). Simultaneously with the development of local acquired resistance (LAR), whole-plant-extending level of resistance can be also provoked which manifests in systemic acquired resistance (SAR) in the distal, pathogen-free organs (Fu and Dong, 2013; Shah and Zeier, 2013). SAR already develops directly beyond the LAR zone, conversely, it can not be clearly distinguished from SAR developed in distal „systemic" leaves (Cordelier et al., 2003).

Nevertheless, in such a fast-moving evolutionary arms race between host and pathogens, abiotic factors have crucial importance (Roden and Ingle, 2009). Among others, the quality, intensity and duration of light can determine the virulence of pathogens, activation of defence responses and therefore survival of plants (Roden and Ingle, 2009; Poór et al., 2018). Roberts and Paul (2006) have assigned prevalent importance to light availability primarily in the initial phase of infection in contrast to circadian rhythm under activation of defence responses. Light exposure after infection can be also a determinant by modulating the extent of methyl-salicylate (MeSA) required for the establishment of SAR (Liu et al., 2011). Moreover, the salicylic acid (SA)-mediated production of reactive oxygen species (ROS) which is essential for the establishment of SAR also seems to be dependent on the availability of light (Poór et al., 2017). In contrast, the accumulation of some other compounds related to local plant defence reactions like jasmonic acid (JA) was found to be light-independent but the role of another gaseous phytohormone ethylene (ET) remained uninvestigated in this process (Zeier et al., 2004).

Oxidative burst triggered by receptor-mediated recognition of pathogens is induced in the apoplast by NADPH respiratory burst oxidase D (RbohD). NADPH oxidase plays an important role in producing superoxide radical ($O_2^{\cdot -}$), however other enzymes or cell compartments can also contribute to ROS production (Mersmann et al., 2010; Czarnocka and Karpiński, 2018). Overproduction of ROS and nitric oxide (NO) not only triggers PCD in HR but also takes part in cell wall strengthening, interferes with pathogens as well as functions as secondary messenger like a long-distance signal for the establishment of SAR through activation of defence-related genes (Floryszak-Wieczorek and Arasimowicz-Jelonek, 2016; Mandal et al., 2019).

Beyond oxidative burst, the accumulation of pathogenesis-related (PR) proteins plays an important role in plant defence reactions by suppressing pathogens via detoxifying virulence factors or degrading cell wall (Kushalappa et al., 2016). PR proteins can be divided into 17 families, from which the PR-3 chitinase group is specifically responsible for preventing microbial infection. Expression of acidic PR genes, like *PR-1*, is strongly

correlated with the SA levels and the development of SAR (Sels et al., 2008; Liu et al., 2011). Accumulation of different PR proteins was also demonstrated to show a tissue-specific pattern in numerous studies, where the content of basic PR proteins was higher in LAR, whereas a low production was observable in the case of acidic PR-1, PR-2 and PR-3 proteins (Brederode et al., 1991; Cordelier et al., 2003).

Demand for excessive production of proteins involved in defence responses against biotic stressors may exceed the folding capacity of the endoplasmic reticulum (ER) under adverse conditions, which can lead to the accumulation of misfolded and unfolded proteins generating ER stress (Afrin et al., 2020). Alleviation of ER stress can be mediated by unfolded protein response (UPR) responsible for up-regulation of components participate in proper folding as well as removing of unfolded proteins by transcriptional and translational processes (Deng et al., 2013). Components of ER quality control system (ERQC) are simultaneously up-regulated, such as various chaperones and enzymes of (N)-linked glycan modification supporting further folding (Iwata and Koizumi, 2012). Lumenal binding protein (BiP) is the main chaperon involved in the (N)-glycan-independent pathway, whose expression is up-regulated under UPR (Wan and Jiang, 2016; Wang et al., 2017). Accumulation of BiP is regulated in multiple ways, among others by SA as a positive modulator, however, the role of different plant hormones, particularly the function of ET is not known (Malerba et al., 2010; Poór et al., 2019a).

Depending on the host-pathogen interaction, plant defence reactions are differently regulated. While SA mainly plays role in plant responses to biotrophic pathogens, JA and ET are basically involved in defence processes against necrotrophs (Glazebrook, 2005). The role of ET in biotic stress responses is rather contradictory since besides functioning as a signalling molecule promoting disease resistance and establishment of SAR, it can also act as a virulence factor of some fungal or bacterial pathogens (Verberne et al., 2003; Chagué et al., 2006; van Loon et al., 2006a). It was also observed, that exogenous ET treatment applied prior to infection promoted survival whereas after that, it contributed to accelerated disease development (van Loon et al., 2006a). Cordelier et al. (2003) confirmed that expression of basic PR1, PR2, PR3 and PR5 proteins is also triggered through an ET-dependent pathway during the establishment of LAR by diffusing out of necrotizing cells under HR. This ET burst is strongly associated with necrotic lesion formation, therefore the development of HR (van Loon et al., 2006a). At the same time, light-regulation of ET production has not been completely clarified yet, but it may be negatively influenced by inhibiting the conversion of 1-aminocyclopropane-1-carboxylic acid (ACC) to ET (Kao and Yang, 1982).

Effects of biotic stressors can be investigated by the application of various elicitors such as chitosan (CHT), a deacetylated derivative of fungal cell wall-composing chitin (Iriti and Faoro, 2009; El Hadrami et al., 2010). Depending on its general properties (degree of acetylation, molecular weight, pH) it can act as a defender molecule contributing defence responses like PCD during HR as well as like an executor by triggering necrotic cell death (Sun et al., 2007; Iriti and Faoro, 2009). Perception of CHT induces different signalling cascades involving ROS, NO and different plant hormones, however, its receptor stills remained unidentified (Malerba and Cerana, 2016). CHT-triggered local defence responses appear in stomatal closure, membrane depolarization, accumulation of PR proteins, activation of mitogen-activated protein kinase (MAPK) cascade, callose deposition as well as oxidative burst associated with H_2O_2 accumulation (Ördög, 2011; Hadwiger, 2013; Malerba and Cerana, 2015; Suarez-Fernandez et al. 2020). Among plant hormones, JA was verified to be a key player in the induction of resistance by CHT against *Botrytis cinerea* infection (Peian et al., 2020). ET was also suggested to behave as a signalling element on the basis of the potential of oligochitosan to induce ET receptor and ET responsive element binding protein

(EREBP) genes (Yin et al., 2006). At the same time, not only the activation but also the de-repression of various defence-related genes mediated by various transcription factors upon ET can be a significant step in this process (McGrath et al., 2005; Agrawal et al., 2012; Pusztahelyi, 2018). Low molecular weight CHT-induced cell death was demonstrated to be associated with DNA fragmentation and cytochrome *c* release from mitochondria as classical hallmarks of PCD (Malerba et al., 2012). Activation of LAR as a consequence of HR results in the accumulation of PR proteins responsive to CHT like PR-1a, chitinase, glucanase or peroxidase contributing to increase the effectiveness of disease resistance upon pathogen infection (Nandeeshkumar et al., 2008; Yafei et al., 2009). At the same time, numerous current studies have verified the role of CHT in inducing SAR (Corsi et al., 2015; Martínez et al., 2018; Rendina et al., 2019; Czékus et al., 2020; Samarah et al., 2020; Suarez-Fernandez et al. 2020). However, there are many gaps in our knowledge regarding the light-dependence of CHT-induced defence reactions and the establishment of local- and systemic defence responses under dark conditions (Czékus et al., 2020). The capability of CHT to trigger ER stress and its contribution to CHT-induced defence responses also remained unanswered (Malerba et al., 2012). Moreover, since the role of ET during pathogen infection is rather contradictory in contrast to SA or JA, its exact function during the pathogen- or elicitor treatment in establishing LAR and SAR needs further clarification (van Loon et al., 2006a).

In this work, the short-time effects of CHT treatments on plant defence reactions were investigated in the presence or absence of light in the agriculturally important tomato plants. Furthermore, light-dependent development of ER stress and UPR after CHT treatment were investigated for the first time. Besides the CHT-induced rapid local defence responses, the possible CHT-induced systemic reactions were also investigated. The light-dependent role of ET in CHT-triggered defence processes, moreover its involvement in the development of local- and systemic responses were also in the focus of our investigation via using ET receptor-deficient *Never ripe* tomato plants.

Materials and methods

Plant material

Wild type (WT) and ET insensitive *Never ripe* (*Nr*) tomato (*Solanum lycopersicum* L. cv. Ailsa Craig) seeds were germinated at 26°C for 3 days at dark. After growing seedlings in perlit for additional 2 weeks, plants were grown hydroponically according to Poór et al. (2011). For growing plants, a constant environment was provided with a photosynthetic photon flux density of 200 μmol m^{-2} s^{-1} [PPFD; White LED (5700 K) supplemented with FAR LEDs; PSI, Drásov, Czech Republic], 12/12-hours light/dark period, 24/22°C of day/night temperatures and 55%–60% of relative humidity during five weeks. The experiments were conducted from intact plants at the 7th or 8th week at 8-9 developed leaf-level stage.

Treatments

The 6th leaf-level of intact plants was foliar-treated with low molecular weight CHT solution (Czékus et al., 2020). The stock solution was prepared as described by Shepherd et al. (1997) by dissolving CHT in acetate (AA) buffer (100 mM, pH 3.6) reaching 10 mg mL^{-1} concentration. Experimental CHT solution was prepared from stock to contain 100 μg mL^{-1} CHT, 1 mM AA, 10 mM KCl and 5 mM 2-(N-morpholino) ethanesulfonic acid (MES) (pH 6.15). Control experimental solution was prepared in the same way without CHT (Ördög, 2011). All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

To study the short-time effects of CHT, the 6th leaf level of plants was treated at 8:00 a.m., then the rapid defence responses were recorded 30 minutes or one hour later. To reveal the

light-dependence of CHT-induced protective reactions plants were also submitted to artificial dark conditions following treatments. To unravel a potential, whole-plant extending systemic defence response of plants after CHT treatments, distal leaves from the 5th leaf level located directly above of treated ones were harvested and the same measurements were carried out.

Epidermal strip preparation

Strips from epidermis were taken immediately after sampling from CHT-treated (6th leaf-level) and systemic, distal (5th leaf-level) leaves with forceps, then transferred to plastic cell culture dishes containing incubation buffer of 10 mM KCl and 5 mM MES (pH 6.15) (Zhang et al., 2001).

Stomatal aperture measurements

Epidermal strips, taken immediately after sampling were examined microscopically (Nikon Eclipse TS-100, Nikon Instruments, Tokyo, Japan) to measure the size of stomatal apertures (Melotto et al., 2006). Width of at least 30-40 stomata of randomly chosen areas from strips of at least three different intact plants in each biological repetition was determined on digital images with Image-Pro Plus 5.1 software (Media Cybernetics, Inc., Rockville, MD, USA).

Determination of superoxide production

100 mg of leaf material was ground in 1 mL of 100 mM sodium phosphate buffer (pH 7.2) containing 1 mM sodium diethyldithiocarbamate trihydrate (SDDT). Samples were centrifuged at 12,000 g, 4°C, for 15 min. The reaction was assembled with 0.65 mL of 0.1 M sodium phosphate buffer (pH 7.2), 50 µL of 12 mM nitroblue tetrazolium (NBT) and 0.3 mL of supernatant. The absorbance of samples was determined at 540 nm after 2 (A0) and 7 (AS) minutes of incubation spectrophotometrically (KONTRON, Milano, Italy). Production of $O_2^{\cdot-}$ was calculated using the following formula: $\Delta A540 = AS - A0$, and it was expressed as $\Delta A540$ [min^{-1} g^{-1} fresh mass (FM)] (Chaitanya and Naithani, 1994). All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Determination of H_2O_2 content

H_2O_2 production of tomato leaves was measured spectrophotometrically based on the method of Velikova et al. (2000). 200 mg of leaf sample was homogenised in 1 mL of 0.1% (w/v) trichloroacetic acid (TCA). After centrifugation of samples (12,000 g, 4°C, 10 min), 0.25 mL of supernatant was added into reaction mixture containing 0.25 mL of 50 mM potassium phosphate buffer (pH 7.0) and 0.5 mL of 1 M potassium iodide (KI). After incubating samples at dark for 10 min, the absorbance of samples was measured at 390 nm spectrophotometrically (KONTRON, Milano, Italy). The concentration of H_2O_2 was determined using a calibration curve based on increasing concentrations of H_2O_2 from the stock solution. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Detection of NO accumulation

Production of NO was visualized via fluorescent staining of leaf disks by infiltrating them for 30 minutes with 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate (DAF-FM DA) dissolved in MES/KCl buffer (5 mM MES, 10 mM KCl, pH 6.15) after the CHT treatments. Samples were rinsed twice than the intensity of fluorescence was detected using a fluorescence microscope (Zeiss Axiowert 200 M, Carl Zeiss Inc., Jena, Germany). A high-resolution digital camera (Axiocam HR, HQ CCD) was used to take digital images from leaf disks. The fluorescence intensity of NO production was measured by using AxioVision Rel.

4.8 (Carl Zeiss Inc., Munich, Germany) software (Czékus et al., 2020). All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Measurement of ET production

ET accumulation was determined according to Poór et al. (2015) with gas chromatograph (GC) (Hewlett-Packard, Avondale PA, USA). 0.5 g of leaf sample was collected into gas-tight flasks containing 0.5 mL of deionized water restraining tissue dehydration closed with a silicone-rubber stopper, then stored for 1 h under darkness. Following that, emitted gas at 2.5 mL volume was collected with a gas-tight syringe and pressed into the GC. ET production generated by leaves were determined via using ET standard sets.

RNA extraction, expression analysis by quantitative real-time PCR

Total RNA was extracted from tomato leaves using TRI reagent (Chomczynski and Sacchi, 1987). Digestion of genomic DNA was achieved by DNase I (Thermo Scientific, Waltham, MA, USA), subsequently, cDNA synthesis from a single-stranded RNA template was catalyzed by MMLV reverse transcriptase (Thermo Scientific, Waltham, MA, USA). Transcript accumulation from examined tomato genes [*SlACO1* (Solyc07g049530): F: 5'-ATGTCCTAAGCCCGATTTGA-3', R: 5'-CCTCCTGCGTCTGTATGAGC-3'; *SlACS6* (Solyc08g008100): F: 5'-AGGGTTTCCTGGATTTAGGG-3', R: 5'-GACAACGGCATCATTGTACG-3'; *SlPR3* (Solyc02g061770): F: 5'-CCATCCACCCGTAGTTTCAT-3', R: 5'- AAAACATTTGCTGCCTTTGG-3'; *SlBiP* (Solyc08g082820): R: 5'-TCAGAAAGACAATGGGACCTG-3', F: 5'-GCTTCCACCAACAAGAACAAT-3'), collected from Sol Genomics Network (SGN; http://solgenomics.net/) database] was determined via quantitative real-time reverse transcription-PCR (qRT-PCR; qTOWER Real-Time qPCR System, Analytik Jena, Jena, Germany) according to Takács et al. (2016). The qPCR reaction was assembled from 1.6 µL cDNA template, 0.2 µL forward and 0.2 µL reverse primers and 5 µL of Maxima SYBR Green qPCR Master Mix (2X) (Thermo Scientific, Waltham, MA, USA) in molecular biology water at a final volume of 10 µL. The qRT-PCR programme following an initial denaturation step for 7 min at 95°C, was assembled by 40 repetitive cycles containing a denaturation step for 15 s at 95°C followed by annealing extension for 60 s at 60°C. Data were analysed by qTOWER Software 2.2 (Analytik Jena, Jena, Germany). As a reference, elongation factor-1α subunit was applied and the relative transcript accumulation was calculated by the $2^{(-\Delta\Delta Ct)}$ formula (Livak and Schmittgen, 2001). Normalization of data was referred to the transcript levels of the reference gene, as well as to control leaves.

Determination of exochitinase activity

Exochitinase activity was evaluated according to Yan and Fong (2018) with minor modifications. Protein extraction was carried out from 500 mg of leaf samples using 1 mL of 50 mM sodium acetate buffer (pH 5.0). After centrifugation (12,000 g, 15 min, 4°C), the supernatant was used to perform the enzymatic assay. The reaction mixture consisted of 0.45 mL of substrate solution [50 mM sodium acetate buffer (pH 5.0) containing 0.5 mg/mL of *p*-nitrophenyl *N*-acetyl-β-D-glucosamidine (*p*-NP-(GlcNAc)n] and 50 µL of enzyme solution. Samples were kept at 37°C for 15 min then the reaction was stopped by adding 1 mL of 0.4 M Na_2CO_3 solution. Colour development due to *p*-nitrophenol release was detected at 405 nm spectrophotometrically (KONTRON, Milano, Italy). One enzyme unit of exochitinase was assumed to release 1.0 µmole of *p*-nitrophenol from *p*-NP-(GlcNAc)n substrate in one minute at pH 5.0 at 37°C. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Determination of soluble protein concentration of samples was evaluated according to Bradford (1976) based on a standard of bovine serum albumin (BSA).

Western blot analysis

Firstly, leaf tissue was ground in liquid nitrogen to a fine powder by pestle and mortar. Proteins were extracted in modified Lacus buffer (25 mM Tris–HCl, pH 7.8, 10 mM $MgCl_2$, 15 mM EGTA, 75 mM NaCl, 1 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 0.05% Triton X-100) (Hurný et al., 2020), following that samples were centrifuged (12,000 g, 20 min, 4°C). The protein concentration of the supernatant was determined according to Bradford (1976). 15 µg of proteins per samples were separated on 12% SDS-PAGE and transferred onto PVDF membrane (Immobilon-P, Millipore, USA). The membranes were blocked with TBS-T buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20) containing 24 mg ml^{-1} bovine serum albumin (BSA) for 1 h at room temperature, then were incubated overnight at 4°C with anti-PR3 (AS07 207, 8 µg/ml), anti-BIP2 (AS09 481, 1:2000) or anti-ACT (AS13 2640, 1:3000) primary (rabbit) antibodies dissolved in the identical TBS-T buffer. After washing 3 times, membranes were incubated in HRP-conjugated goat-anti-rabbit IgG secondary antibody solution (AS09 602, 1:12000) at room temperature for 1 h. Subsequently washing the membranes four times, proteins were visualized using Western Chemiluminescent HRP Substrate (Immobilon, Millipore, USA) where the chemiluminescent signal was detected by using a C-DiGit western blot scanner system (LI-COR Biotechnology, Lincoln, NE, USA) (Meng et al., 2016). All antibodies were purchased from Agrisera (Vännäs, Sweden).

Statistical analysis

The experiments were replicated at least 3 times in each treatment. Data expressed are means $\pm$SE. Statistical analysis was performed by using Sigma Plot 12 software (Systat Software Inc. Erkrath, Germany) where results were analysed by one-way ANOVA, with Duncan's multiple comparison test and differences were considered significant if $P \leq 0.05$.

Results

Effects of CHT on stomatal movement locally and systemically

Despite the indispensable importance of plant hormones in defence responses is obvious, the exact role of ET in fungal elicitor CHT-induced defence reactions has remained less studied. Our work focuses not only on how the rapid defence responses of intact tomato plants are regulated by the plant hormone ET but also on the light-dependency of CHT-triggered processes via keeping plants in light or under continuous darkness.

From the aspect of preventing further pathogen invasion after infection, closure of stomata has elementary importance as one of the main components of the first line of plant defence reaction. Based on our work, CHT was able to close stomata significantly after 30 min as well as 1 h in WT plants under the light condition which was not only extended to treated leaves but was also detectable at systemic level. In the absence of light, local and systemic stomatal closure was also observable in the leaves of WT plants. At the same time, ET-insensitive mutants seemed to be impaired in managing defence responses based on the effectiveness of CHT in the induction of stomatal closure (Fig. 1).

Variation in ROS levels after CHT

Enhanced ROS production is an essential concomitant of the effectiveness of plant defence responses not only from the aspect of the manifestation of local cell death near to the infected leaf area but can also act as potent signalling molecules activating the protective plant

responses. Production of $O_2^{\cdot -}$ was only slightly elevated after 30 min following the CHT treatment in WT leaves (Fig. 2A). However, after 1 h, it was further increased in distal leaves from the treated ones, but this tendency was more pronounced in the light as compared to dark conditions where a slight decrease was observable in local CHT-treated leaves compared to the distal tissue. In contrast to WT plants, in *Nr* mutants CHT did not change significantly the levels of $O_2^{\cdot -}$ (Fig. 2B).

Production of H_2O_2 was also determined as an important participant of cell damage as well as activation of defensive signalling processes however significant changes were not observable locally and systemically nor in WT, neither in *Nr* plants after 30 min or one hour following the CHT treatments (Fig. 2 C, D).

Changes in NO production upon CHT

Not only ROS but also NO can play a determinant role in cell death as well as defence processes in plants submitted to biotic stress conditions. After 30 min, CHT treatment did not change significantly the NO production in any of the genotypes (Fig. 3A). However, after 1 h, NO level was increased significantly after CHT treatment under all conditions except in the distal leaves of WT plants kept in darkness (Fig. 3B).

CHT induced ET emission

In order to gain more information about the hormonal regulation of plant defence responses triggered by CHT, the production of ET was studied in leaves of intact tomato plants following the elicitor treatment. While CHT in leaves of WT plants triggered high ET production locally in light, a similar tendency was not observable neither in plants kept under continuous darkness, nor in *Never ripe* mutants at all. Remarkable changes in systemic leaves of CHT-treated plants did not happen in any of the examined genotypes (Fig. 4).

Changes in the expression of ET biosynthesis genes upon CHT

Induction of ET production was also examined by analyzing the expression pattern of *SlACS6* and *SlACO1* ET biosynthesis-related genes. Expression of *SlACS6* increased significantly upon CHT treatment in WT plants independently of the availability of light, but only a slight increase was observable in the distal leaves from the CHT-treated ones. *SlACS6* was also induced significantly in *Nr* mutants locally both in light and under darkness, however at much lower levels when compared to WT plants (Fig. 5A).

CHT induced also significant transcript accumulation of *SlACO1* locally both in WT and *Nr* plants in the light as well as under darkness however *SlACO1* was unchanged for the WT plants and repressed for the *Nr* plants in the distal tissues (Fig. 5B).

CHT-induced changes in chitinase levels and activity

Immune responses of plants triggered by fungal pathogen infection, in general, are accompanied by the increased expression of different pathogenesis-related (PR) proteins like PR3 encoding chitinase enzyme that plays role in the degradation of the fungal cell wall. For this consideration, both the expression of *SlPR3* and protein level of PR3 were monitored after CHT treatments. Application of elicitor significantly elevated *SlPR3* transcript levels not only in CHT-treated leaves of WT plants but also in untreated distal ones which showed a strong correlation with changes in protein levels of PR3. This increment was also observable under darkness where it was more significant (Fig. 6). In *Nr* mutants, *SlPR3* expression and PR3 accumulation were basally higher as compared to WT plants, both in light and dark conditions in control plants. Enhanced expression of *SlPR3* in *Nr* plants upon CHT was only observable locally in plants kept in light, whereas it was decreased in distal leaves under light.

At protein level, PR3 accumulation was significant both in light and under darkness locally and systemically in the ET-insensitive mutant plants (Fig. 6).

Based on our results regarding the elevated expression of chitinase-coding *SlPR3* and PR3 accumulation by CHT treatments, a chitinase activity assay was also performed, however, CHT did not provoke significant changes in enzyme activity level in any of the examined genotypes at this time-point (Fig. 7).

CHT-triggered UPR

Enhanced demand for the production of defence-related proteins in a high amount necessarily requires the accumulation of certain ER-localized chaperones like BiP providing correct protein folding. In our experiments, ER stress generated by CHT was verified not only in gene expression level by analyzing the transcript level of *SlBiP*, but also by the accumulation of BiP proteins monitored by Western blot analysis. In *Nr* mutants *SlBiP* levels were basally higher as compared to WT plants. CHT induced *SlBiP* transcript accumulation both in WT and *Nr* plants independently of the availability of light, however, this increase in whole-plant level was only significant in WT plants. In the distal leaves of *Nr* mutants, the expression of *SlBiP* was decreased under darkness. The changes in BiP levels largely correlated with the transcriptional data but were too subtle to make significant conclusions (Fig. 8).

Discussion

In this article, the microbial elicitor CHT-induced light- and ET-dependent local- and systemic defence responses were investigated in intact tomato plants. Despite the fact, that CHT is probably one of the best-characterized MAMPs in biological researches, there are many gaps in our knowledge regarding its exact mechanism especially on a whole-plant level (Narula et al., 2020). It is noteworthy, that almost all of these experiments were carried out in detached leaves closing the doors in front of further exploration of long-term defence mechanisms activated in the whole-plant level that can manifest in SAR (Czékus et al., 2020). It has been well-documented that regulation of plant defence responses is strongly determined not only by the circadian clock but also by daytime and abiotic environmental factors such as light (Karapetyan and Dong, 2018). Based on our preliminary results, we also obtained that light directly regulates defence responses of plants upon CHT exposure (Czékus et al., 2020). For this consideration, in our experimental setup plants were kept at the light and under continuous darkness until sampling making it possible to examine the direct effect of light on the activation of defence responses independently of the obvious influencing role of the circadian clock. Many publications have revealed that CHT treatments possess a long-term effect lasting even for days (Ben-Shalom et al., 2003; Manjunatha et al., 2009). However, some of them are also in accordance with our previous observations assigning crucial importance to the first hours after treatment in the establishment and maximal activity of defence responses particularly the accumulation of ROS and NO (Yin et al., 2013; Devireddy et al., 2020). Taking these observations into account, rapid plant defence responses were recorded after the CHT application.

Closure of stomata is one of the earliest processes induced after recognition of pathogens or elicitor molecules that can be recorded even after minutes (Srivastava et al., 2009; Koers et al., 2011; Devireddy et al., 2020). The application of CHT on plants was demonstrated to trigger stomatal closure, moreover, its inhibitory role on stomatal opening was also described (Ördög, 2011; Wu et al., 2017; Czékus et al., 2020). In our results, significant and rapid stomatal closure was also observable already in 30 min and 1 h after CHT treatments in WT plants, moreover, it was extended to the upper, systemic leaves from the elicitor-treated ones confirming the role of CHT in establishing systemic response in

whole-plant level in accordance with our previous results (Czékus et al., 2020). Darkness already causes significant closure of stomata, however, CHT was able to further enhance it. In contrast to WT plants, in ET-insensitive mutants, CHT did not cause any significant change in the size of stomatal apertures suggesting a pivotal role of ET in the activation of defence signalling triggered by CHT as well as in the development of systemic responses. Inhibitors of NO and ROS production, as well as Ca^{2+} chelators, restrict CHT-induced stomatal closure confirming their crucial role in that process (Srivastava et al., 2009). Accumulation of ROS like H_2O_2 and availability of cytosolic NAD(P)H were demonstrated to be necessary for stomatal closure induced by CHT, in which levels of ROS started to increase not more than after 5 min, while NO in 10 min in guard cells (Iriti et al., 2009; Li et al., 2009; Srivastava et al., 2009). Oxidative burst triggered by CHT, in general, shows a peak in the first hours after treatment. Generation of H_2O_2 reached a maximum in short-time following CHT application; after 12 min in wheat cell culture, 30 min in *Brassica napus* leaves and 50 min in rice suspension cell culture (Lin et al., 2005; Paulert et al., 2010; Yin et al., 2013). However, Rossard et al. (2010) have revealed that H_2O_2 production after CHT treatment cannot be restricted exclusively to the first phase of infection. Based on experiments with *Beta vulgaris* leaf disks it was demonstrated that accumulation of H_2O_2 shows a biphasic pattern with a maximum 1 h after treatment and a prolonged, second peak after 4 h lasting even hours, partially due to constitutive Cu/Zn superoxide dismutase (SOD) activity (Rossard et al., 2010). Interestingly, in our experiment H_2O_2 levels did not show any significant change 30 min or 1 h after CHT treatments neither in WT, nor in *Nr* plants, however $O_2^{\cdot-}$ started to accumulate already after 30 min which increment was more pronounced in distal, systemic leaves one hour later. Production of $O_2^{\cdot-}$ was not only negatively influenced by darkness, but also by the lack of active ET signalling since it was completely inhibited in *Nr* mutants suggesting a pivotal role of ET in ROS signalling during the initial phase of resistance development. The direct effect of ET on $O_2^{\cdot-}$ production was also described earlier using ACC ET biosynthesis precursor (Borbély et al., 2019). Based on our results, the locally generated ET emission by CHT can contribute to the accumulation of superoxide in distal parts of intact plants activating systemic defence responses. Temporal variations in the local production of different ROS have been also described in *Artemisia annua* plants, treated foliar with CHT, where similarly to our results, rapid and continuous $O_2^{\cdot-}$ production was observable immediately after treatment whereas H_2O_2 content started to increase only after hours and peaked at 24 h, assigning elementary importance to $O_2^{\cdot-}$ rather than H_2O_2 in defence responses activated 1 h after elicitor application (Lei et al., 2011). Early and high $O_2^{\cdot-}$ production was proved to due to the direct activation of NADPH oxidase upon CHT treatment locally, whereas simultaneous up-regulation of the antioxidant system such as glutathione peroxidase (GPX), catalase (CAT) and ascorbate peroxidase (APX) can also reduce the production of ROS that led to relatively low H_2O_2 generation in maize seedlings treated with CHT (Prasad et al., 2017; Turk, 2019; Xu et al., 2020). Rapid $O_2^{\cdot-}$ burst followed by delayed H_2O_2 production can be also explained by the relatively slow activation of SOD (Lei et al., 2011). A recent study has revealed, that ROS level remaining high in systemic leaves after high light stress treatment can function in 'systemic stress memory' thereby keeping defence mechanisms in an upregulated state (Devireddy et al., 2020).

Generation of NO similarly to ROS is also observable immediately after elicitor treatment locally, which can function in the activation of early defence responses (Manjunatha et al., 2009; Yin et al., 2013). NO generation was triggered by CHT not only in WT but also in *Nr* plants independently of the availability of light after the treatments, even in the systemic, distal leaves. This suggests that NO production is not dependent on the active ET signalling. Other phytohormones, such as JA or SA can also promote rapid NO generation

under stress (Mur et al., 2013; Takács et al., 2016). Nevertheless, the activity of nitrate reductase that can directly produce NO is inhibited in the dark, moreover, enzymes displaying nitric oxide synthase (NOS)-like activity in plants have not been identified yet, therefore the source of NO remains an open question (Malerba et al., 2012; Poór et al., 2019b).

The gaseous ET has been known as a plant hormone regulating development as well as plant defence responses under biotic stress, however, its role is rather contradictory. It can be concluded that ET in closely associated pathways with JA, in general, contributes to resistance against necrotrophic pathogens whereas SA mainly plays role in preventing infection upon biotrophic pathogen attack (van Loon et al., 2006a). In incompatible plant-pathogen interactions, the establishment of HR is generally accompanied by enhanced ET production promoting resistance against pathogens. However, it seems uncertain being involved in necrotic lesion formation based on experiments of *Nr* mutant tomato plants infected with avirulent *Xanthomonas campestris* pv. *vesicatoria* (Ciardi et al., 2000; van Loon et al., 2006a). *Nr* mutants are impaired in ET perception however are able to synthesize it even in a higher amount than WT plants due to negative ET feedback response (Lanahan et al., 1994; Borbély et al., 2020; Nascimento et al., 2020). The importance of ET in rapid defence responses induced by CHT was verified by Yin et al. (2006) where oligochitosan treatment increased the expression of two EREBP- and an ET receptor gene already 1 h after treatment in leaves of oilseed rape plants. ET perception is also required for SAR signalling under Tobacco Mosaic Virus (TMV) infection, however, its role in mediating systemic resistance in whole plant level upon CHT treatment remained unknown (Verberne et al., 2003). The direct effect of CHT in the production of ET was firstly demonstrated in pine cell suspension culture where the elicitor was capable to induce ET production (Popp et al., 1997). We also found that CHT triggered significant ET accumulation locally in leaves of WT tomato plants, however, this was not observable in *Nr* mutants or in plants kept in dark. Systemic induction of ET production was neither triggered by CHT. Expression of both *SlACO1* and *SlACS6* ET biosynthesis-related genes was significantly induced by CHT in WT as well as *Nr* plants, however, it was significantly higher in the leaves of WT plants. It is in good correspondence with the observations of Castagna et al. (2007) who also found that genes involved in ET biosynthesis showed delayed expression in *Nr* plants as compared to WT suggesting a delayed ET response due to impaired ET perception. Despite a previous observation where the light directly inhibited the endogenous ET production, in our results ET accumulation seemed to be restrained under darkness (Kao and Yang, 1982). This can help explain how the development of HR could be suppressed not only by the lack of ET accumulation but also in the absence of light based on experiments in *Arabidopsis thaliana* plants kept under continuous darkness immediately after Turnip Crinkle Virus (TCV) infection (Chandra-Shekara et al., 2006; van Loon et al., 2006a).

Accumulation of PR proteins in response to pathogen attack or exogenous application of elicitor molecules induced the prevention from further pathogen invasion as well as improvement of systemic responses that can be strongly regulated by plant hormones like ET (Hadwiger, 2013). Formerly we found that CHT rapidly induced the expression of SAR marker gene *PR1* both locally and systemically in intact WT tomato plants (Czékus et al., 2020). Other members of the PR protein family such as chitinases can hydrolyze chitin or CHT polymers degrading fungal cell wall, thereby are important markers of activation of defence reactions (Grover, 2012; Xing et al., 2015). Tomato *SlPR3* encodes a class II endochitinase that can be involved both in HR and SAR (Sol Genomics Network; Grover, 2012). PR3 is basically considered to be activated in an ET- and JA-dependent pathway, moreover, it can be induced by ET or ACC applied exogenously (van Loon et al., 2006b; Mazarei et al., 2007; Chen et al., 2008; Zhu et al., 2014). Expression of chitinase was also

restricted in ET-insensitive *Nr* tomato plants providing evidence for the ET-dependence of defence response regulation under *Xanthomonas campestris* pv. *vesicatoria* infection (Ciardi et al., 2000). It is known that low - rather than high - molecular weight CHT has a stronger effect in inducing the expression of PR genes, moreover, CHT primarily induces accumulation of endochitinase *PR3b*, not more than in 1 h (Dubin et al., 2020). In our experiments, *SlPR3* transcript levels in *Nr* plants were significantly higher when compared to WT plants even under the control conditions. We observed rapid induction of *SlPR3* in the local tissue of both WT and *Never ripe* plants kept in the light which was also significant and more increased under darkness in WT plants. Interestingly, this elevation was significant in the untreated, distal leaves of WT but not of *Nr* plants verifying the ET-dependence of activation of systemic responses upon CHT treatment. A similar tendency was also observable regarding the changes in PR3 protein levels based on Western blot analysis confirming further that rapid and enhanced chitinase production is indispensable in plant defence reactions induced by CHT not only in local-, but similarly in systemic defence responses. Class I chitinase has two isoforms (CHN A, CHN B) which have a very similar structure, however, due to deletion and substitution of specific amino acids, their molecular mass can differ even by 1500-2000 Da (Sticher et al., 1993). That could be the reason for a double-band appearance on Western blot upon analyzing PR3 protein accumulation that was also observed earlier (Munger et al., 2012; Faliconi et al., 2014). These results confirmed the possible role of CHT in establishing SAR observed previously in tomato plants by enhanced chitinase protein accumulation after foliar treatment with the fungal elicitor (Atia et al., 2005).

Several studies have demonstrated that increased chitinase expression not only resulted in PR transcript- or protein accumulation, but chitinase activity also increased simultaneously (Aziz et al., 2006; Jayaraj et al., 2009). Despite that in our experiments we detected both increased *SlPR3* expression as well as significant PR3 protein accumulation, 1 h after CHT treatments chitinase activity did not change remarkably in any of the examined genotypes. This can be a consequence of delayed enzyme-activation which was earlier observed in rice suspension cell culture where CHT increased chitinase activity however it reached a maximum only after 48 hours (Lin et al., 2005). Nevertheless, the critical importance of activation of chitinase enzyme in the course of the establishment of resistance was also refuted whereas chitinases can also contribute to the generation of signalling molecules participating in defence responses that can be more relevant, than their direct enzymatic activity (Ciardi et al., 2000; van Loon et al., 2006b).

It is well-known, that PR proteins are synthesized through the rough ER, where increased BiP accumulation is necessarily induced before elevated PR translation under biotic- or abiotic stress conditions (Carvalho et al., 2014). ER stress-triggered accumulation of misfolded or unfolded proteins can be mitigated via UPR in which accumulation of BiP proteins plays a pivotal role thereby functioning as an important UPR marker (Cheng et al., 2015). BiP accumulation triggered by CHT was firstly reported in plants by Malerba et al. (2012) that also caused remarkable modifications in the architecture of ER. Application of COS-OGA (elicitor formed by cationic chitosan and anionic pectin oligomers) elicitors also positively regulated the expression of certain HSP70 chaperon genes (Van Aubel et al., 2016). We observed enhanced *SlBiP* transcript accumulation following CHT treatments both in WT and *Nr* plants suggesting induction of ER stress following CHT treatment where UPR was also strongly activated only 1 h after treatment. Others also found rapid *BiP* expression both locally and systemically which was transient and occurred before the other PR member *β-1,3-glucanase* induction under biotic stress in tobacco (Jelitto-Van Dooren et al., 1999). Accumulation of BiP, in general, showed a large correlation with gene expression data, however, at this time point differences in protein levels were not pronounced enough to create

significant conclusions. The enhanced expression of *SlBiP* in CHT-treated leaves was neither influenced by the defectiveness in ET signalling of *Nr* mutants nor by the absence of light, however, it was decreased in untreated leaves of ET-insensitive mutants. This suggests that the CHT-induced local expression of *BiP* is independent of the lack of ET perception or the absence of light. Systemic *SlBiP* and BiP protein accumulation neither have been described yet previously providing further evidence of ET-dependent activation of SAR as a part of CHT-induced defence signalling.

Conclusions

Our results highlight the importance of ET as well as the crucial role of light in local and systemic short-time defence responses induced by CHT. Foliar treatment with CHT not only induced significant ET emission and stomatal closure locally but also rapid production of $O_2^{\cdot-}$ which were observable in whole-plant level in WT plants kept in light, however, these were inhibited in *Nr* mutants. ET as well as $O_2^{\cdot-}$ accumulation were also inhibited under darkness suggesting the pivotal importance of ET and light in inducing resistance both locally and systemically upon CHT treatment. Production of NO seemed to be unaffected by that two factors after application of CHT. Based on experiments of *PR3* and *BiP* expression we can conclude that ET has an essential role in CHT-induced systemic response because it was inhibited in the absence of active ET signalling. We observed first time, that CHT-induced ER stress, as well as UPR, were also activated in intact plants which were not dependent on the availability of light but establishment UPR systemically is mediated by ET.

Author contributions

Conceptualization P.P.; investigation, Z.C., N.I., B.P., A.M., A.Ö., P.P.; writing—original draft preparation, Z.C. and P.P.; writing—review and editing, P.P. and A.Ö.

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Conflict of Interest

No conflict of interest is declared.

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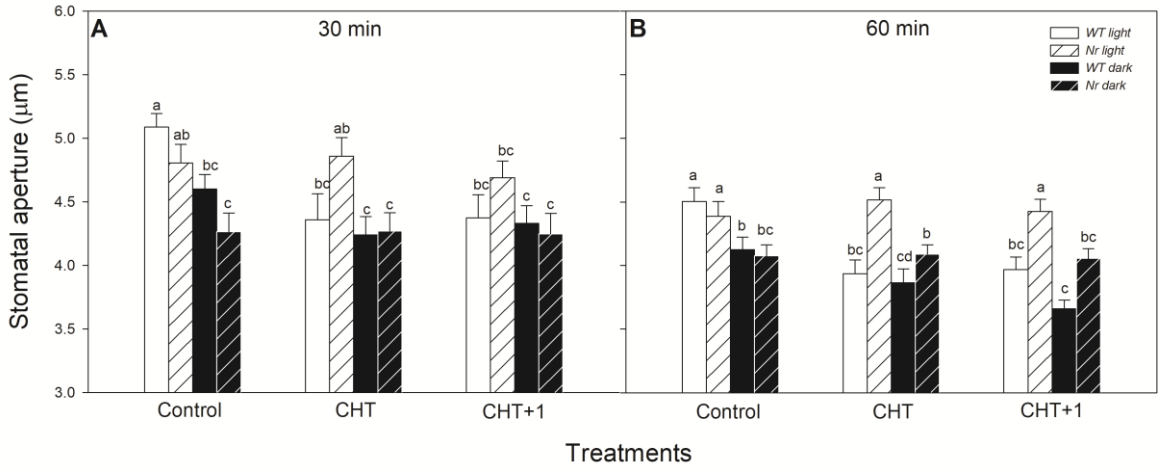

Figure 1. Effect of chitosan (CHT) treatment applied in 100 μg mL^{-1} concentration on the stomatal closure on the abaxial epidermal strips in leaves of intact wild type (WT) and ET-insensitive *Never ripe* (*Nr*) tomato. Plants were treated at 8:00 p.m. then kept under light or continuous darkness until measurements at 8:30 (A) or 9:00 p.m.(B). Data are represented as means ± SE (n=3) after analysis by one-way ANOVA and Duncan's test. Distinct letters were used to sign mean values considered to be significantly different upon $P< 0.05$. (Control: 1 mM acetate buffer (AA) treatment; CHT: 100 μg mL^{-1} CHT treatment; CHT+1: untreated, distal leaf).

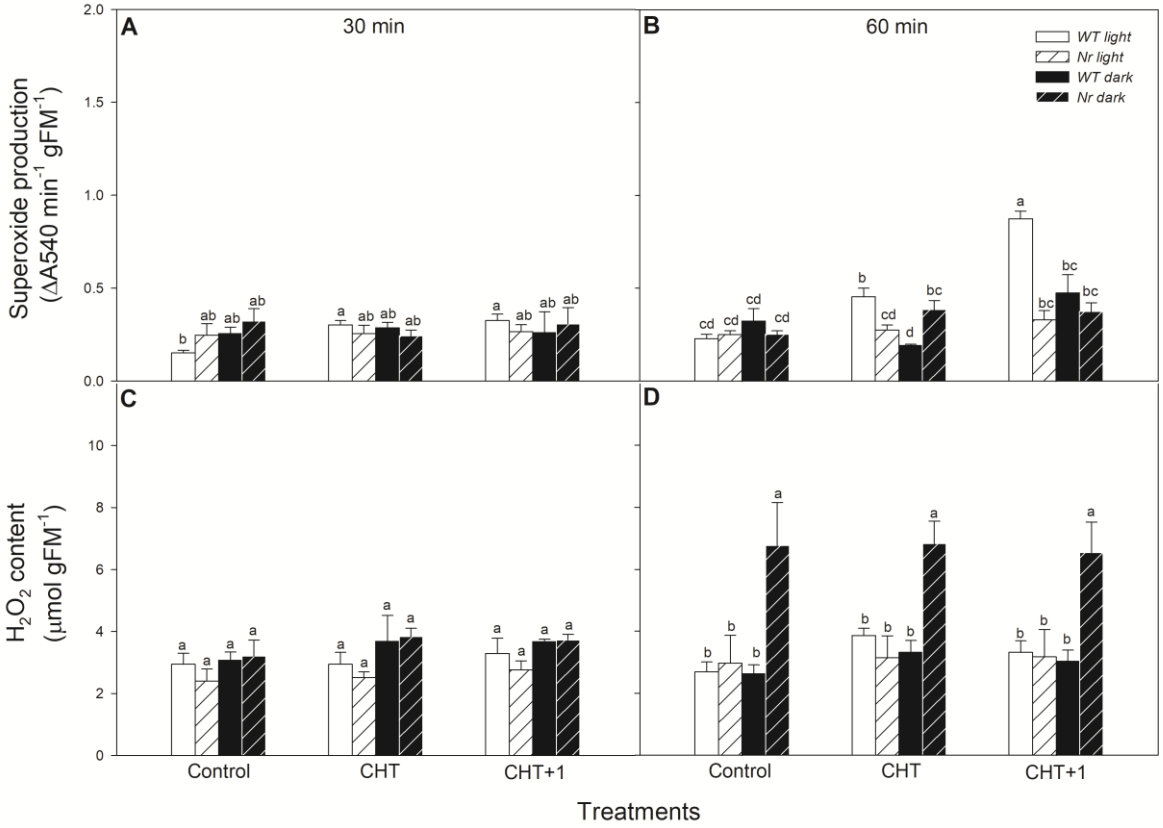

Figure 2. Effect of chitosan (CHT) treatment applied in 100 μg mL^{-1} concentration on the superoxide ($O_2^{\cdot-}$) production (A, B) and accumulation of H_2O_2 (C, D) in leaves of intact wild type (WT) and ET-insensitive *Never ripe* (*Nr*) tomato. Plants were treated at 8:00 p.m. then kept under light or continuous darkness until measurements at 8:30 or 9:00 p.m. Data are represented as means ± SE (n=3) after analysis by one-way ANOVA and Duncan's test. Distinct letters were used to sign mean values considered to be significantly different upon $P< 0.05$. (Control: 1 mM acetate buffer (AA) treatment; CHT: 100 μg mL^{-1} CHT treatment; CHT+1: untreated, distal leaf).

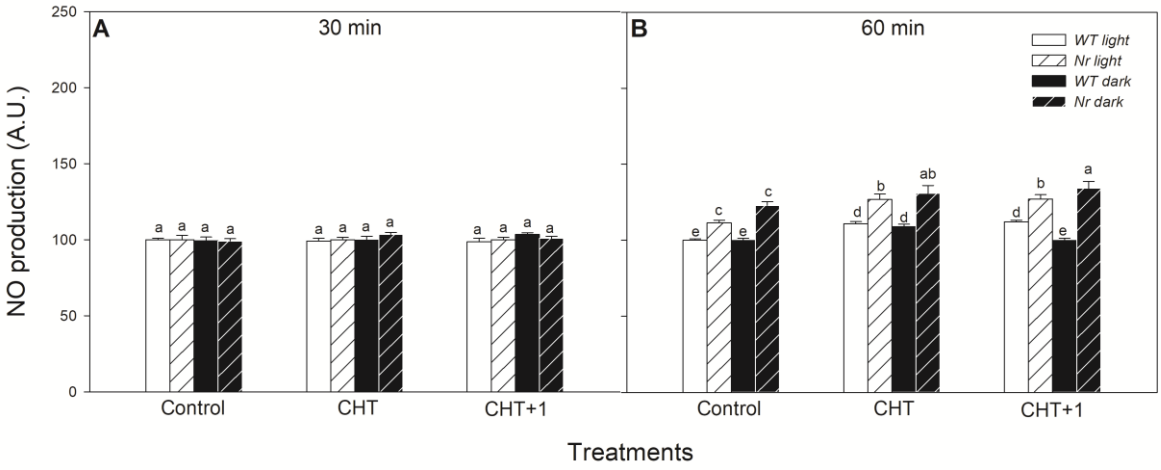

Figure 3. Effect of chitosan (CHT) treatment applied in 100 μg mL^{-1} concentration on the nitric oxide (NO) production in leaves of intact wild type (WT) and ET-insensitive *Never ripe* (*Nr*) tomato. Plants were treated at 8:00 p.m. then kept under light or continuous darkness until measurements at 8:30 (A) or 9:00 p.m. (B). Data are represented as means ± SE (n=3) after analysis by one-way ANOVA and Duncan's test. Distinct letters were used to sign mean values considered to be significantly different upon $P< 0.05$. (Control: 1 mM acetate buffer (AA) treatment; CHT: 100 μg mL^{-1} CHT treatment; CHT+1: untreated, distal leaf).

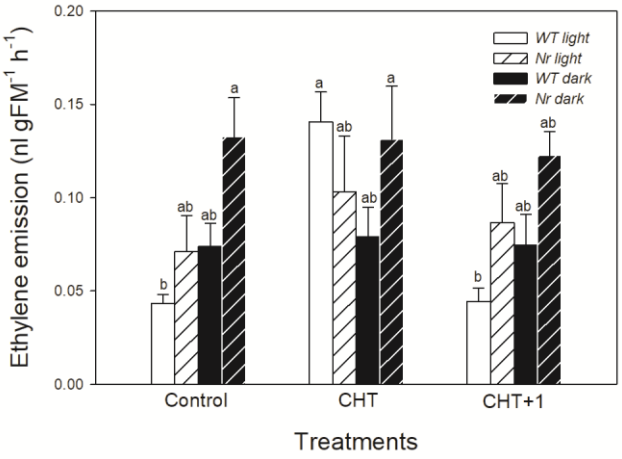

Figure 4. Effect of chitosan (CHT) treatment applied in 100 µg mL^{-1} concentration on the ethylene (ET) production in leaves of intact wild type (WT) and ET-insensitive *Never ripe* (*Nr*) tomato. Plants were treated at 8:00 p.m. then kept under light or continuous darkness until measurements at 9:00 p.m. Data are represented as means ± SE (n=3) after analysis by one-way ANOVA and Duncan's test. Distinct letters were used to sign mean values considered to be significantly different upon $P< 0.05$. (Control: 1 mM acetate buffer (AA) treatment; CHT: 100 µg mL^{-1} CHT treatment; CHT+1: untreated, distal leaf).

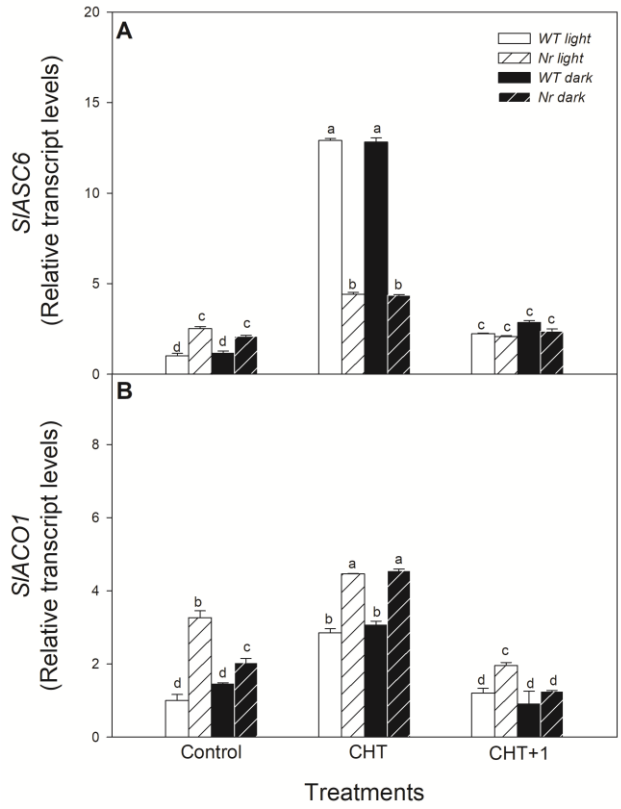

Figure 5. Effect of chitosan (CHT) treatment applied in 100 μg mL^{-1} concentration on the expression of *SlACS6* (A) and *SlACO1* (B) genes in leaves of intact wild type (WT) and ET-insensitive *Never ripe* (*Nr*) tomato. Plants were treated at 8:00 p.m. then kept under light or continuous darkness until measurements at 9:00 p.m. Data are represented as means ± SE (n=3) after analysis by one-way ANOVA and Duncan's test. Distinct letters were used to sign mean values considered to be significantly different upon $P< 0.05$. (Control: 1 mM acetate buffer (AA) treatment; CHT: 100 μg mL^{-1} CHT treatment; CHT+1: untreated, distal leaf).

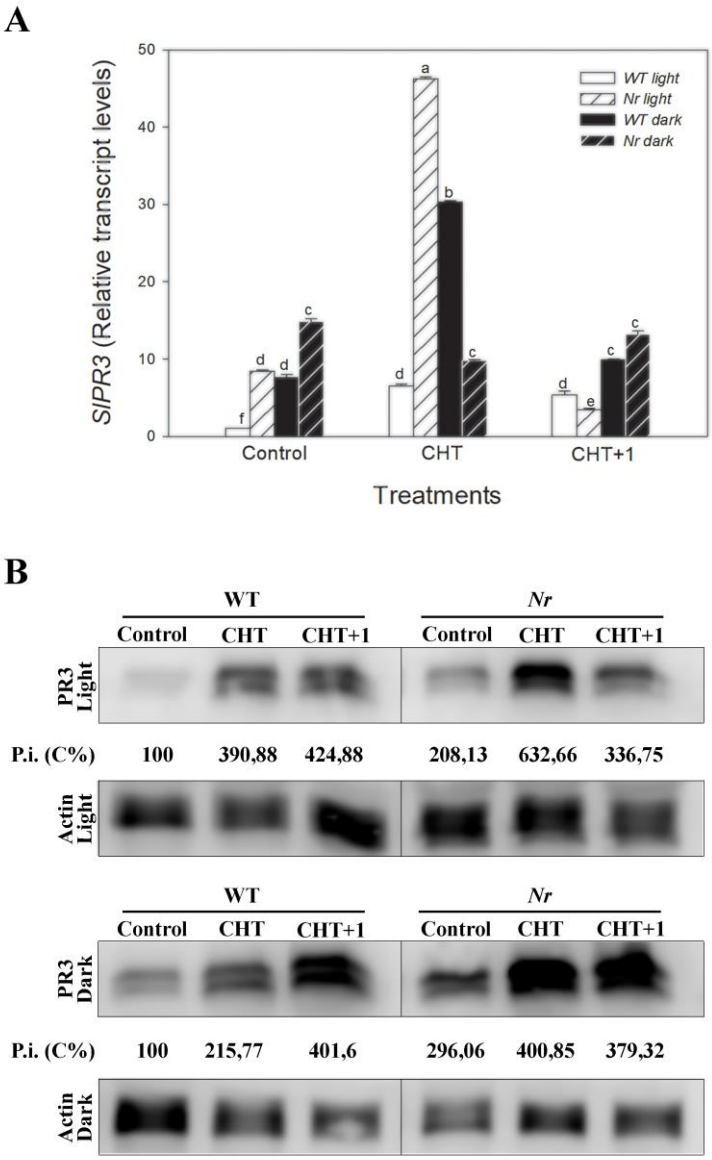

Figure 6. Effect of chitosan (CHT) treatment applied in 100 μg mL^{-1} concentration on the expression of *SlPR3* gene (A) and content of Pathogenesis-related 3 (PR3) protein (B) in leaves of intact wild type (WT) and ET-insensitive *Never ripe* (*Nr*) tomato. Plants were treated at 8:00 p.m. then kept under light or continuous darkness until measurements at 9:00 p.m. Data are represented as means ± SE (n=3) after analysis by one-way ANOVA and Duncan's test. Distinct letters were used to sign mean values considered to be significantly different upon $P< 0.05$. Pixel intensity (P.i.) is expressed as control % (C%). (Control: 1 mM acetate buffer (AA) treatment; CHT: 100 μg mL^{-1} CHT treatment; CHT+1: untreated, distal leaf).

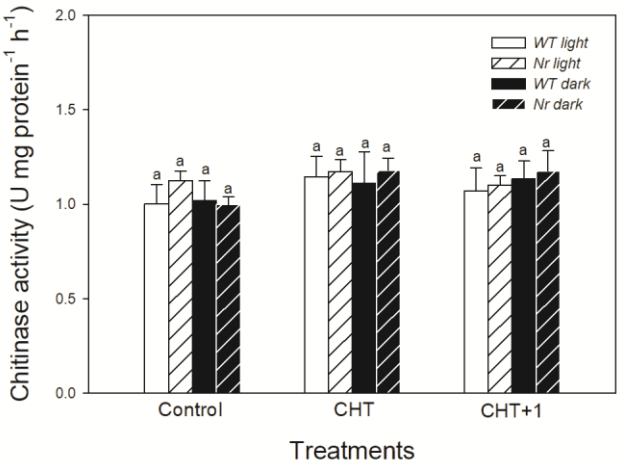

Figure 7. Effect of chitosan (CHT) treatment applied in 100 µg mL^{-1} concentration on the chitinase activity in leaves of intact wild type (WT) and ET-insensitive *Never ripe* (*Nr*) tomato. Plants were treated at 8:00 p.m. then kept under light or continuous darkness until measurements at 9:00 p.m. Data are represented as means ± SE (n=3) after analysis by one-way ANOVA and Duncan's test. Distinct letters were used to sign mean values considered to be significantly different upon $P< 0.05$. (Control: 1 mM acetate buffer (AA) treatment; CHT: 100 µg mL^{-1} CHT treatment; CHT+1: untreated, distal leaf).

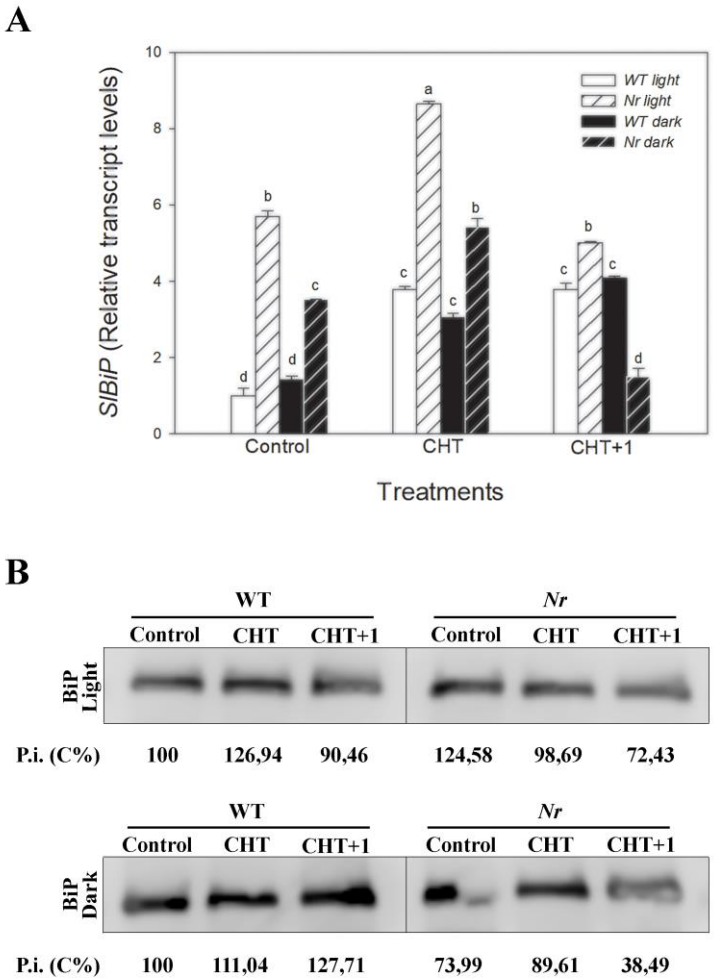

Figure 8. Effect of chitosan (CHT) treatment applied in 100 µg mL^{-1} concentration on the expression of *SlBiP* gene (A) and content of Binding Protein (BiP) protein (B) in leaves of intact wild type (WT) and ET-insensitive *Never ripe* (*Nr*) tomato. Plants were treated at 8:00 p.m. then kept under light or continuous darkness until measurements at 9:00 p.m. Data are represented as means ± SE (n=3) after analysis by one-way ANOVA and Duncan's test. Distinct letters were used to sign mean values considered to be significantly different upon $P< 0.05$. Pixel intensity (P.i.) is expressed as control % (C%). (Control: 1 mM acetate buffer (AA) treatment; CHT: 100 µg mL^{-1} CHT treatment; CHT+1: untreated, distal leaf).