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The role of photosynthetic activity in the regulation of flg22-induced local and systemic defence reaction in tomato

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Abstract

Flagellin (flg22) induces rapid and long-lasting defence responses. It may also affect the photosynthetic activity depending on several internal and external factors, such as the phytohormone ethylene or the day/night time. Based on the results, flg22 treatment, neither in the light phase nor in the evening, caused any significant change in chlorophyll fluorescence induction parameters in the leaves of wild-type and ethylene-receptor mutant *Never ripe* tomato plants measured the next morning. However, flg22 in the light phase decreased the effective quantum yield and the photochemical quenching both locally and systemically in guard cells. In parallel, the production of reactive oxygen species and nitric oxide increased, which contributed to the stomatal closure and a decrease in CO_2 assimilation the next day. A decrease in sugar content and elevated hexokinase activity measured after flg22 exposure can also contribute to local defence responses in intact tomato plants.

Keywords: assimilation; flagellin; mycotoxin; photosystem II; stomatal conductance.

Introduction

The presence or absence of light and the circadian clock mediate various molecular, biochemical, and physiological processes in living organisms such as the defence mechanisms (Chen *et al.* 2004, Roberts and Paul 2006, Ballaré 2014, Reddy and Rey 2014). The light-driven photosynthesis serves the generation of energy and reducing power not only under normal conditions but also contributes to the successful defence mechanism, *e.g.*, by partitioning assimilates or by the production of chloroplastic reactive oxygen species (ROS) during the day (Dodd *et al.* 2005, Berger *et al.* 2007, Kangasjärvi *et al.* 2012). It can be crucial because the day/night time of

Highlights

- Flg22 decreased the effective quantum yield of PSII in tomato guard cells
- Flg22 induced local and systemic stomatal closure which was dependent on ethylene
- Hexokinase activity and expression of SlHXK3 were elevated locally by flg22

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Abbreviations: ABA – abscisic acid; Chl – chlorophyll; ET – ethylene; Flg22 – flagellin 22; F_0 – minimal fluorescence yield in dark-adapted state; F_m – maximal fluorescence yield in dark-adapted state; FM – fresh mass; F_v/F_m – maximum quantum yield of PSII; HXK – hexokinase; JA – jasmonic acid; NO – nitric oxide; NPQ – nonphotochemical quenching; *Nr* – *Never ripe*; PAM – pulse amplitude modulation; P_N – net photosynthetic rate; q_P – photochemical quenching coefficient; ROS – reactive oxygen species; SA – salicylic acid; WT – wild type, $Y_{(II)}$ – effective quantum yield of PSII photochemistry.

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the infection can determine the outcome of the successful defence reaction of plants. Interestingly, reduced and delayed systemic acquired resistance (Karpiński *et al.* 2003) and lesion formation were found in response to avirulent pathogens in the dark (Zeier *et al.* 2004, Griebel and Zeier 2008). Thus, the importance of the energy-producing processes during the day and especially in the dark phase could be very significant (Poór *et al.* 2021). In plants, the degradation of photosynthetic products such as starch is under circadian control to ensure the maintenance of carbohydrate availability until the next anticipated dawn and sustain plant productivity (Graf *et al.* 2010, Lu *et al.* 2017). However, not only starch but other photosynthetic products, the soluble sugars (glucose, fructose, and sucrose), as well as ROS interacting with defence-related phytohormones, such as salicylic acid (SA) and ethylene (ET), play an important role in regulating stress responses in complex- and daytime-dependent manner (Couée *et al.* 2006, Rosa *et al.* 2009, Wind *et al.* 2010, Ballaré 2014).

The photosynthetic activity of mesophyll cells and the photosynthesis of guard cells in the epidermis play a crucial role in the regulation of defence against various bacterial pests (Lawson 2009). It is well known that stomata serve the transpiration and CO_2 accumulation for plants but also provide an entry site to pathogens (Melotto *et al.* 2017). The regulation of stomatal pore size is under strong light and circadian control (Chen *et al.* 2012). Blue light stimulates stomatal opening at dawn and together with red light facilitates transpiration and CO_2 uptake for photosynthetic CO_2 fixation during the light period in C_3 plants (Suetsugu *et al.* 2014, Matthews *et al.* 2020). During dark periods, stomata are closed, providing the first line of defence against several pathogens, which are mostly infective in the dark (Roberts and Paul 2006, Shimazaki *et al.* 2007, Matthews *et al.* 2020). At the same time, stomatal closure not only plays role in the fast local defence response of plants but is also an integral part of systemic whole-plant response upon stress or pathogen infection coordinated by ROS and phytohormones (Zandalinas 2020). This stomatal closure as a part of the systemic response was detected at least 6-h-long in *Arabidopsis* in the light phase (Devireddy *et al.* 2020). At the same time, data are scarce on the effects of the daytime on the local and the systemic response of plants (Czékus *et al.* 2020).

Rapid- and long-term local and systemic defence responses can be dependent on photosynthesis and sugar metabolism (Kangasjärvi *et al.* 2012, Rojas *et al.* 2014). It was found that the rapid increase in sugar contents, especially sucrose and glucose in the systemic leaves of plants is also a crucial step of systemic signalling under stress stimuli (Choudhury *et al.* 2018). Earlier it was found that exogenously added sucrose or glucose stimulated the stomatal closure mediated by guard-cell hexokinase (HXK) in tomato leaves (Kelly *et al.* 2013). Recently, the role of hexose-phosphorylating and sugar-sensing enzyme HXK was detected in the induction of stomatal closure promoting ROS and nitric oxide (NO) production in guard cells of poplar (Shen *et al.* 2021). Thus, the photosynthetic activity during the day and sugar accumulation can influence plant defence reaction by regulating stomatal closure, respectively (Granot and Kelly 2019). At the same time, these processes are controlled by defence-related phytohormones, such as abscisic acid (ABA), salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) (Pieterse *et al.* 2012, Derksen *et al.* 2013). Among these hormones, the gaseous ET plays a fine-regulator role in plant defence (Broekgaarden *et al.* 2015) by promoting local (Zhang *et al.* 2021) and systemic stomatal closure (Czékus *et al.* 2021a), regulating photosynthesis (Müller and Munné-Bosch 2021) and sugar metabolism (Depaepe *et al.* 2021). Based on these findings, understanding the role of ET in local and systemic responses of plants to pathogens in different day/night times provides an important perspective for plant stress physiology research and plant protection.

Infection of pathogens can be mimicked using the bacterial elicitor flagellin (flg22) (Felix *et al.* 1999). The perception of flg22 by RLK receptor complex FLS2-BAK1 leads to the phosphorylation of BIK1 kinase which activates the plasma membrane-localized NADPH oxidase AtRBOHD in *Arabidopsis* (Kadota *et al.* 2014, Li *et al.* 2014). Flg22-induced ROS production by NADPH oxidase activates the plasma membrane-localized Ca^{2+} channels in guard cells (Thor and Peiter 2014), the SLAC1 anion channel, and the aquaporin PIP2;1 which leads to rapid stomatal closure (Deger *et al.* 2015, Rodrigues *et al.* 2017). In addition, quick production of ET and high expression of ET biosynthetic genes were measured after flg22 treatments showing the crucial role of this hormone in the defence responses and local stomatal closure of plants (Felix *et al.* 1999, Denoux *et al.* 2008, Mur *et al.* 2008, Mersmann *et al.* 2010, Park *et al.* 2015). Although the role of ET in flg22-induced rapid defence responses is well known, the effects of this gaseous phytohormone in the systemic response of intact plants were less investigated. Moreover, the potential impact of the different day/night times on the plant responses upon flg22 and the role of photosynthesis in this process have not been elucidated. Investigation of the role of these factors in the defence responses of plants could be significant because the flg22-induced signalling is highly dependent on the presence of light (Sano *et al.* 2014). In addition, not only ET emission and signalling but also ROS production and metabolism are different in the dark compared to the light (Liebsch and Keech 2016, Poór *et al.* 2017).

In this work, the daytime- and ET-dependent effects of flg22 were investigated in intact leaves of tomato plants. Our experiments focused on whether flg22 could induce local and systemic stomatal closure in the following light phase after the treatments in different day/night time. In addition, long-term defence responses can be regulated by photosynthesis which was examined in leaves of intact wild-type (WT) and ET-receptor mutant *Never ripe* (*Nr*) plants.

Materials and methods

Plant material: Wild-type (WT) and ET-receptor mutant *Never ripe* (*Nr*) tomato plants (*Solanum lycopersicum* L. Ailsa Craig) were grown hydroponically for 6 weeks in the greenhouse [12/12-h light/dark (light starting from 06:00

until 18:00 h and 12-h dark period during the remaining night time); 24/22°C; 50–60% relative humidity; 200 µmol(photon) m^{-2} s^{-1} light flux density (*5700 K* white LED supplemented with FAR LEDs; *PSI*, Drásov, Czech Republic)] after the germination in the dark. The nutrient solution (pH 5.8) containing 2 mM $Ca(NO_3)_2$, 1 mM $MgSO_4$, 0.5 mM KH_2PO_4, 0.5 mM Na_2HPO_4, 0.5 mM KCl, 0.02 mM Fe(III)-EDTA, and micronutrients [1 µM $MnSO_4$, 5 µM $ZnSO_4$, 0.1 µM $CuSO_4$, 0.1 µM $(NH_4)_6Mo_7O_{24}$, 10 µM H_3BO_4], was changed three times a week (Iqbal *et al.* 2021). Thereafter, 6- to 7-week-old intact tomato plants at 7–8 developed leaf-level stage were used for the experiments.

Flagellin treatments: flg22 (*Genscript Biotech Corporation*, Piscataway, NJ, USA) in 5 µM concentration was used to treat the abaxial side of leaves of intact tomato plants at the 6th leaf level in the late afternoon (17:00 h) or in the evening (21:00 h) with squirrel hairbrush without wounding the leaves (Zhang *et al.* 2008, Korneli *et al.* 2014). Local and systemic effects of flg22 were detected on the 6th and the distal 5th leaf levels from the shoot apex in the next light phase at 09:00 h. Sterile distilled water was used as a control without flg22.

Photosynthetic activity: Chlorophyll (Chl) fluorescence of leaves and guard cells in epidermal strips from the abaxial side of intact plants was analysed with pulse amplitude modulation (PAM) chlorophyll fluorometer (*PAM-2000*; *Heinz Walz*, Effeltrich, Germany) and with a *Microscopy-PAM* chlorophyll fluorometer (*Heinz Walz*, Effeltrich, Germany) mounted on a *Zeiss Axiovert 40* inverted epifluorescence microscope (*Carl Zeiss Inc*., Jena, Germany) described earlier by Goh *et al.* (1999) and Poór and Tari (2012). Abaxial epidermal strips were rapidly prepared from the treated and distal leaves of intact WT or *Nr* plants then immediately transferred to glass-bottom culture dishes (*MatTek Co*., Ashland, MA) containing 3.5 mL of buffer solution [10 mM 2-(N-morpholino) ethanesulfonic acid (MES), 10 mM KCl, pH 6.15] based on Zhang *et al.* (2001). Before measuring the minimal fluorescence yield of the dark-adapted state (F_0), leaves were dark-adapted for 15 min. Firstly, the maximal fluorescence in the dark-adapted state (F_m) was measured after the dark incubation. During the experiments, the following parameters were calculated: the maximal quantum efficiency of PSII photochemistry [$F_v/F_m = (F_m - F_0)/F_m$], the actual quantum yield of PSII electron transport in the light-adapted state [$Y_{(II)} = (F_m' - F_s)/F_m'$] and the photochemical quenching coefficient [$q_P = (F_m' - F_s)/(F_m' - F_0')$]. Finally, the light-induced photoprotection through thermal dissipation of energy was determined as NPQ = [$(F_m - F_m')/F_m'$] based on Genty *et al.* (1989) and Kramer *et al.* (2004). Four leaves from four different plants were measured in the case of all treatments which were repeated three times ($n = 3$). Means ± SE were calculated based on all data of the three biological repetitions.

The stomatal conductance and the net photosynthetic rate (P_N) were detected in the leaves of intact tomato plants using a portable photosynthesis system (*LI-6400*; *LI-COR Inc*., Lincoln, NE) described earlier by Poór *et al.* (2011). Leaves were illuminated (PPFD of 200 µmol m^{-2} s^{-1}) and data were recorded after 10 min under constant environment (25°C, 65 ± 10% relative humidity, and controlled CO_2 supply of 400 µmol mol^{-1}) during the measurements. Six leaves from four different plants were measured in the case of all treatments which were repeated three times ($n = 3$). Means ± SE were calculated based on all data of the three biological repetitions.

Detection of stomatal ROS and NO production: ROS production was detected using 2',7'-dichlorofluorescein diacetate (H_2DCFDA) as described earlier by Suhita *et al.* (2004). Epidermal strips were loaded with 10 µM H_2DCFDA for 20 min, in the 10 mM MES/KCl buffer (pH 6.15) in the dark at room temperature. NO accumulation in guard cells of tomato epidermal strips was detected using 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate (DAF-FM DA) as described earlier by Bright *et al.* (2006) by the same way. Samples were rinsed twice with 10 mM MES/KCl buffer (pH 6.15), then the intensity of fluorescence was detected by *Zeiss Axiowert 200 M* type fluorescence microscope (*Carl Zeiss Inc*., Jena, Germany). Digital images were taken from stomata with a high-resolution digital camera (*Axiocam HR*, HQ CCD camera). The fluorescence intensity of ROS and NO production was measured by using *AxioVision Rel. 4.8* (*Carl Zeiss Inc*., Munich, Germany) software (Czékus *et al.* 2021a). Stomata (30–40) from four leaves of different plants were measured in the case of all treatments and were repeated three times ($n = 3$). Means ± SE were calculated based on all data of the three biological repetitions. All chemicals originated from *Sigma-Aldrich* (St. Louis, MO, USA).

Measurement of sugar content: Sugar content was measured based on Hansen and Møller (1975). Fresh mass (FM; 100 mg) was ground in liquid N_2 and boiled in 1 ml of 80% ethanol at 80°C for 30 min. Then samples were centrifuged at 2,600 × *g* for 10 min and the supernatant was used for the measurements. Sugar content was determined spectrophotometrically at 630 nm (*Kontron*, Milano, Italy) after reaction with anthrone using glucose (*Normapur*, *VWR Int*., Leuven, Belgium) dissolved in 80% ethanol as a standard. Three samples from four different plants were measured in the case of all treatments which were repeated three times ($n = 3$). Means ± SE were calculated based on all data of the three biological repetitions.

Measurements of hexokinase (HXK) enzyme activity: HXK (EC 2.7.1.1) enzyme activity was measured with glucose substrate based on Whittaker *et al.* (2001). Leaf samples (0.5 g) were ground under liquid N_2 and then 1 ml of cold extraction buffer (20 mM KH_2PO_4, pH 7.5; 0.5 mM Na EDTA, 5 mM dithiothreitol) was added to the samples. The homogenate was centrifuged (12,000 × *g* for 20 min at 4°C). HXK enzyme activity was detected in a reaction mixture containing 100 mM KH_2PO_4 buffer (pH 7.5), 2 mM $MgCl_2$, 1 mM Na EDTA, 1 mM ATP, 10 mM glucose, 1 U of glucose-6-phosphate dehydrogenase (EC 1.1.1.49, G6PDH), 1 U of phosphoglucose isomerase

(EC 5.3.1.9, PGI) from baker's yeast, and 100 µl of plant extract. The absorbance of the reaction mixture was measured at 340 nm for 5 min at 25°C by spectrophotometer (*Kontron*, Milano, Italy). Three samples from four different plants were measured in the case of all treatments which were repeated three times (n = 3). Means ± SE were calculated based on all data of the three biological repetitions. The amount of enzyme producing 1 µmol min^{-1} of phosphorylated glucose was defined as one unit (U) and the enzyme activities were expressed as U mg^{-1}(protein). Soluble protein concentration was determined based on Bradford (1976) using bovine serum albumin as a standard.

Detection of the relative transcript levels of tomato *HXKs*: Quantitative real-time reverse transcription-PCR (qRT-PCR) using *qTOWER 2.0* (*Analytik Jena*, Jena, Germany) was used to detect the expression pattern of the selected tomato *HXK* genes mined from *Sol Genomics Network* (*SGN*; http://solgenomics.net/) database based on Poór *et al.* (2018). After the total RNA extraction by TRI reagent method (Chomczynski and Sacchi 1987), the genomic DNA was digested using *DNase I* (*Thermo Scientific*, Waltham, MA, USA), and then cDNA was synthesized using MMLV reverse transcriptase (*Thermo Scientific*, Waltham, MA, USA). The PCR reaction mixture contained 10 ng of cDNA template, 400 nM forward and 400 nM reverse primers, and 5 µL of *Maxima SYBR Green qPCR Master Mix* (*Thermo Scientific*, Waltham, MA, USA) in nuclease-free water at a final volume of 10 µL. After the PCR (95°C for 7 min, followed by 40 repetitive cycles of denaturation at 95°C for 15 s and annealing extension at 60°C for 60 s), data were analysed by using *qTOWER Software 2.2* (*Analytik Jena*, Jena, Germany). As a reference, elongation factor-1α subunit was used and the expression data were calculated by the $2^{(-\Delta\Delta CT)}$ formula (Livak and Schmittgen 2001). Data were normalized to the transcript levels of tomato reference genes, and the transcript levels of untreated control leaves. Four leaves from four different plants were measured in the case of all treatments which were repeated three times (n = 3). Means ± SE were calculated based on all data of the three biological repetitions.

Statistical analysis: All experiments were repeated three times in each treatment. The data presented are means ± SE. Statistical analysis was performed by using *Sigma Plot 11* software (*Systat Software Inc*., Erkrath, Germany) where results were analysed by analysis of variance (*ANOVA*) and *Duncan*'s multiple comparison test and differences were considered significant if $P \leq 0.05$.

Results

Photosynthetic activity: To test the daytime- and ET-dependent effects of 5 µg mL^{-1} flg22 on the photosynthetic activity of intact tomato plants, changes in chlorophyll (Chl) fluorescence parameters were investigated in wild-type (WT) and *Nr* plants. After flg22 treatments at different daytimes, experiments were carried out in the next light phase at 09:00 h to reveal the role of long-term- and daytime-dependent local and systemic effects of the bacterial elicitor. During the investigation, the flg22-treated (6th leaf levels from the shoot apex) and the distal leaves (5th leaf levels from the shoot apex) of intact tomato plants were analysed. The treatment with flg22 did not induce any significant changes in the photosynthetic activity of intact tomato plants in the morning based on the changes in F_v/F_m, $Y_{(II)}$, q_P, and NPQ (Fig. 1).

In contrast to the changes in the photosynthetic activity of intact leaves of tomato plants, $Y_{(II)}$ and q_P significantly decreased after flg22 treatments in the light period (17:00 h) locally and systemically in guard cells of leaves of WT plants at 09:00 h (Fig. 2*C–E*). NPQ increased only slightly but not significantly in the guard cells of these plants (Fig. 2*G*). At the same time, this decrease in $Y_{(II)}$ was neither detected in plants treated in the evening (Fig. 2*D*) nor in *Nr* leaves at all (Fig. 2*C*,*D*); it suggested the daytime- and ET-dependent effects of flg22 on stomatal photosynthesis.

Stomatal ROS and NO production: Significantly higher ROS production was also observed in the guard cells of flg22-treated and distal leaves from flg22-treated plants (Fig. 3*A*) compared to the control. These changes were not dependent on the daytime of the flg22 application but were not detectable in the *Nr* leaves (Fig. 3*A*,*B*). In contrast, NO production was significant only after the flg22 treatment at 17:00 h in the guard cells of WT plants (Fig. 3*C*) and did not change after the nocturnal treatment or in the *Nr* plants (Fig. 3*C*,*D*).

Stomatal conductance and net photosynthetic rate: The stomatal conductance was significantly reduced after flg22 application locally and systemically in WT plants treated at 17:00 h (Fig. 4*A*). Similarly, the net photosynthetic rate decreased in these plants upon the application of flg22 (Fig. 4*C*). In contrast, stomatal conductance and the net photosynthetic rate changed significantly neither in the nocturnal treated plants (Fig. 4*B–D*) nor in *Nr* leaves (Fig. 4).

Sugar content and HXK activity: Only the local treatment with flg22 at 17:00 h in the light period resulted in a significant decrease in the sugar content of the leaves of WT plants (Fig. 5*A*). Neither *Nr* leaves nor the dark-treated plants or the systemic leaves did significantly change the contents of sugars in the next light phase (Fig. 5*A*,*B*).

In parallel, the application of flg22 resulted in a significantly higher HXK activity locally in WT tomato compared to the control (Fig. 5*C*). In other cases, the enzyme activity of HXK did not change significantly in either WT or *Nr* leaves but WT showed higher HXK activity compared to *Nr* plants (Fig. 5*C*,*D*).

Gene expression of tomato *HXKs*: Based on the qRT-PCR analysis of the selected tomato HXK genes, *SlHXK3* was induced significantly by flg22 application at 17:00 h

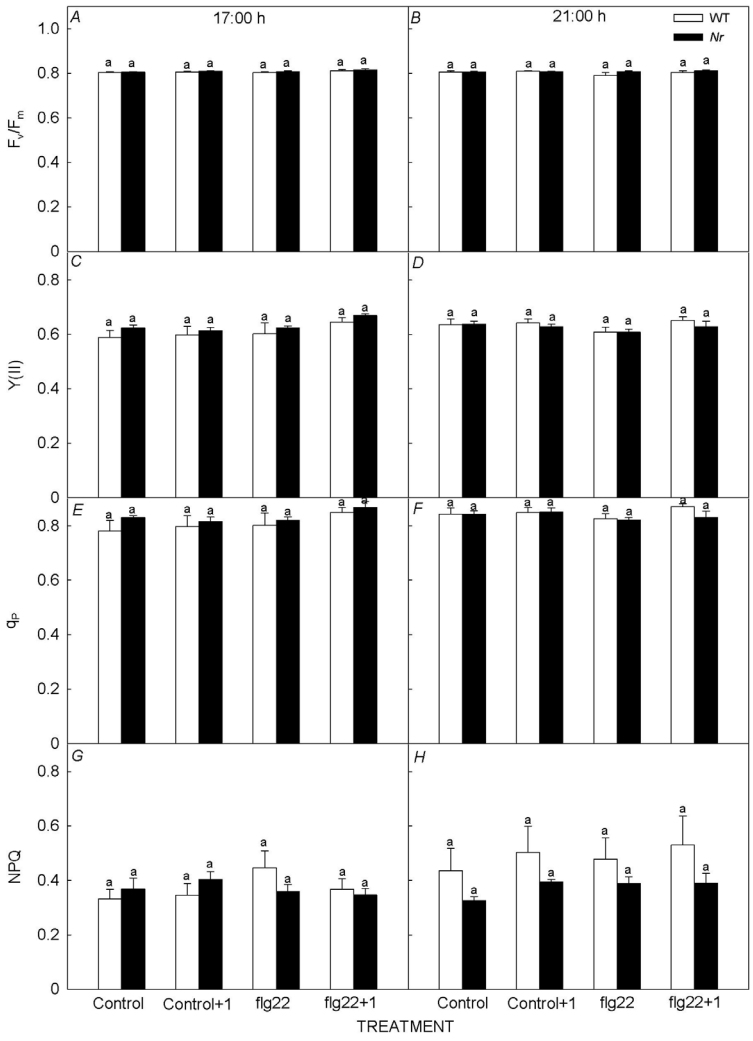

Fig. 1. Changes in the maximum quantum yield of PSII (F_v/F_m) (*A*,*B*), the effective quantum yield of PSII [$Y_{(II)}$] (*C*,*D*), the photochemical quenching coefficient (q_P) (*E*,*F*), and the nonphotochemical quenching (NPQ) (*G*,*H*) in leaves of wild-type (WT; *white columns*) and ethylene-insensitive *Never ripe* (*Nr*; *black columns*) tomato plants foliar-treated with 5 μg mL^{-1} flagellin (flg22) in the afternoon under lightness (at 17:00 h) or at night under darkness (at 21:00 h). Measurements were carried out in the next light phase at 09:00 h. Means ± SE, $n = 3$. Bars denoted by *different letters* are significantly different at $P \leq 0.05$ as determined by *Duncan*'s test. Control – treatment with sterile distilled water; Control+1 – untreated distal leaf level from the control; flg22 – treatment with 5 μg mL^{-1} flagellin dissolved in sterile distilled water; flg22+1 – untreated distal leaf level from the flg22-treated one.

in the elicitor-treated WT leaves (Fig. 6*E*). In addition, relative transcript levels of *SlHXK3* decreased in *Nr* leaves (Fig. 6*E*). In other cases, the gene expression of tomato *HXKs* did not change significantly in either WT or *Nr* leaves upon flg22 in the next light phase (Fig. 6).

Discussion

Chloroplasts not only play a crucial role in photosynthesis but also the synthesis of several phytohormones and the generation of ROS. Thus, chloroplasts significantly contribute to the successful defence responses of plants locally and systemically, respectively (Littlejohn *et al.* 2021). Pathogen infection alters the normal molecular and physiological processes in the host plants influencing the photosynthetic activity which is vital for plants (Kuźniak and Kopczewski 2020). In this work, flg22 was used to study the long-term daytime- and ET-dependent effects of bacterial pathogens in intact leaves of tomato plants focusing on the local and systemic effects of the elicitor and the role of photosynthesis in this process.

Bacteria- and flg22-induced signalling and plant defence responses are also highly dependent on the presence of the light during day/night time (Zeier *et al.* 2004, Griebel and Zeier 2008, Sano *et al.* 2014). Two closest time points were selected for the treatments in the late light and early dark period of the day (17:00 and 21:00 h) based on our previous work (Czékus *et al.* 2021b) to distinguish the direct effect of external light/darkness from the internal effect of circadian rhythm on plants and measurements were accomplished in the next light phase at 09:00 h. These mimic the natural environmental conditions and make it possible to compare and describe plant defence responses under natural light/dark conditions instead of artificial darkening. Natural light/dark conditions have crucial importance from the aspect of defence as most of the plant bacteria are more active at night (Santamaría-Hernando *et al.* 2018). At 17:00 h, stomata are open and the accumulation of photoassimilates is usually finished (Lawson 2009). At 21:00 h (3 h after the end of the light period), the light-dependent processes of photosynthesis and active phytochrome signalling are already inactivated (Graf *et al.* 2010, Medzihradszky *et al.* 2013). At the same time, these time points for treatments are close to each other providing almost the same availability of carbohydrates and starch for metabolic

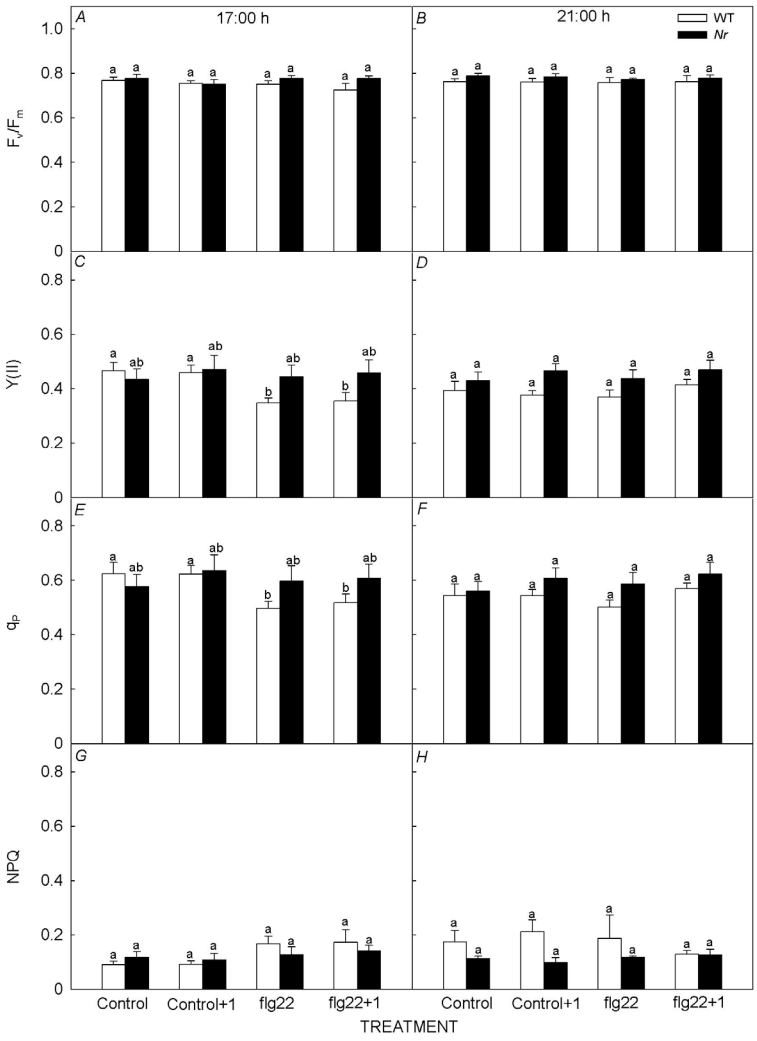

Fig. 2. Changes in the maximum quantum yield of PSII (F_v/F_m) (*A*,*B*), the effective quantum yield of PSII [$Y_{(II)}$] (*C*,*D*), the photochemical quenching coefficient (q_P) (*E*,*F*), and the nonphotochemical quenching (NPQ) (*G*,*H*) in the stomata of intact leaves of wild-type (WT; *white columns*) and ethylene-insensitive *Never ripe* (*Nr*; *black columns*) tomato plants foliar-treated with 5 μg mL^{-1} flagellin (flg22) in the afternoon under lightness (at 17:00 h) or at night under darkness (at 21:00 h). Measurements were carried out in the next light phase at 09:00 h. Means ± SE, $n = 3$. Bars denoted by *different letters* are significantly different at $P \leq 0.05$ as determined by *Duncan*'s test. Control – treatment with sterile distilled water; Control+1 – untreated distal leaf level from the control; flg22 – treatment with 5 μg mL^{-1} flagellin dissolved in sterile distilled water; flg22+1 – untreated distal leaf level from the flg22-treated one.

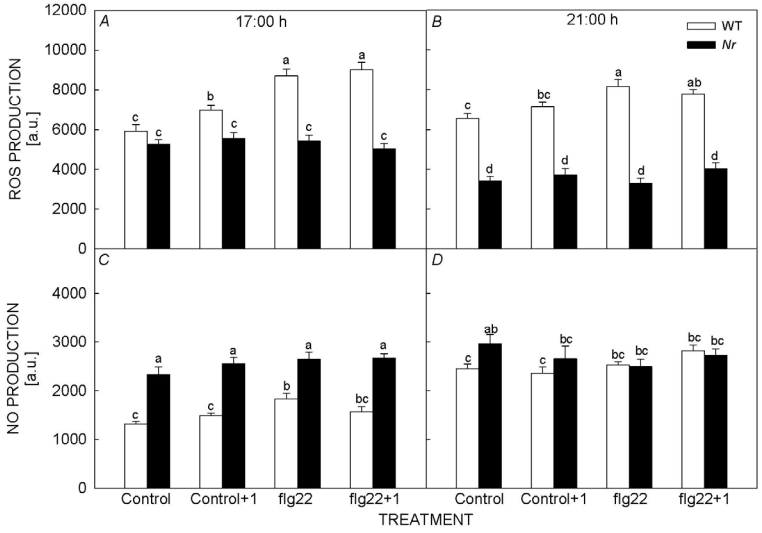

Fig. 3. Changes in the production of reactive oxygen species (ROS) (*A*,*B*) and nitric oxide (NO) (*C*,*D*) in stoma of intact leaves of wild-type (WT; *white columns*) and ethylene-insensitive *Never ripe* (*Nr*; *black columns*) tomato plants foliar-treated with 5 μg mL^{-1} flagellin (flg22) in the afternoon under lightness (at 17:00 h) or at night under darkness (at 21:00 h). Measurements were carried out in the next light phase at 09:00 h. Means ± SE, $n = 3$. Bars denoted by *different letters* are significantly different at $P \leq 0.05$ as determined by *Duncan*'s test. Control – treatment with sterile distilled water; Control+1 – untreated distal leaf level from the control; flg22 – treatment with 5 μg mL^{-1} flagellin dissolved in sterile distilled water; flg22+1 – untreated distal leaf level from the flg22-treated one.

energy (Graf and Smith 2011) which are significant in the aspect of the plant defence responses. The sampling at 9:00 h (3 h after the end of the dark period) was selected because light signalling is activated, stomata are opened, and photosynthesis is active at this time point (Czékus *et al.* 2020).

In our previous work, it was found that H_2O_2 contents increased locally within 30 min by flg22, and superoxide

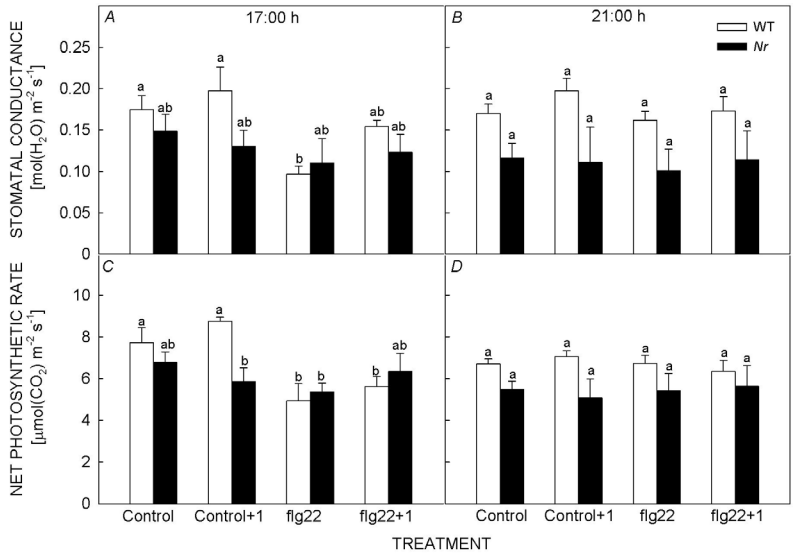

Fig. 4. Changes in the stomatal conductance (*A*,*B*) and the net photosynthetic rate (*C*,*D*) in leaves of wild-type (WT; *white columns*) and ethylene-insensitive *Never ripe* (*Nr*; *black columns*) tomato plants foliar-treated with 5 µg mL^{-1} flagellin (flg22) in the afternoon under lightness (at 17:00 h) or at night under darkness (at 21:00 h). Measurements were carried out in the next light phase at 09:00 h. Means ± SE, n = 3. Bars denoted by *different letters* are significantly different at $P \leq 0.05$ as determined by *Duncan*'s test. Control – treatment with sterile distilled water; Control+1 – untreated distal leaf level from the control; flg22 – treatment with 5 µg mL^{-1} flagellin dissolved in sterile distilled water; flg22+1 – untreated distal leaf level from the flg22-treated one.

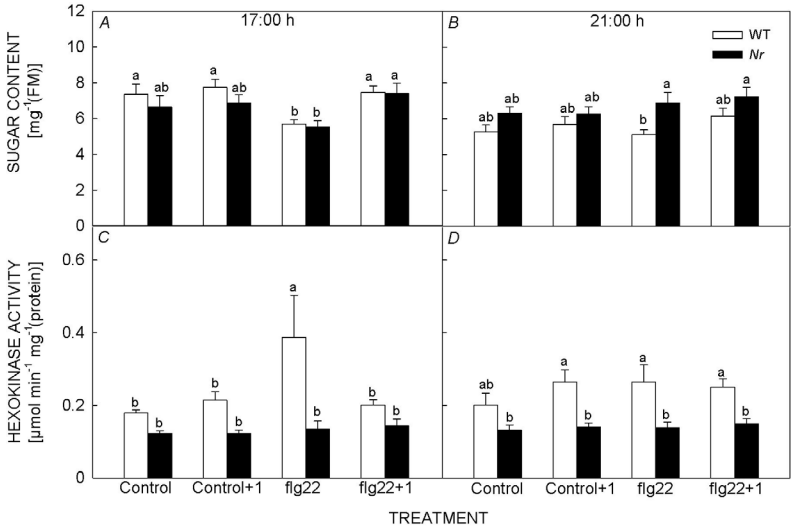

Fig. 5. Changes in the sugar content (*A*,*B*) and the hexokinase activity (*C*,*D*) in leaves of wild-type (WT; *white columns*) and ethylene-insensitive *Never ripe* (*Nr*; *black columns*) tomato plants foliar-treated with 5 µg mL^{-1} flagellin (flg22) in the afternoon under lightness (at 17:00 h) or at night under darkness (at 21:00 h). Measurements were carried out in the next light phase at 09:00 h. Means ± SE, n = 3. Bars denoted by *different letters* are significantly different at $P \leq 0.05$ as determined by *Duncan*'s test. Control – treatment with sterile distilled water; Control+1 – untreated distal leaf level from the control; flg22 – treatment with 5 µg mL^{-1} flagellin dissolved in sterile distilled water; flg22+1 – untreated distal leaf level from the flg22-treated one.

production was significantly higher in systemic leaves of WT tomato plants after 1 h in the light period of the day promoting the rapid stomatal closure in both leaf levels (Czékus *et al.* 2021b). At the same time, not only ROS but also ET, JA, and SA accumulation was observed after 1 h in flg22-treated WT tomato leaves while such changes were not detected in *Nr* plants and in the case of the night treatments at 21:00 h (Czékus *et al.* 2021b). These rapid changes in leaves can determine the long-lasting defence responses of plants. Nevertheless, long-term- and day/night-time-dependent effects of flg22 on physiological responses of intact plants in the morning of the next day have not been investigated. In this process, the role of photosynthesis has neither been examined thus we focused on that in this manuscript. Changes in photosynthesis are crucial under pathogenesis because it serves energy and reducing power to the successful defence process of plants (Dodd *et al.* 2005, Berger *et al.* 2007). At the same time, pathogens can suppress photosynthesis and the photosynthesis-related gene expression as was observed in the case of *Pseudomonas syringae* (Bonfig *et al.* 2006) or *Xanthomonas oryzae* infection (Yu *et al.* 2014). In addition, the presence or absence of light highly influences photoinhibition and photodegradation in the infected leaves. It was found that the damage of the photosynthetic apparatus was greater in the dark after the 3-d-long *P. syringae* infection in tobacco leaves (Cheng *et al.* 2016). However, more rapid changes (30 min and 2 h) in photosynthesis-related genes and the rapid induction of various phytohormone-mediated signalling components were observed after flg22 treatments in the light or dark. Similar results were observed after the application of photosynthesis inhibitor 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) suggesting that photosynthesis plays a role in controlling the light-dependent expression of flg22-inducible defence genes (Sano *et al.* 2014). Göhre *et al.* (2012) investigated firstly the short- and long-term effects of flg22 on the photosynthetic activity using *Arabidopsis* seedlings grown in liquid media. A rapid and significant decrease in the NPQ was

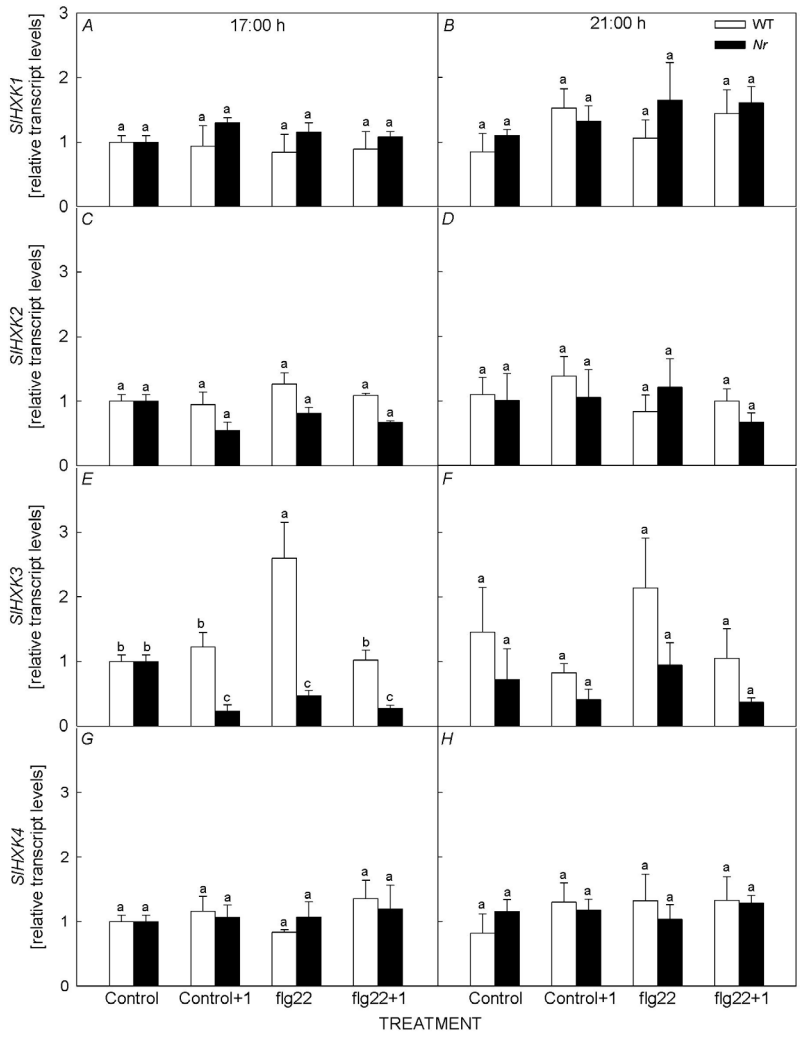

Fig. 6. Changes in the relative transcript levels of tomato hexokinase (*HXK*) genes (*A–H*) in leaves of wild-type (WT; *white columns*) and ethylene-insensitive *Never ripe* (*Nr*; *black columns*) tomato plants foliar-treated with 5 μg mL^{-1} flagellin (flg22) in the afternoon under lightness (at 17:00 h) or at night under darkness (at 21:00 h). Measurements were carried out in the next light phase at 09:00 h. Means ± SE, $n = 3$. Bars denoted by *different letters* are significantly different at $P \leq 0.05$ as determined by *Duncan*'s test. Control – treatment with sterile distilled water; Control+1 – untreated distal leaf level from the control; flg22 – treatment with 5 μg mL^{-1} flagellin dissolved in sterile distilled water; flg22+1 – untreated distal leaf level from the flg22-treated one.

observed already after 20 min, reached a minimum after 1 h but then recovered after 4 h despite the constant presence of flg22 in the liquid media (Göhre *et al.* 2012). In parallel, flg22 induced high ROS production with the maximum after 20 min (Göhre *et al.* 2012). Interestingly, the authors detected the long-term adaptation to flg22 in *Arabidopsis* seedling and found that $Y_{(II)}$ significantly decreased while NPQ significantly increased after 7 d upon flg22 (Göhre *et al.* 2012). These results suggested that flg22 has long-term effects on the photosynthetic activity of seedlings mediating plant defence responses but the day/night-time or systemic effects of flg22 treatments were not investigated. We first measured the effects of different day/night-time applications of flg22 on the photosynthetic activity of various leaves of intact tomato plants in the morning of the next light period. Surprisingly, flg22 treatments did not induce any significant changes in the photosynthetic activity of mesophyll cells based on the analysis of Chl fluorescence parameters. This suggests that the PSII activity of mesophyll is less sensitive or later affected by the effects of flg22 in leaves of intact and developed plants as compared to seedlings exposed to the constant presence of flg22 (Göhre *et al.* 2012).

There are some important differences between chloroplasts of guards cells and palisade mesophyll cells such as the lower number and size of chloroplasts in guard cells as compared to the palisade mesophyll cells which can affect their photosynthetic activity. Other important differences can be found in the case of starch metabolism of guard cell chloroplasts influencing the stomatal movement during the day (Lawson 2009). At the same time, guard cells localized in the epidermis are exposed to flg22 first, thus the effects of flg22 on the photosynthetic activity of guard cells could be very significant in determining the long-lasting stomatal closure as a part of the defence responses of plants. Based on our results, flg22 significantly decreased $Y_{(II)}$ and q_P in guard cells of local and systemic leaves of WT plants treated in the light period (17:00 h). In parallel, NPQ increased only slightly in the guard cells of these plants. This suggests that stomatal photosynthetic activity is much more sensitive to flg22 locally and systemically which can contribute to stomatal closure and the limitation of CO_2 assimilation at the whole plant level systemically, as well as to the inhibition of pathogen entry in the morning of the next day. These effects of flg22 on stomatal photosynthetic activity can be dependent due to

the rapid ROS production within minutes mediated by NADPH oxidase (Zhang *et al.* 2008, Ranf *et al.* 2011, Thor and Peiter 2014) and later to the ROS production by chloroplasts in a self-amplifying loop (Kangasjärvi *et al.* 2012) which contributed to the rapid stomatal closure and which cannot be detected in the dark (Czékus *et al.* 2021b). At the same time, these changes in the photosynthetic parameters were neither observed in the case of the evening treatment in the dark nor *Nr* leaves suggesting the daytime (light)- and ET-dependent effects of flg22 on stomatal photosynthesis. Based on these results daytime (light) and ET can play a role in the regulation of the early detection of flg22 by FLS2 and the rapid signaling of flg22 mediated by ROS (Mersmann *et al.* 2010, Czékus *et al.* 2021b) in the absence of which there is no long-term effect of flg22 on the photosynthetic activity. This is in a good correlation with the results of Borbély *et al.* (2019) who found that ET in a time- and concentration-dependent manner can induce a rapid production of superoxide and H_2O_2 in the leaves and parallelly can decrease $Y_{(II)}$ and increase NPQ. Moreover, ET production, sensing, and signalling are not only highly dependent on the developmental stages of plants but also on the light (Harkey *et al.* 2019). Our previous result also confirmed that flg22 induced rapid ET production and signalling only during the day in the light which was not detectable at night in the dark (Czékus *et al.* 2021b). These differences between the two-time points confirm the long-term effects of flg22 in the next light phase at 9:00 h.

Treatment with flg22 at 17:00 h not only induced significant changes in the photosynthetic activity of stomata but also induced high stomatal ROS production which can promote chloroplastic ROS generation in a self-amplifying feedback loop contributing to the inhibition of photosynthetic activity (Kangasjärvi *et al.* 2012) and thus maintaining the long-lasting and systemic stomatal closure after flg22 treatments. Moreover, flg22 exposure induced significantly high NO production in the guard cells of the flg22-treated leaves in the light period which was not detected in guard cells of *Nr* leaves. At the same time, *Nr* plants accumulate higher NO as compared to WT plants whose NO contents were not influenced by flg22, thus suggesting the potential role of ET in this process. Increased ROS and NO production by flg22 in guard cells promoted the activation of plasma membrane-localized Ca^{2+} channels and subsequently of the SLAC1 anion channel and aquaporin PIP2;1 in guard cells contributing to stomatal closure after the bacterial elicitor treatments locally in the light phase of the day (Zhang *et al.* 2008, Ranf *et al.* 2011, Thor and Peiter 2014, Deger *et al.* 2015, Toum *et al.* 2016, Rodrigues *et al.* 2017). However, this investigation confirmed the long-term effects of flg22 on stomata of intact plants in the next light phase. Mersmann *et al.* (2010) found that flg22 did not induce stomatal closure mediated by ROS in ET-insensitive *Arabidopsis* mutants. Here, we measured also the absence of stomatal closure in the case of ET-receptor mutant *Nr* plants whose closure can be dependent on the light- and ET-dependent ROS and NO production. At the same time, flg22 did not induce ET production and ET signalling at night in the dark (Czékus *et al.* 2021b) which can also contribute to the lack of ROS wave in flg22-treated plants at this time point and the inhibition of stomatal closure in the next morning. Stomatal closure induced by flg22 at 9:00 h could contribute to the significant decrease in the net photosynthetic rate of tomato leaves which was measured by us. This decrease in CO_2 assimilation can negatively influence the Calvin cycle and sugar biosynthesis resulting in the reduction of biomass production. Göhre *et al.* (2012) observed also growth inhibition and biomass reduction upon 7-d-long flg22 exposure in *Arabidopsis*. This process is also dependent on the light/dark phase and the active ET signalling mediated by ROS/NO production based on our measurements.

Recently, it was found that plant HXKs not only play a role in sugar metabolism (Granot *et al.* 2013) or in the regulation of stomatal closure (Shen *et al.* 2021) or in mitochondrial cell death by regulating ROS (Poór *et al.* 2019) but also influence plant immune reactions *via* responding to glucose (Jing *et al.* 2020). Sugars such as glucose not only play a role as an energy source but also as a signalling molecule interacting with phytohormones such as ET (Sheen 2014, Li and Sheen 2016). Reduction in disease symptoms of *Pseudomonas syringae* was found in the *hxk1 Arabidopsis* mutant, indicating that HXKs play role in plant immune responses (Jing *et al.* 2020). We measured that flg22 treatment in the light phase of the day elevated HXK activity and the expression of mitochondrial *SlHXK3* in the treated leaves of WT tomato plants, but changes were not detected systemically or in *Nr* leaves. Our results suggest that a decrease in sugar content and elevated HXK activity in a light- and ET-dependent manner could contribute to local stomatal closure and the activation of defence in intact tomato plants.

In conclusion, flg22 is responsible for the induction of rapid and long-term defence reactions in plants but its effects on photosynthesis are less studied especially in the case of stomata. Based on our results, it can be concluded that flg22 treatment neither in the light phase nor in the evening caused any significant change in chlorophyll fluorescence induction parameters in the mesophyll cells of tomato plants measured in the next morning at the beginning of the next light cycle. However, treatment in the light phase decreased the effective quantum yield [$Y_{(II)}$] and the photochemical quenching (q_P) both locally and systemically in guard cells of epidermal peels prepared from the flg22-treated leaves. In parallel, the production of ROS and NO increased in these guard cells, which may have contributed to the next day's long-term stomatal closure and a decrease in CO_2 assimilation in these plants. These processes can be dependent on the daytime (light) and ET, which take part in the regulation of flg22 sensing and signalling *via* the rapid ROS and NO production in guard cells. Our results demonstrated also that flg22-induced ET- and light-dependent decrease in sugar content and elevated hexokinase activity can also contribute to local defence responses in intact tomato plants.

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