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Salicylic acid- and ethylene-dependent effects of the ER stress-inducer tunicamycin on the photosynthetic light reactions in tomato plants

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ABSTRACT

Plant hormones such as ethylene (ET) and salicylic acid (SA) have an elementary role in the regulation of ER stress and unfolded protein response (UPR) in plants via modulating defence responses or inducing oxidative stress. Chloroplasts can be sources and targets of reactive oxygen species (ROS) that affect photosynthetic efficiency, which has not been investigated under tunicamycin (Tm)-induced ER stress. In this study, the direct and indirect effects of Tm on chloroplastic ROS production were first investigated in leaves of wild-type tomato (*Solanum lycopersicum* L.) plants. Secondly changes in activities of photosystem II and I were analysed under Tm exposure and after application of the chemical chaperone 4-phenylbutyrate (PBA) in different genotypes, focusing on the regulatory role of SA and ET Tm treatments significantly but indirectly induced ROS production in tomato leaves and in parallel it decreased the effective quantum yield of PSII [Y(II)] and PSI [Y(I)], as well as the photochemical quenching coefficient (qP) and the quantum yield of non-photochemical energy dissipation in PSI due to acceptor-side limitation [Y(NA)]. At the same time, Tm increased non-photochemical quenching (NPQ) and cyclic electron flow (CEF) in tomato leaves after 24 h. However, the photosynthetic activity of the SA hydroxylase-overexpressing NahG tomato plants was more severely affected by Tm as compared to wild-type and ET-insensitive *Never ripe* (*Nr*) plants. These results suggest the protective role of SA in the regulation of photosynthetic activity contributing to UPR and the survival of plants under ER stress. Interestingly, the activation of photoprotective mechanisms by NPQ was independent of SA but dependent on active ET signalling under ER stress, whereas CEF was reduced by ET due to its higher ratio in *Nr* plants.

1. Introduction

Plants are sessile organisms, which makes it necessary for them to defend themselves against various abiotic and biotic stresses (Saijo and Loo, 2020). To eliminate the adverse effects of these stressors, sophisticated defence responses are activated under the control of the defence-related phytohormones salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) (Peleg and Blumwald, 2011). These phytohormones can regulate plant defence responses such as the synthesis of enzymes involved in antioxidant mechanisms, chaperones, and proteins with antimicrobial activity (e.g. pathogenesis-related (PR) proteins, defensins) through various signalling pathways (Saleem et al., 2021). At the same time, under stress conditions, the overproduction of defence-related proteins can disrupt and overwhelm the folding capacity and protein trafficking of the endoplasmic reticulum (ER), resulting in ER stress in plant cells (Afrin et al., 2020). Under ER stress, unfolded or misfolded proteins accumulate in the ER lumen. These changes trigger a specific cellular response known as the unfolded protein response (UPR). The UPR can alleviate ER stress by reducing the protein load in the ER, upregulating specific genes participating in the regulation of protein synthesis, such as chaperones like Binding Proteins (BiP), as well as promoting ER-associated protein degradation (ERAD) or inducing autophagy (Sun et al., 2021). Two main arms of the plant UPR have been identified, the inositol-requiring enzyme 1 (IRE1)-mediated pathway and the other is regulated by the basic leucine zipper 28 (bZIP28) and bZIP17 transcription factors, each of which is mediated by specific proteolysis steps prior to the transcriptional activation of UPR-related genes (Simoni et al., 2022). The role of SA (Wang et al., 2005; Nagashima et al., 2014; Poór et al., 2019a), JA (Xu et al., 2019; Czékus et al., 2020) and ET (Czékus et al., 2022) in the UPR has already been reported,

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e.g. by controlling ER stress-induced signalling and metabolism of reactive oxygen species (ROS).

Extensive ROS increase under stress can have a signalling function at low levels, but can also lead to lethal oxidative stress in cells by oxidising proteins, lipids or nucleic acids (Mittler et al., 2022). ROS can be generated by NADPH oxidase in the apoplast, in mitochondria and chloroplasts via the electron transport chains, and in the ER by ER luminal oxidoreductase 1 (ERO1) (Soares et al., 2019). Thus, potential links between the ER and other organelles such as chloroplasts, in the progression and/or elimination of oxidative processes can be supposed. In this context, phytohormones such as SA and ET could also be important signalling compounds, as they basically determine ROS levels as well as the activation of antioxidant-mediated defence responses in these cell compartments in a time- and concentration-dependent manner (Borbély et al., 2019; Poór et al., 2019b). The contribution of these phytohormones to the regulation of the UPR under ER stress has been analysed applying the widely-used ER stress-inducer tunicamycin (Tm), which inhibits N-glycosylation of secreted glycoproteins and also causes high ROS production within hours (Watanabe and Lam, 2008), resulting in protein oxidation and lipid peroxidation (Czékus et al., 2022). At the same time, this effect of Tm can be effectively alleviated by the exogenous application of chemical chaperones such as 4-phenylbutyrate (PBA) (Howell, 2013). The study by Ozgur et al. (2014) first suggested that application of Tm induced significant H_2O_2 accumulation and altered the activities of superoxide dismutase, catalase, ascorbate peroxidase and glutathione reductase in the shoot of Arabidopsis. This suggests the involvement of other cell organelles in the regulation or ER stress-induced oxidative stress. Nevertheless, the relationship between the ER and other organelles, such as chloroplasts, which can also be a source or target of ROS under ER stress, remained unknown in detail, especially in crops. The strong interaction between chloroplasts and ER membranes was shown by Schattat et al. (2011) under stromula formation, suggesting interacting surfaces to exchange metabolites such as H_2O_2 between the two organelles (Ozgur et al., 2018). Other data confirmed that inhibition of chloroplastic electron transport by methyl viologen (MV) resulted in high superoxide production and *BiP3* expression, whereas application of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) caused high ROS accumulation but decreased *BiP3* expression in Arabidopsis (Ozgur et al., 2015). Thus, there may be an interaction between different ROS, the changes in photosynthetic activity and the ER, which may be differentially dependent on the activity of the two photosystems (PS) as ROS targets under stress conditions. Recently, only the role of the metalloprotease FtsH2 has been reported as part of the UPR involved in the repair of the ROS-degraded D1 protein of PSII (Dogra et al., 2019) in which SA can act as a retrograde signalling molecule (Dogra and Kim, 2019). Recently, SA has been reported to regulate ER stress-induced ROS levels through glutathione metabolism (Czékus et al., 2023), and the role of other phytohormones in controlling ROS levels under ER stress such as JA (Czékus et al., 2020) or ET (Czékus et al., 2022) has also been confirmed. At the same time, the potential role of these key defence-related phytohormones in the regulation of ER stress-induced oxidative stress responses needs further investigation, particularly in the case of the activity of energy-producing organelles such as chloroplasts.

In this work, the effect of ER stress on photosynthetic light reactions and the role of SA and ET in its regulation and modulation of the UPR were studied in leaves of intact tomato plants. First, the direct and indirect effects of Tm on chloroplastic ROS production were investigated. Secondly, changes in the activity of both PSII and PSI were then analysed under Tm exposure and after the addition of the chemical chaperone PBA. Our experiments also focused on the SA- and ET-mediated defence processes, such as changes in the non-photochemical quenching (NPQ) and cyclic electron flow (CEF).

2. Materials and methods

2.1. Plant growing conditions

Seeds of ET-insensitive *Never ripe* (*Nr*) (Lanahan et al., 1994) and SA hydroxylase-overexpressing (NahG) tomato plants (Muñoz-Espinoza et al., 2015) as well as their respective controls (*Solanum lycopersicum* L. cv. Ailsa Craig and Moneymaker) were germinated for 3 d at 27 °C. The seedlings were grown in perlite before being transferred to pots and grown in a hydroponic system under a constant environmental conditions of 12 h light/12 h dark period, 200 μmol m^{-2} s^{-1} photosynthetic photon flux density, 24 °C day/22 °C night temperature and a relative humidity between 55 and 60%. The nutrient solution used for plant growing was 3 times changed in every week (Poór et al., 2019b).

2.2. Isolation of chloroplasts and ROS staining

Leaves of WT tomato plants (15 g) were homogenised in 30 mL of ice-cold isolation buffer (0.3 M sucrose, 50 mM Tricine/NaOH (pH 7.8), 10 mM NaCl, 5 mM $MgCl_2$ and freshly added 0.2% bovine serum albumin (BSA)) according to the method of Grabsztunowicz and Jackowski (2013). After filtration of the homogenate, the samples were centrifuged (1000 g, 7 min, 4 °C). The supernatant was discarded and the green pellet was carefully suspended in 10 mL of isolation buffer. The chloroplast suspension was then loaded onto a 40%–80% Percoll gradient and centrifuged in a swing-out rotor (8000 g, 40 min, 4 °C) according to Bhattacharya et al. (2020). Intact chloroplasts were collected from the 40–80% Percoll interface, diluted in 30 mL isolation buffer without BSA and centrifuged (1000 g, 2 min, 4 °C). The supernatant was discarded and the washed chloroplast pellet was collected and the washing step was repeated.

ROS production of isolated chloroplasts was visualised by using 10 μM 2′,7′-dichlorofluorescein diacetate (H_2DCFDA) in the chloroplast isolation buffer without BSA under control conditions and adding 0.5 μg mL^{-1} Tm for 60 min in a final volume of 1 mL (Poór et al., 2013). H_2O_2 treatment was used as a positive control (data not shown). The green fluorescence intensity of the dye and the red autofluorescence of the chloroplasts were detected using a Zeiss Axiowert 200 M fluorescence microscope (Carl Zeiss Inc., Jena, Germany) equipped with a 20X objective. Digital photographs of the samples were taken with a high-resolution digital camera (Axiocam HR, HQ CCD camera; Carl Zeiss Inc., Jena, Germany). Fluorescence intensity was measured using AXIOVISION REL. 4.5 software (Carl Zeiss Inc., Munich, Germany). The microscope fields of each different sample were randomly selected.

2.3. Treatments of intact plants

Tomato plants at the five-leaf stage were treated with 0.5 μg mL^{-1} Tm in the nutrient soulution (Watanabe and Lam, 2008). To assess the effects of Tm in inducing ER stress, both in the presence or absence of Tm, the nutrient solution was supplemented with 1 mM PBA (Czékus et al., 2022). The pots were covered with aluminium foil and the final volume of the solutions was 400 mL for 6 plants. All chemicals were purchased from Sigma-Aldrich (St. Louis MO, USA). For all measurements, treatments were carried out at 9 a.m. and samples from the 3rd or 4th leaf levels were collected 24 h later. All experiments were replicated at least three times.

2.4. Determination of H_2O_2 content

H_2O_2 content was measured after homogenisation of leaf samples (0.2 g) with 1 mL trichloroacetic acid (TCA; 0.1%) according to Velikova et al. (2000). After centrifugation (12000 g, 10 min, 4 °C), 0.25 mL of the supernatant was added to the reaction mixture containing 0.25 mL of 10 mM phosphate buffer (pH 7.0) and 0.5 mL of 1 M potassium iodide (KI). The absorbance of the samples was determined spectrophotometrically

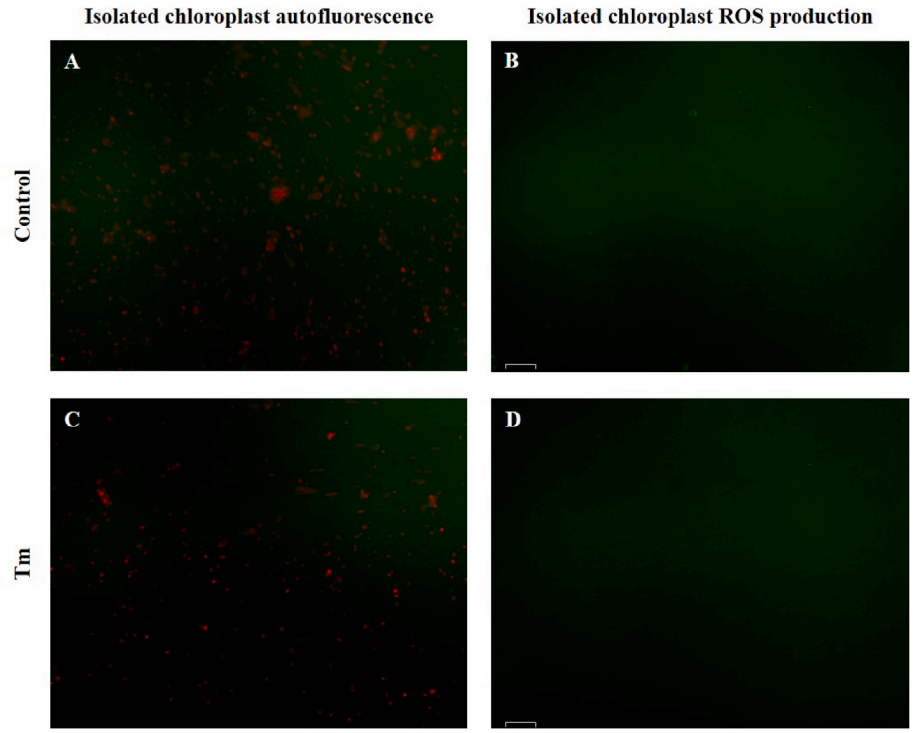

Fig. 1. The representative image of the autofluorescence (A, C) and the changes in the production of reactive oxygen species (ROS; B, D) in the isolated chloroplast of wild-type (WT) plants under control conditions or after a 60 min treatment with 0.5 μg mL^{-1} tunicamycin (Tm) (ruler: 50 μm; green colour shows ROS production, red colour shows autofluorescence of chloroplasts). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

at 390 nm (KONTRON, Milan, Italy) after 10 min incubation in the dark. H_2O_2 content was calculated from a standard curve generated from the H_2O_2 stock solution.

2.5. RNA extraction and gene expression analysis by quantitative real-time PCR

RNA extraction and gene expression analysis were performed as described by Takács et al. (2018). Genomic DNA was eliminated by digestion with DNase I enzyme. cDNA was synthesised from single-stranded RNA using MMLV reverse transcriptase enzyme. Quantitative real-time (qRT)-PCR (Piko Real-Time qPCR System, Thermo Fisher Scientific, Waltham, MA, USA) was used to investigate the relative expression of SlBiP (R: 5′-TCAGAAAGACAATGGGACCTG-3′, F: 5′-GCTTCCACCAACAAGAACAAT-3′) selected from the National Centre for Biotechnology Information (NCBI; https://www.ncbi.nlm.nih.gov/) database. The reaction mixture for qRT-PCR analysis contained 400-400 nM forward and reverse primers, 5 μL Maxima SYBR Green qPCR Master Mix (2X), 10 ng cDNA template and molecular biology water in a final volume of 10 μL. After an initial denaturation step (7 min, 95 °C), the reaction was built up by 40 repeated reaction cycles (denaturation for 15 s at 95 °C, annealing extension for 1 min at 60 °C). The qRT-PCR instrument software (PikoReal Software 2.2; Thermo Fisher Scientific, Waltham, MA, USA) was used to analyse the data. Expression data were calculated using the tomato elongation factor 1α subunit gene as a reference and the formula of $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001). All chemicals for molecular work were purchased from Thermo Fisher Scientific (Waltham, MA, USA).

2.6. Subcellular detection of H_2O_2 using confocal microscopy

Leaf discs prepared from control and 0.5 μg mL^{-1} Tm treated WT plants were imaged using a Leica Stellaris 5 laser scanning confocal microscope (Leica Microsystems, Heidelberg, Germany) after incubation in 50 μM Amplex™ Red (AR; Thermo Fisher Scientific, Waltham, MA, US) for 30 min in TRIS/HCl buffer (pH 7.4) based on Poór et al. (2015). For AR imaging, 586 nm white light laser excitation (2% intensity) and 595–640 nm emission range were used, and for chlorophyll imaging, 501 nm laser excitation (at 2% intensity) and 650–750 nm detection range were used with a 20X HC PC PL APO CS2 objective (N.A. 0.75). Identical excitation and detection settings were used to image AR-labelled and control samples. AR and chlorophyll images were pseudocoloured green and red, respectively.

2.7. Determination of the photosynthetic activity

Chlorophyll fluorescence and the redox state of P700 were measured with Dual-PAM-100 (Heinz-Walz, Effeltrich, Germany) (Klughammer and Schreiber, 1994). Before measuring the minimum fluorescence yield of the dark-adapted state (F_0), dark adaptation of the leaves was carried out for 15 min at room temperature in the dark. F_0 was determined by weak measuring light with open reaction centres (RC). Maximum dark-adapted fluorescence (F_m) was measured using a pulse (800 ms) of saturating light (12,000 μmol m^{-2} s^{-1}). Leaves were illuminated with actinic light (220 μmol m^{-2} s^{-1}) for the determination of the light-adapted steady-state fluorescence (F_s), whereas to record the maximum fluorescence level (Fm') in the light-adapted state, saturating pulses were applied. After switching off the actinic light, leaves were illuminated with far-red light (5 μmol m^{-2} s^{-1}) for 3 s to determine the minimum fluorescence level in the light-adapted state (F_0'). Via these measurements the maximum quantum yield of PSII (variable fluorescence (F_v)/maximum fluorescence (F_m)), the minimum fluorescence yield in the dark-adapted state (F_0), the maximum fluorescence yield in the dark-adapted state (F_m), the fraction of open PSII RC (q_L), the quantum yields of PSI [Y(I)] and PSII [Y(II)], the non-photochemical quenching (NPQ), the quantum yield of non-photochemical energy dissipation due to acceptor-side limitation [Y (NA)], the quantum yield of non-photochemical energy dissipation due to donor-side limitation [Y

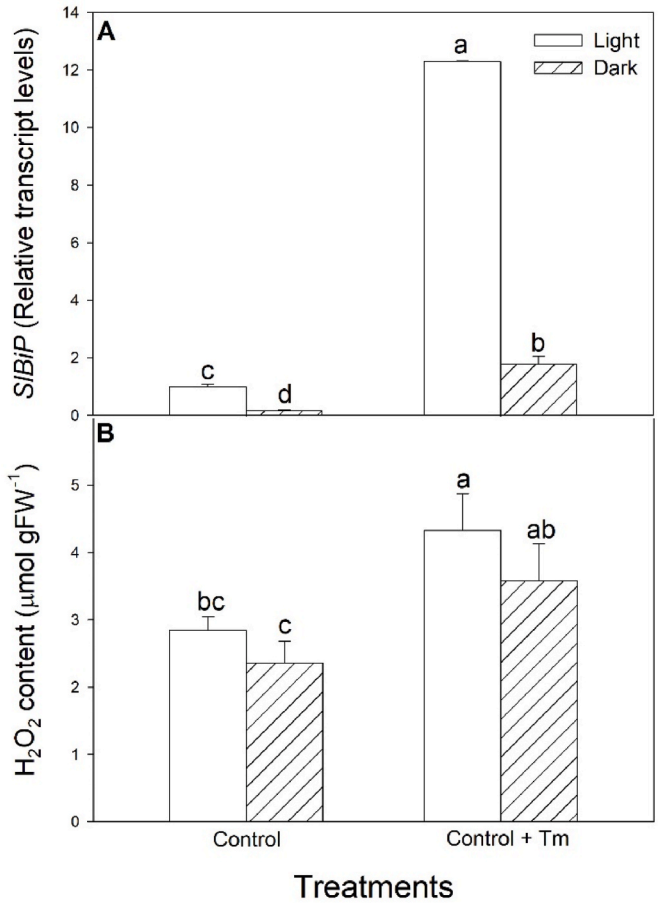

Fig. 2. Changes in relative transcript levels of *SlBiP* (A) and H_2O_2 contents (B) in the leaves of wild-type (WT) tomato plants after 24 h of treatment with 5 µg ml-1 Tm under the normal light/dark cycle or prolonged dark conditions. Means ± SE, n = 3. Bars are denoted with different letters if $P \leq 0.05$ based on the Duncan's test.

(ND)], and the photochemical quenching coefficient (qP) were determined (Zhang et al., 2014; Poór et al., 2019b). The cyclic electron flow (CEF) was calculated as Y(CEF) = [Y(I) – Y(II)] (Lei et al., 2014).

2.8. Statistical analysis

Each experiment was replicated at least three times independently (at least six plants were measured per treatment). Mean values were expressed ± SE. SigmaPlot 11.0 software (Systat Software GmbH, Erkrath, Germany) was used for statistical analysis. For the statistical analysis of the significant differences between all treatments and genotypes ANOVA was used with Duncan's test. Differences between mean values were considered to be significant if $p \leq 0.05$.

3. Results and discussion

Under Tm-induced ER stress, high ROS production and activation of key antioxidant enzymes such as chloroplastic superoxide dismutase or ascorbate peroxidase have been reported (Ozgur et al., 2014), suggesting the potential role of chloroplasts and chloroplastic ROS in the propagation of ROS signalling and oxidative stress leading to cell death in plants. The strong interaction between chloroplasts and ER by ROS was further confirmed by using photosynthesis inhibitors such as MV or DCMU (Ozgur et al., 2015). At the same time, the potential direct and/or indirect effects of the ER stress inducer Tm on chloroplasts and photosynthetic light responses as sources and targets of ROS remained unclear.

First, the direct effects of the widely used Tm (Watanabe and Lam, 2008), which inhibits N-glycosylation of secreted glycoproteins in the ER, on the ROS production of isolated tomato chloroplasts were investigated (Fig. 1). The application of H_2DCFDA probe is widely used to visualise ROS in plants (Fichman et al., 2019). It can react primarily with H_2O_2, but also with hydroxyl radical, superoxide and peroxynitrite in various cellular compartments and extracellularly (Dikalov and Harrison, 2014). Based on the results, Tm did not induce significant ROS production in the isolated chloroplasts (361 ± 14 A.U.) compared to the control (394 ± 19 A.U.) within 1 h. These results suggest that Tm may only have an indirect effect on chloroplasts, as suggested by the changes in chloroplastic antioxidants (Ozgur et al., 2014, 2015).

To analyse the potential role of chloroplasts in Tm-induced ER stress and UPR, intact tomato plants were treated with Tm for 24 h under light and dark conditions (Fig. 2). Based on the results, the expression of the UPR marker gene *SlBiP* was significantly induced by treatment with Tm under normal light conditions, but remained significantly lower in the dark compared to the light condition (Fig. 2A). These results suggest a potential role for chloroplasts in the activation of a more robust UPR in intact plants under normal light/dark conditions. In the activation of more significant ER stress and UPR, ROS such as H_2O_2 may be a key component It is known that large amounts of ROS can be generated by chloroplasts under normal light conditions (Foyer and Hanke, 2022). To investigate the potential role of chloroplastic H_2O_2 under Tm exposure, plants were treated in darkness in addition to normal light/dark conditions (Fig. 2B). Significant H_2O_2 levels were measured after Tm treatments under normal growth conditions compared to control plants, but H_2O_2 contents were lower in the dark (Fig. 2B). These results suggest that chloroplasts and the inhibition of photosynthetic light responses may be a source of ROS under ER stress.

To determine the role of chloroplasts in ROS production under ER stress, leaves of treated plants were analysed by confocal microscopy after H_2O_2 staining (Fig. 3). Based on the results, significant H_2O_2 production was detected in the leaves of Tm-treated plants (165 ± 22 A.U.) as compared to the control (57 ± 5.7 A.U.), which was significantly derived from chloroplasts, as confirmed by the merged images of the H_2O_2 probe and chloroplast autofluorescence (Fig. 3D–F). These and the results obtained with isolated chloroplasts confirmed that Tm treatment led to chloroplastic ROS production, but indirectly. In this process, ERO1-induced ROS (Soares et al., 2019) could be a source of initiator to provoke further rapid oxidative bursts within the cell, e.g. by chloroplasts. In addition, inhibition of the synthesis of proteins involved in PSII repair, such as FtsH2 (Dogra et al., 2019), could also lead to inhibition of photosynthetic electron flow and thus increase chloroplastic ROS production.

Secondly, if photosynthetic light responses are inhibited under ER stress, it is also an important question whether phytohormones such as SA and ET may play a role in activating plant defence responses, since the role of both phytohormones has been confirmed in ER stress and the UPR (Czékus et al., 2022, 2023), as well as in photosynthesis (Borbély et al., 2019; Poór et al., 2019b). Based on the analysis of chlorophyll fluorescence parameters in leaves of wild type (WT) and transgenic/mutant plants, the F_v/F_m parameter under ER stress did not change in the PSII after 24 h in the WT and ET-insensitive *Nr* tomato leaves (Fig. 4A). However, Tm significantly decreased F_v/F_m in SA hydroxylase-overexpressing NahG leaves as compared to the control (Fig. 4A), suggesting the protective function of SA promoting UPR (Wang et al., 2005; Nagashima et al., 2014). Lower concentrations of SA (Poór et al. 2019) and ET (Borbély et al., 2019) also did not alter F_v/F_m in tomato leaves. In addition, F_0 and F_m parameters did not change significantly following Tm and PBA treatments neither in WT nor in *Nr* plants (Fig. 4B and C). However, F_0 and F_m were significantly lower when exposed to PBA compared to Tm + PBA in NahG leaves (Fig. 4B and C). Interestingly, the q_L parameter was basically higher in *Nr* leaves and significantly lower in NahG plants compared to WT and it decreased

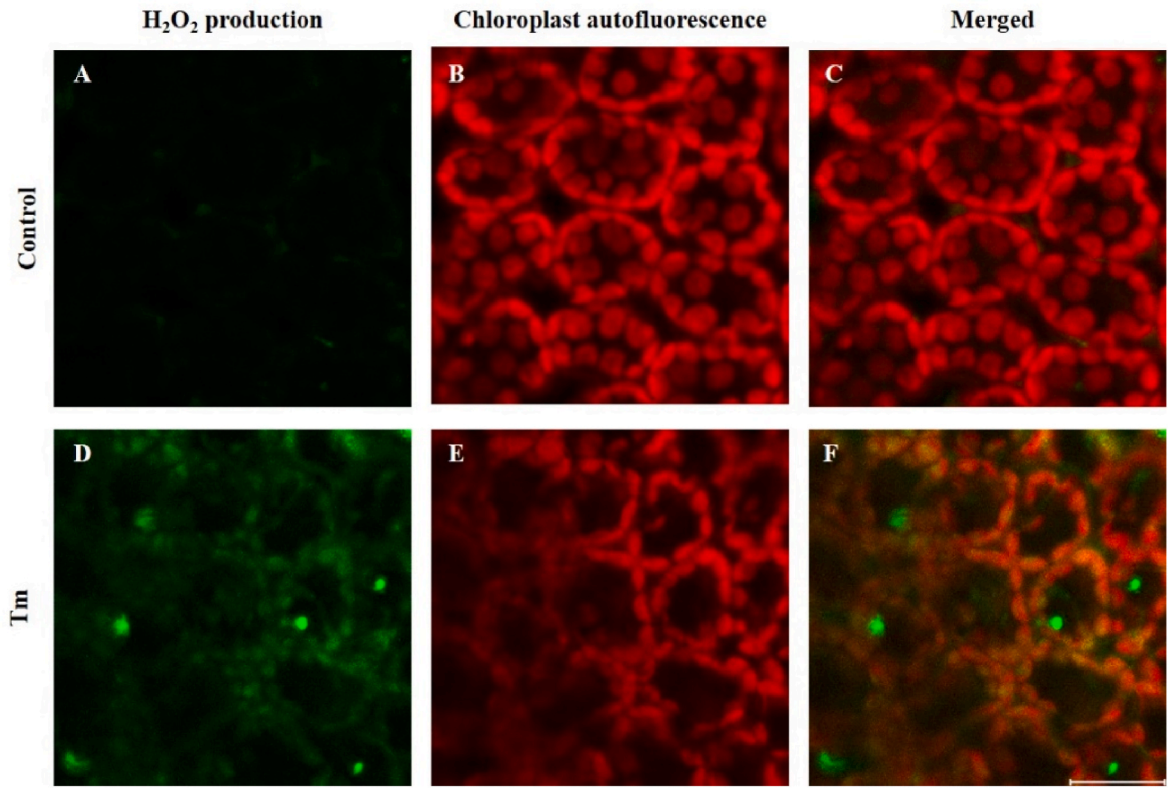

Fig. 3. The representative image of H_2O_2 production (A, D), chloroplast autofluorescence (B, E) and merged images (C, F) in leaves of wild-type (WT) plants under control conditions or after 24 h treatment with 0.5 μg mL^{-1} tunicamycin (Tm) (ruler: 20 μm).

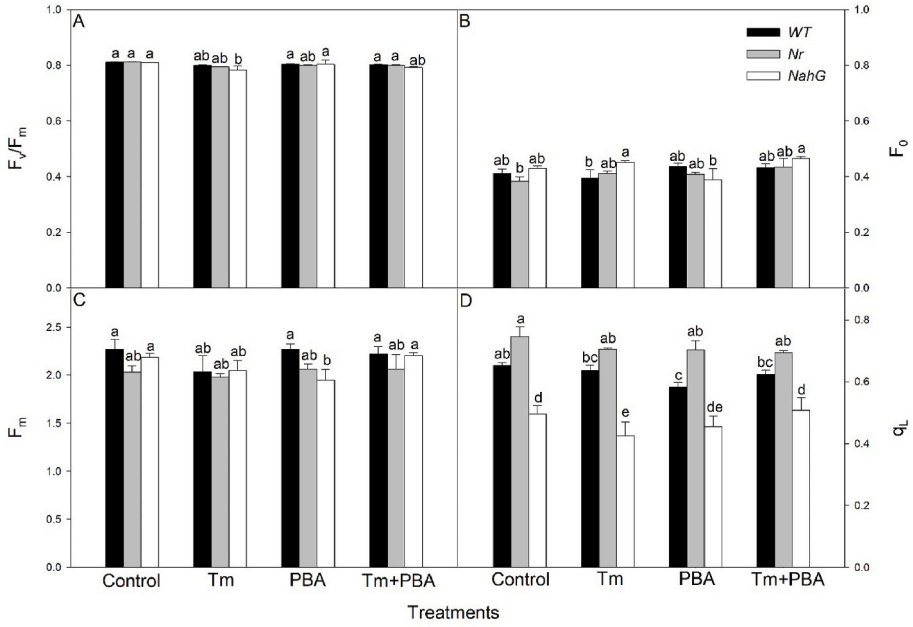

Fig. 4. Changes in the maximum quantum yield of PSII [F_v/F_m; A], the minimum fluorescence yield of the dark-adapted state [F_0; B], the maximum fluorescence in the dark-adapted state [F_m; C] and the fraction of open PSII reaction centres [q_L; D] measured in the leaves of wild-type (WT; black columns), ethylene receptor mutant (*Nr*; grey columns) and salicylic acid hydroxylase-overexpressing (NahG; white columns) tomato plants following 0.5 μg mL^{-1} tunicamycin (Tm) or 1 mM 4-phenylbutyrate (PBA) treatments alone or in combination for 24 h. Means ± SE, n = 6. Bars are denoted with different letters if $P \leq 0.05$ based on the Duncan's test.

significantly after Tm treatment in NahG leaves as compared to the control in this tomato genotype (Fig. 4D). Earlier it was also found that changes in q_L were dependent on exogenous SA concentrations (Poór et al. 2019).

The effective quantum yield of PSII [Y(II)] was significantly decreased by Tm in all tomato genotypes as compared to their respective controls, and Y(II) showed the most significant decrease in NahG tomato leaves (Fig. 5A). Interestingly, treatment with PBA increased Y(II) in the

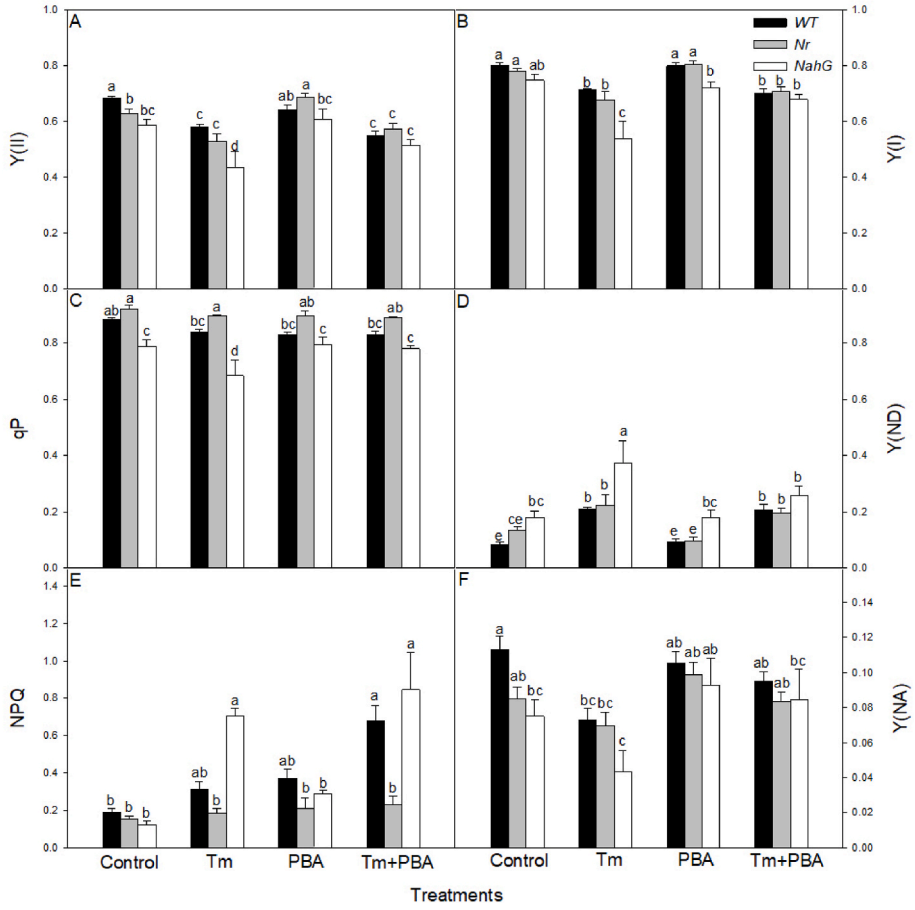

Fig. 5. Changes in the effective quantum yield of PSII [Y(II); A], the quantum yield of PSI [Y(I); B], the photochemical quenching coefficient [qP; C], the quantum yield of non-photochemical energy dissipation in PSI caused by donor side limitation [Y(ND); D], the non-photochemical quenching [NPQ; E], and the quantum yield of non-photochemical energy dissipation in PSI caused by acceptor side limitation [Y(NA); F] in the leaves of wild-type (WT; black columns), ethylene receptor mutant (*Nr*; grey columns) and salicylic acid hydroxylase-overexpressing (NahG; white columns) tomato plants following 0.5 μg mL^{-1} tunicamycin (Tm) or 1 mM 4-phenylbutyrate (PBA) treatments alone or in combination for 24 h. Means ± SE, n = 6. Bars are denoted with different letters if $P \leq 0.05$ based on the Duncan's test.

Nr genotype as compared to its control, but the decrease in Y(II) induced by Tm was not alleviated by PBA in either genotype (Fig. 5A). Similar to PSII, Y(I) of PSI also showed a significant decrease under Tm exposure not only in WT but also in *Nr* and especially in NahG leaves, in which it was significantly lower as compared to Tm-treated WT plants (Fig. 2B). Interestingly, the photochemical quenching coefficient (qP) was basically lower in NahG as compared to WT and *Nr* plants (Fig. 5C). Nevertheless, Tm decreased qP significantly only in NahG plants (Fig. 5C). These results suggest that Tm has an inhibitory effect on the function of both PSII and PSI. However, this effect was not dependent on active ET signalling, but it was dependent on SA. Inhibition of electron transport and overexcitation of PSII can result in the accumulation of ROS, causing protein damage, such as to the D1 protein in PSII (Li and Kim, 2021). To protect the photosynthetic apparatus, plants have evolved various defence strategy, such as the antioxidant system and non-photochemical quenching (NPQ). NPQ is involved in dissipating the excess energy captured by light-harvesting complex II (LHCII) and preventing the formation of ROS that would damage the photosynthetic apparatus (Miyake, 2010; Ghosh et al., 2022). NPQ was elevated by Tm and Tm + PBA in NahG plants compared to the control in this species, and the application of Tm + PBA also increased NPQ in the leaves of WT plants (Fig. 5E). At the same time, NPQ remained unchanged in *Nr* plants under Tm-induced ER stress (Fig. 5E). These results suggest that the photoprotective mechanisms via NPQ were activated by Tm and Tm + PBA independently of SA, but these were dependent on active ET signalling. In accordance, it was earlier found that treatment with ET precursor 1-aminocyclopropane-1-carboxylic acid elevated NPQ in tomato leaves (Borbély et al., 2019). The quantum yield of non-photochemical energy dissipation in PSI caused by donor side limitation [Y(ND)] in all tomato genotypes increased significantly by Tm, especially in NahG plants (Fig. 5D). Y(ND) was also significantly higher upon Tm + PBA co-treatments in all genotypes but was lower in NahG as compared to only Tm-treated transgenic plants (Fig. 5D). At the same time, the quantum yield of nonphotochemical energy dissipation in PSI caused by acceptor side limitation [Y(NA)] was decreased by Tm in all genotypes. Y(NA) was basically lower in NahG as compared to WT plants (Fig. 5F) and upon application of Tm it was significantly decreased in the leaves of both WT and NahG plants, however it remained significantly lower in NahG plants (Fig. 5F). At the same time, there were not detected significant effects of PBA on Y(NA) in any of the examined genotypes as compared to their respective controls (Fig. 5F). Based on these results, Tm application caused PSI photoinhibition and PSI acceptor side reduction depending on SA. The overreduction of the PSI acceptor side can lead to the generation of chlorophyll triplets. Moreover, accumulation of NADPH at PSI also contributes to accelerate the Mehler reaction that results in the generation of superoxide radicals. Thus, ER stress-induced ROS accumulation in the chloroplasts can lead to photoinhibition and damage to PSI (Huang et al., 2011; Zhang et al., 2014). Similar effects of exogenous SA treatments were also reported earlier (Poór et al. 2019).

Interestingly, cyclic electron flow (CEF) was only slightly increased by Tm in WT but it significantly increased in Nr leaves compared to the

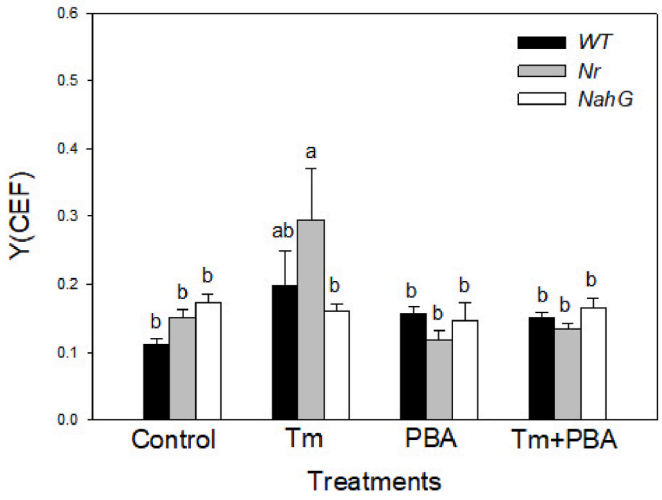

Fig. 6. Changes in the ratio of the quantum yield of cyclic electron flow (CEF) in the leaves of wild-type (WT; black columns), ethylene receptor mutant (*Nr*; grey columns) and salicylic acid hydroxylase-overexpressing (NahG; white columns) tomato plants following 0.5 μg mL^{-1} tunicamycin (Tm) or 1 mM 4-phenylbutyrate (PBA) treatments alone or in combination for 24 h. Means ± SE, n = 6. Bars are denoted with different letters if $P \leq 0.05$ based on the Duncan's test.

control (Fig. 6). At the same time, CEF showed no significant difference in NahG plants under Tm-induced ER stress (Fig. 6). Previously, higher Y(CEF) was found after exogenous SA treatments in tomato (Poór et al. 2019) which suggested the protective function of SA in defence responses of plants during the UPR. Namely, CEF helps to protect PSII against photoinhibition by creating a proton gradient across the thylakoid membrane via electron transfer from PSI to the plastoquinone (Takahashi and Murata, 2008). In addition, CEF also has a role in preventing PSI photoinhibition by diverting the excess electron flow to oxygen, that results in the accumulation of superoxide anion radicals that can be utilised under the process of water-water cycle (Ort and Baker, 2002). Thus, Tm-induced ET can contribute to photosynthetic damage via ROS production and CEF limitation. In accordance with this, Tm-induced ET caused higher ROS production in WT compared to *Nr* tomato plants, resulting in higher lipid peroxidation and electrolyte leakage under ER stress in WT tomatoes (Czékus et al., 2022).

4. Conclusions

ER stress induced significant SA and ET production in tomato leaves, phytohormones that play a crucial role in the regulation of the UPR (Czékus et al., 2022). Both phytohormones significantly influence ROS metabolism and photosynthesis in plants, where chloroplasts can be both sources and targets of ROS (Mittler et al., 2022). At the same time, the effects of ER stress on photosynthetic activity and the role of SA and ET in this process have not been elucidated. Based on our results, it can be concluded that Tm treatments significantly induce H_2O_2 accumulation in tomato leaves, originating mainly from chloroplasts. This indirect effect of Tm on chloroplast can be both an important source and target of ROS by inhibiting photosynthetic light responses. Our results firstly confirmed that, in parallel with significantly high H_2O_2 production in the chloroplast, Tm decreased the values of Y(I) and Y(II) parameters and increased Y(ND), NPQ and CEF in tomato leaves after 24 h. Nevertheless, the photosynthetic activity of the NahG genotype was more severely affected by Tm than that of WT or *Nr* leaves based on the changes in F_v/F_m, qL, Y(I), Y(II), qP, and CEF parameters. These results suggest a protective function of SA in the regulation of photosynthetic activity as determining factor of UPR and defence reactions of plants under ER stress. At the same time, the activation of photoprotective mechanisms via NPQ was independent of SA, but dependent on active ET signalling under ER stress. However, Tm-induced ET may also contribute to photoinhibition processes by reducing CEF due to its higher ratio in *Nr* mutants.

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CRediT authorship contribution statement

Nadeem Iqbal: Investigation. **Attila Ördög:** Investigation. **Péter Koprivanacz:** Investigation. **András Kukri:** Investigation. **Zalán Czékus:** Investigation. **Péter Poór:** Writing – review & editing, Writing – original draft, Investigation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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