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The role of salicylic acid on glutathione metabolism under endoplasmic reticulum stress in tomato

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ABSTRACT

The endoplasmic reticulum (ER) stress and the unfolded protein response (UPR) are highly dependent on phytohormones such as salicylic acid (SA). In this study, the effect of SA supplementation and the lack of endogenous SA on glutathione metabolism were investigated under ER stress in wild-type (WT) and transgenic SA-deficient NahG tomato (*Solanum lycopersicum* L.) plants. The expression of the UPR marker gene *SlBiP* was dependent on SA levels and remained lower in NahG plants. Exogenous application of the chemical chaperone 4-phenylbutyrate (PBA) also reduced tunicamycin (Tm)-induced *SlBiP* transcript accumulation. At the same time, Tm-induced superoxide and hydrogen peroxide production were independent of SA, whereas the accumulation of reduced form of glutathione (GSH) and the oxidised glutathione (GSSG) was regulated by SA. Tm increased the activity of glutathione reductase (GR; EC 1.6.4.2) independently of SA, but the activities of dehydroascorbate reductase (DHAR; EC 1.8.5.1) and glutathione S-transferases (GSTs; EC 2.5.1.18) were increased by Tm in a SA-dependent manner. *SlGR2, SlGGT* and *SlGSTT2* expression was activated in a SA-dependent way upon Tm. Although expression of *SlGSH1*, *SlGSTF2*, *SlGSTU5* and *SlGTT3* did not change upon Tm treatment in leaves, *SlGR1* and *SlDHAR2* transcription decreased. PBA significantly increased the expression of *SlGR1*, *SlGR2*, *SlGSTT2,* and *SlGSTT3*, which contributed to the amelioration of Tm-induced ER stress based on the changes in lipid peroxidation and cell viability. Malondialdehyde accumulation and electrolyte leakage were significantly higher in WT as compared to NahG tomato leaves under ER stress, further confirming the key role of SA in this process.

1. Introduction

Salicylic acid (SA) is one of the best-characterised phytohormones involved in a concentration- and time-dependent manner in plant defence responses, such as against biotrophs and hemibiotrophs during their biotrophic phase (Rivas-San Vicente and Plasencia, 2011; Saleem et al., 2021; Khan et al., 2022). In these processes, SA transcriptionally regulates numerous defence-related and antimicrobial genes, such as the Pathogenesis-Related (PR) genes, e.g. its marker gene *PR-1*, *PR-2* (β-1, 3-glucanase), *PR-3* (chitinase) and *PR-5* (thaumatin-like protein) (Durrant and Dong, 2004). These genes are partially regulated by the cytoplasmic non-expressor of PR gene 1 (NPR1) and various TGA transcription factors, which interact with NPR1, as important components of the SA signalling pathway (Zhang and Li, 2019). Under pathogen infection, SA-induced redox changes led to the reduction and monomerisation of NPR1 and its subsequent translocation to the nucleus (Ghanta et al., 2011). Thus, SA-induced redox changes, effects of SA on the metabolism of reactive oxygen (ROS, e.g. superoxide radical and H_2O_2) and nitrogen species (RNS, e.g. nitric oxide, peroxynitrite) are key components of both rapid and long-lasting plant defence responses such as hypersensitive response (HR) and systemic acquired resistance (SAR) (Saleem et al., 2021; Kaur et al., 2022).

ROS levels are regulated by various enzymatic and non-enzymatic antioxidants, such as glutathione and ascorbate (Noctor et al., 2012). Changes in glutathione metabolism may be at the core of SA-regulated stress responses in plants (Poór, 2020; Gallé et al., 2021; Mahajan et al., 2023). It has been reported several times that SA increases ROS levels, but in parallel elevates glutathione levels as a reducing power, which in turn can be involved in ROS scavenging under different stress conditions (Görgényi Miklósné Tari et al., 2015; Kaya et al., 2020; Saleem et al., 2021). At the same time, increment of the reduced form of

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List of abbreviations

BiP	Luminal binding protein
bZIP	Basic leucine zipper
DHAR	Dehydroascorbate reductase
ER	Endoplasmic reticulum
ERAD	ER-associated protein degradation
ERO1	ER oxidoreductase 1
ERSE	ER stress elements
ET	Ethylene
FM	Fresh mass
GGC	γ-glutamyl cyclotransferase
GGT	γ-glutamyl transpeptidase
GSH	Reduced form of glutathione
GSSG	Oxidised form of glutathione
GPOX	Glutathione peroxidases
GR	Glutathione reductase
GRX	Glutaredoxin
GSH	Glutathione synthetase
GST	Glutathione S-transferase
HR	Hypersensitive response
IRE1	Inositol-requiring enzyme 1
JA	Jasmonic acid
NahG	Salicylic acid hydroxylase-overexpressing plant
NPR1	Non-expressor of PR gene 1
5-OP	5-oxoproline
OXP	5-oxoprolinase
PBA	4-phenylbutyric acid
PDI	Protein disulfide isomerase
PERK	RNA-like ER kinase
PR	Pathogenesis-Related
RIDS	Regulated IRE1-dependent splicing
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
Tm	Tunicamycin
UPR	Unfolded protein response
S2P	Site-2TF protease
SA	Salicylic acid
SAR	Systemic acquired resistance
WT	Wild type

glutathione (GSH) is known to result in increased expression of NPR1-dependent genes such as *PR1* in tobacco (Ghanta et al., 2011). GSH is a low molecular weight tripeptide (γ-Glu-Cys-Gly) that can be oxidised by ROS in thiol-disulfide interactions (GSSG) in the Asada-Halliwell-Foyer cycle (Hasanuzzaman et al., 2019). In this cycle, GSSG can be converted back to GSH by the glutathione reductase (GR) using NADPH (Noctor et al., 2012). In addition to GR, dehydroascorbate reductase (DHAR), glutathione S-transferases (GSTs), glutaredoxins (GRXs) and glutathione peroxidases (GPOXs) also use GSH as a co-substrate and significantly influence glutathione metabolism in plant cells (Hasanuzzaman et al., 2019). Briefly, GSH is synthesised in two steps in *Arabidopsis*: the first step is catalysed by γ-EC synthetase (GSH1) in the plastids, and the second step is catalysed by glutathione synthetase (GSH2) in the cytoplasm (Noctor et al., 2012). GSH catabolism is regulated by γ-glutamyl cyclotransferase (GGC), resulting in 5-oxoproline (5-OP) formation in the cytoplasm, and 5-OP is converted to Glu by 5-oxoprolinase (OXP) (Masi et al., 2015). Another pathway of GSH catabolism is mediated by γ-glutamyl transpeptidase (GGT) enzymes in the apoplast and vacuole (Masi et al., 2015). GSH in relatively high, millimolar concentrations can be found in different cellular compartments such as cytosol, chloroplasts, mitochondria, peroxisomes, vacuole and apoplast (Noctor et al., 2012). At the same time, a key role of GSH has been reported in regulating the unfolded protein response (UPR) in the endoplasmic reticulum (ER) of *Saccharomyces cerevisiae* (Ponsero et al., 2017). In addition, there is increasing evidence that GSH transporters play a role in the exchange of GSH between the ER and the cytosol (Chakraborty et al., 2021), which may contribute to increase the reducing power of ER oxidoreductase 1 (ERO1) and subsequently protein disulfide isomerase (PDI), a thioredoxin-like protein that catalyses the formation of disulfide bonds in the ER lumen (Uzilday et al., 2017; Ozgur et al., 2018). Moreover, hydrogen peroxide is produced during disulfide bond formation by ERO1 (Ozgur et al., 2018). Thus, glutathione metabolism plays a crucial role in the regulation of the UPR and plant defence responses under various stresses.

Under stress, the damaging effects of ROS and the overproduction of defence-related and antimicrobial proteins (e.g. PRs) can disrupt and overwhelm the folding capacity and protein trafficking of the ER, promoting ER stress (Ozgur et al., 2018; Czékus et al., 2022). The accumulation of unfolded or misfolded proteins in the ER triggers the UPR to alleviate ER stress. The UPR can rescue protein homeostasis by reducing the protein load in the ER, by upregulating various genes related to defend protein synthesis, such as chaperones, and by promoting ER-associated protein degradation (ERAD) or by inducing autophagy (Sun et al., 2021). Two main arms of the UPR have been described in plants: in the first arm, inositol-requiring enzyme 1 (IRE1) mediates the nonconventional splicing of the basic leucine zipper (bZIP) 60 transcription factor in the cytosol. In the second arm, the bZIP28 and bZIP17 transcription factors regulate intramembrane sequential proteolysis (Nawkar et al., 2018; Simoni et al., 2022). During ER stress, the ER-resident chaperone, luminal binding protein (BiP) dissociates from IRE1, and the released IRE1 is dimerised in the ER membrane. After this step, the RNAse function of IRE1 is activated which leads to the splicing of bZIP60 mRNA through a process called regulated IRE1-dependent splicing (RIDS). The spliced bZIP60 then translocates to the nucleus and upregulates UPR-responsive genes (Nawkar et al., 2018). The second arm of plant UPR signalling is regulated by bZIP28/bZIP17, which can dissociate from its BiP-associated form upon ER stress and then undergo regulated intramembrane proteolysis (RIP). bZIP28 released from the ER translocates to the Golgi, where is cleaved by the site-2TF protease (S2P). This cleaved protein translocates to the nucleus and upregulates UPR downstream genes by binding to ER stress element (ERSE) cis-regulatory motifs in their promoter regions (Simoni et al., 2022). Although the UPR is well described in *Arabidopsis*, only a few cases have been investigated in other crops such as tomato (Park and Park, 2019; Czékus et al., 2022).

In addition, despite the fact that UPR is crucial in plant defence responses against pathogens, the regulatory roles of defence-related phytohormones such as SA, jasmonic acid (JA) and ethylene (ET) have been less studied (Nawkar et al., 2018). Wang et al. (2005) reported for the first time that SA regulates ER stress and the UPR during SAR in *Arabidopsis* in an NPR1-dependent manner. They found that the expression of *BiP2* was induced before the increase in *PR1* transcript accumulation Moreover, they reported that SA and tunicamycin (Tm) induced not only *BiP2* but also *BiP3* expression, however Tm increased the transcript levels of both selected chaperone-encoding genes more significantly than SA in *Arabidopsis* (Wang et al., 2005). Later, it was shown that SA induced the activation of the IRE1-bZIP60 signalling pathway to regulate *BiP3* expression in *Arabidopsis*, which was independent of NPR1 signalling (Nagashima et al., 2014). Interestingly, others found that exogenous application of SA can override the Tm-induced UPR in *Arabidopsis*, as the applied concentrations of SA reduced the expression of both *AtBiP3* and *AtbZIP60* after 3 h in root tissues, similarly to treatment with the widely-used chemical chaperone 4-phenylbutyric acid (PBA) (Hayashi and Takaiwa, 2015). Furthermore, it has been shown that SA

activates not only bZIP60 but also bZIP28 independently of NPR1, which was time-dependent (Nagashima et al., 2014). However, it has been shown that Tm-induced ER stress is regulated by NPR1 and causes a more negative redox potential of the cytosol as compared to SA (Lai et al., 2018). In this context, SA could be a crucial signalling component as it has a significant effect on ROS metabolism in a time- and concentration-dependent manner (Poór et al., 2017; Saleem et al., 2021). However, the fine-tuning role of SA during ER stress and the UPR, together with its effect on glutathione metabolism have not been investigated.

The main objective of this work is to elucidate how SA affects ROS- and glutathione metabolism under ER stress in tomato leaves. To better understand the role of SA on ROS levels and glutathione-related enzymes and coding sequences in the regulation of ER stress and the UPR, SA supplementation and an SA-deficient NahG transgenic tomato line were used in addition to ER stress modulators.

2. Materials and methods

2.1. Plant growth conditions

Wild-type (WT; Suttons, Paignton, UK) and SA hydroxylase-overexpressing (NahG; John Innes Centre, Norwich, UK) tomato seeds (*Solanum lycopersicum* L. cv. Moneymaker) were used for the experiments. The NahG transgenic tomato line constantly overexpresses the NahG gene from *Pseudomonas putida*, resulting in the degradation of SA to catechol. Seeds were germinated for 3 days in the dark and the seedlings were then transferred to perlite. After 2 weeks, healthy plants were grown in pots with nutrient solution [2 mM $Ca(NO_3)_2$, 1 mM $MgSO_4$, 0.5 mM KCl, 0.5 mM KH_2PO_4, 0.5 mM Na_2HPO_4, 0.001 mM $MnSO_4$, 0.005 mM $ZnSO_4$, 0.0001 mM $(NH_4)_6Mo_7O_{24}$, 0.01 mM H_3BO_4, and 0.02 mM Fe(III)-EDTA; pH 6] under a controlled environment (200 μmol m^{-2} s^{-1} photosynthetic photon flux density, 12/12 h light/dark period, 24/22 °C day/night temperature and relative humidity of 55–60%) (Poór et al., 2017). The nutrient solution was changed three times per week.

2.2. Treatments

6–7 week old plants were treated exogenously with 1 mM SA (Poór et al., 2017) and/or with the ER stress-inducer Tm at a concentration of 0.5 μg mL^{-1} by addition to the nutrient solution (Czékus et al., 2020). In addition, the chemical chaperone PBA was also added to the nutrient solution to estimate the direct effects of Tm in inducing ER stress (Czékus et al., 2020). All chemicals were purchased from Sigma-Aldrich (St. Louis MO, USA). In the case of all experiments, plants were treated at 9 a.m. and sampled 24 h later. Leaves were collected from the 3rd and 4th fully-expanded leaves. Each treatment was replicated three times.

2.3. RNA extraction and analysis of gene expression via quantitative real-time PCR

RNA extraction and gene expression analysis were performed based on Poór et al. (2017). Genomic DNA was eliminated by digestion with DNase I enzyme (Thermo Scientific, Waltham, MA, USA). cDNA was synthesised from single-stranded RNA using MMLV reverse transcriptase enzyme (Thermo Scientific, Waltham, MA, USA). Quantitative real-time (qRT)-PCR (Piko Real-Time qPCR System, Thermo Scientific, Waltham, MA, USA) was applied to investigate the relative expression of the selected tomato genes obtained from the National Centre for Biotechnology Information (NCBI; https://www.ncbi.nlm.nih.gov/) and Sol Genomics Network (SGN; http://solgenomics.net/) databases (Table 1). The reaction mixture for qRT-PCR analysis contained 400-400 nM forward and reverse primers, 5 μL Maxima SYBR Green qPCR Master Mix (2X) (Thermo Scientific, Waltham, MA, USA), 10 ng cDNA template and molecular biology water in a final volume of 10 μL. After an initial denaturation step for 7 min at 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 Scientific, Waltham, MA, USA) was used to analyse the data. The expression data of each gene were calculated using reference genes and the $2^{(-\Delta\Delta Ct)}$ formula.

2.4. Determination of superoxide production

Leaf tissue (0.1 g) was homogenised in 1 mL of sodium phosphate buffer (100 mM, pH 7.2) containing 1 mM sodium diethyldithiocarbamate trihydrate (SDDT). This mixture was first centrifuged (13,000 g for 15 min at 4 °C) and the supernatant (300 μL) was added to the reaction mixture containing 650 μL of 100 mM sodium phosphate buffer (pH 7.2) and 50 μL of 12 mM nitro blue tetrazolium (NBT). The absorbance of the samples was determined at 540 nm before incubation (A_0) and after the 5-min incubation period (A_S) using a spectrophotometer (KONTRON, Milano, Italy). Superoxide production was calculated using the following formula $A_{540} = A_S - A_0$ and was expressed as A_{540} (min^{-1} gFM^{-1}) (Chaitanya and Naithani, 1994).

2.5. Determination of hydrogen peroxide levels

H_2O_2 levels were measured in tomato leaves based on Velikova et al. (2000). After homogenisation of the samples (0.2 g) with 1 mL of trichloroacetic acid (TCA; 0.1%), they were centrifuged (12,000 g at 4 °C for 10 min). The supernatant in a volume of 0.25 mL was added to the reaction mixture [(0.25 mL of 10 mM phosphate buffer (pH 7.0); 0.5 mL of 1 M potassium iodide (KI)]. After incubation in the dark for 10 min, the absorbance of the samples was determined spectrophotometrically at 390 nm (KONTRON, Milano, Italy). H_2O_2 was calculated from a standard curve generated from the H_2O_2 stock solution.

Table 1
Primer pairs used for qRT-PCR.

Primer pair sequences (5'–3')			
Name and genome locus identifier of selected tomato genes		Reverse	Forward
SlPR1	Solyc01g106620	CCCCAGCACCAGAATGAAT	CATCCCGAGCACAAAACTATG
SlBiP	Solyc08g082820	GCTTCCACCAACAAGAACAAT	TCAGAAAGACAATGGGACCTG
SlGSH1	Solyc08g081010	CTGCGACCCATGAAGTATGA	TGCTTTGCTTTCCCTGTTTC
SlGR1	Solyc09g065900	TTCGGAATTGCCTTCACTTATT	GTTGTACTGAGCTTTGGTGAGC
SlGR2	Solyc09g091840	TGGTGCGATTTTGTTTTTGT	TCTGTTGGTCCTCACAAACG
SlDHAR2	Solyc05g054760	TTCCTACCTTCGTCTCATTTCTG	AACAAACATTCTGCCCATTGA
SlOXP	Solyc09g010560	CGGGATTGAAACAGACAAGG	CGGCTAACATCAGTGGAGGT
SlGGT	Solyc05g051780	GGACAGTGAACGAAATGCTG	GCAGGAGCGGAGAAGTCAT
SlGSTF2	Solyc06g009020	TGAAAGGAAGGGGGAACAAT	TTTTGCTTTGTGGTGTGCTC
SlGSTU5	Solyc01g086680	CCCTCTTGCCTAAACATCCA	TCTCCCTTTCTTCTCCTTTGC
SlGSTT2	Solyc08g080900	GGTGAGTTCGTCGGAGTTAATTT	CGAGAAGGTTGGGACATACG
SlGSTT3	Solyc08g080910	TGAAGTGGCTTGATGATACGA	TACAATCAACCCTCGCTGG

2.6. Determination of glutathione contents

Leaf samples (250 mg) were homogenised with 1 mL of 5% (w/v) TCA and then centrifuged (12,000 g at 4 °C for 20 min). The glutathione concentration was determined by an enzymatic assay using the supernatant, 100 mM phosphate buffer (pH 7.5), 1 mM 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB), 1 mM NADPH, 1 U glutathione reductase (baker's yeast; Sigma-Aldrich, St. Louis, MO, USA) and water (total glutathione assay) which was also supplemented with 4-vinylpyridine to mask GSH (GSSG assay) in 1 mL volume. Glutathione concentrations were measured spectrophotometrically at 412 nm (KONTRON, Milano, Italy) and GSH content was calculated from the difference between the concentrations of total glutathione and GSSG (Görgényi Miklósné Tari et al., 2015).

2.7. Determination of the activity of enzymes related to glutathione metabolism

Leaf tissue (250 mg) from tomato plants was homogenised in 1 mL of 100 mM phosphate buffer (pH 7.0) containing 1 mM phenylmethylsulphonyl fluoride (PMSF) and 1% (w:v) polyvinylpolypyrrolidone (PVPP) on ice, then the homogenate was centrifuged (12,000 g at 4 °C for 20 min) and used for enzymatic activity assays.

Glutathione reductase (GR; EC 1.6.4.2) activity was measured from the increase in absorbance at 412 nm using spectrophotometer (KONTRON, Milano, Italy) when 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB) was reduced by GSH generated from GSSG (Görgényi Miklósné Tari et al., 2015).

The activity of dehydroascorbate reductase (DHAR; EC 1.8.5.1) was determined by the method of Edwards and Dixon (2005). In the reaction mixture, 0.5 mM dehydroascorbate was added to 100 mM K phosphate buffer (pH 7.0) and the reaction was started with the addition of 5 mM GSH. The change in absorbance at 265 nm was calculated from the values between the first and second minutes ($\varepsilon 265 = 14.0\ mM^{-1}\ cm^{-1}$) measured spectrophotometrically (KONTRON, Milano, Italy). 1 U enzyme was expressed from the amount of ascorbate produced in 1 min.

Glutathione peroxidase (GPOX; EC 1.11.1.9) activity was measured using cumene hydroperoxide (CHP) as a substrate according to Horváth et al. (2015). The reaction mixture contained 4 mM GSH, 0.2 mM NADPH, 0.05 U GR (from baker's yeast; Sigma-Aldrich; St. Louis, MO, USA), 100 μL enzyme extract and 0.5 mM substrate in phosphate buffer (0.1 M, pH 7.0) in a total volume of 1 mL. The loss of NADPH was detected at 340 nm using a spectrophotometer (KONTRON, Milano, Italy). 1 U is the activity capable of oxidising 1 nmol NADPH in 1 min ($\varepsilon 340 = 6.22\ mM^{-1}\ cm^{-1}$).

Glutathione transferase (GST; EC 2.5.1.18) activity was measured using 1-chloro-2,4-dinitrobenzene (CDNB) and GSH as substrate (Horváth et al., 2015). After the addition of CDNB to the reaction mixture, the increase in absorbance was detected at 340 nm for 3 min using a spectrophotometer (KONTRON, Milano, Italy). One unit of GST activity is the amount of enzyme that produces 1 μmol conjugated product in 1 min ($\varepsilon 340 = 9.6\ mM^{-1}\ cm^{-1}$).

The concentration of soluble proteins in the samples was determined by the Bradford method (1976) using bovine serum albumin (BSA) as standard.

2.8. Measurement of malondialdehyde (MDA) content

Leaf samples (0.1 g) were homogenised in 1 mL of 0.1% TCA and 0.1 mL of 4% butylated hydroxytoluene (BHT). After centrifugation (11,500 g for 20 min at 4 °C), 0.5 mL of the supernatant was added into 2 mL of 0.5% thiobarbituric acid (TBA) dissolved in 20% TCA. The samples were then incubated at 98 °C for 30 min and cooled on ice. Absorbance was measured spectrophotometrically at 532 nm and 600 nm (KONTRON, Milano, Italy). Malondialdehyde (MDA) content was quantified using the extinction coefficient of 155 $mM^{-1}\ cm^{-1}$ according to Gallé et al., 2021.

2.9. Measurement of electrolyte leakage (EL)

Electrolyte leakage (EL) was measured using the method of Czékus et al. (2020). Leaf discs (1 cm^2) were placed into 20 mL of ultrapure distilled water and incubated for 2 h at room temperature in the dark. After 2 h, the electrolyte leakage was first time determined using a conductivity meter (HANNA Instruments, Woonsocket, Rhode Island, USA) (C1), then the total conductivity (C2) was also measured after incubation at 95 °C for 40 min and recooling. Finally, the relative EL [(C1/C2) x 100] in % was determined.

2.10. Statistical analysis

Results are expressed as mean ± S.E. (n = 5). Statistical analysis was performed using Sigma Plot 11.0 software (Systat Software Inc., Erkrath, Germany). Results were analysed using Duncan's multiple range test by analysis of variance (ANOVA), with differences considered to be significant at $P < 0.05$.

3. Results

3.1. The influence of SA under ER stress on the expression of SlPR1 and SlBiP genes

First, the effects of the different treatments on the expression of the SA signalling marker gene *SlPR1* and the UPR signalling marker gene *SlBiP* were analysed. In WT plants, Tm induced SA signalling after 24 h, based on the increase in *SlPR1* expression, which was not affected by the co-application of the chemical chaperone PBA in tomato leaves (Fig. 1A). Exogenous application of SA also promoted the increase in *SlPR1* transcript level alone, but further enhanced it upon co-application with Tm or PBA (Fig. 1A). The ER stress-inducer Tm slightly increased *SlPR1* expression in NahG plants, but this was negligible as compared to the other treatments (Fig. 1A).

The expression of the UPR marker gene *SlBiP* was significantly increased by Tm, which was ameliorated by co-treatment with PBA (Fig. 1B). SA itself significantly increased *SlBiP* expression, but reduced it in combination with Tm treatment as compared to application of Tm alone (Fig. 1B). The most effective treatment which reduced the Tm-induced increase in *SlBiP* transcripts was PBA (Fig. 1B). Tm-induced *SlBiP* expression was the lowest in NahG as compared to WT plants, but PBA increased *SlBiP* transcript level both alone and in combination with Tm in these transgenic plants (Fig. 1B).

3.2. The effect of SA on the ROS metabolism under ER stress

Among the ROS, Tm induced superoxide production in WT leaves, which was inhibited by PBA (Fig. 2A). SA treatments generally promoted superoxide accumulation, which was not affected by Tm but was reduced by PBA (Fig. 2A). The lowest superoxide production was detected in NahG leaves as compared to WT, but all treatments increased it in this genotype (Fig. 2A).

In the case of H_2O_2, Tm increased its level in the WT leaves as compared to the control, and PBA reduced the effects of Tm (Fig. 2B), similarly to the changes in superoxide production (Fig. 2A). SA treatment increased H_2O_2 levels in the leaves of WT plants, similarly to SA + Tm co-treatment, but PBA reduced it (Fig. 2B). In NahG leaves, no significant changes were detected between treatments, but their H_2O_2 content was generally higher as compared to WT leaves (Fig. 2B).

3.3. Interaction between SA and glutathione levels under ER stress

GSH levels were significantly increased by Tm and PBA treatments in

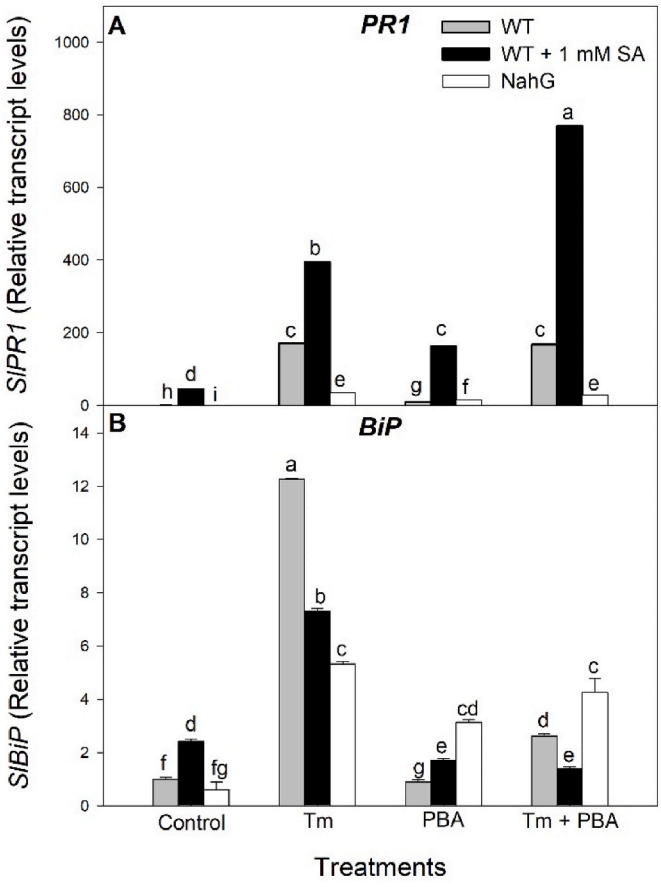

Fig. 1. Changes in relative transcript levels of *SlPR1* (A) and *SlBiP* (B) in the leaves of wild-type (WT) and NahG tomato plants after 24 h of treatment with 5 μg ml^{-1} Tm, 1 mM PBA and 1 mM SA (mean ± SE, n = 5). Columns with different letters are significantly different from each other at P ≤ 0.05 based on the Duncan's test.

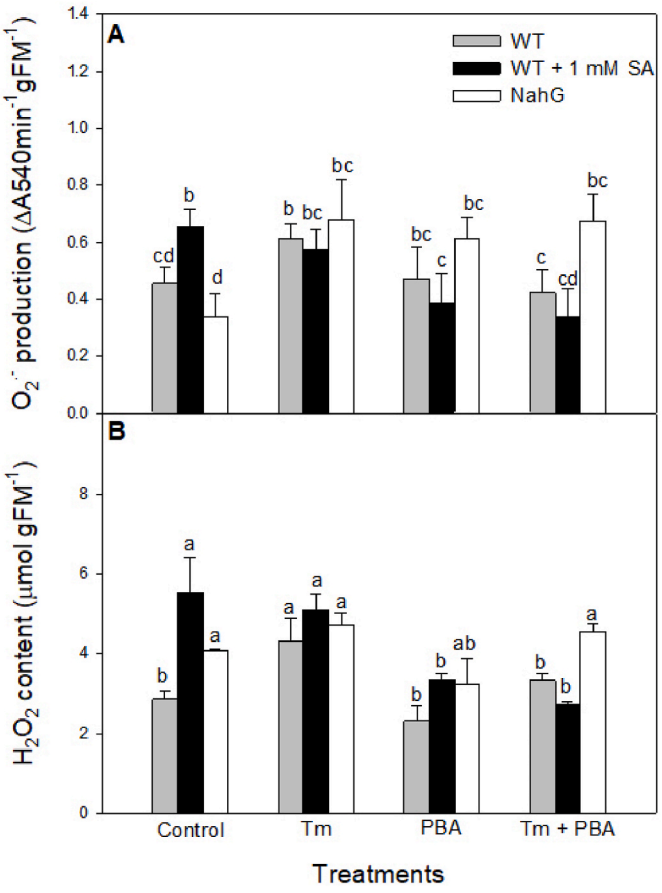

Fig. 2. Changes in superoxide production (A) and hydrogen peroxide contents (B) in the leaves of wild-type (WT) and NahG tomato plants after 24 h of treatment with 5 μg ml^{-1} Tm, 1 mM PBA and 1 mM SA (mean ± SE, n = 5). Columns with different letters are significantly different from each other at P ≤ 0.05 based on the Duncan's test.

WT leaves (Fig. 3A). Treatment with SA resulted in higher GSH accumulation as compared to Tm, but did not differ from the co-treatment with SA + Tm (Fig. 3A). The lowest GSH levels were detected in NahG leaves in general, independently of the treatments that did not result in significant changes in the leaves of transgenic plants (Fig. 3A).

GSSG levels were increased by Tm and PBA in WT tomato leaves, similarly to SA exposure (Fig. 3B). SA application resulted in the highest GSSG levels in WT plants as compared to untreated control leaves, but Tm and PBA did not affect this increase in GSSG (Fig. 3B). The lowest GSSG was detected in NahG as compared to WT leaves, but PBA treatments both alone and in combination with Tm increased it in this genotype (Fig. 3B).

3.4. SA action on the activity of enzymes related to glutathione metabolism

Based on the analysis of the activities of key enzymes involved in glutathione metabolism, GR activity was increased by Tm, which was ameliorated by PBA treatments in WT leaves (Fig. 4A). SA alone and in any combination with Tm and PBA significantly increased GR activity in WT leaves (Fig. 4A). GR activity was the highest in NahG leaves as compared to WT control as well as other treatments and did not change with any treatment (Fig. 4A).

DHAR activity was increased by Tm and was not significantly altered by co-treatment with PBA in WT leaves (Fig. 4B). SA supplementation resulted in the highest DHAR activities in WT leaves in general and it was increased significantly in all ER stress-related treatments independently of SA co-treatment (Fig. 4B). The lowest DHAR activity was exhibited by NahG leaves in general and in all treatments without any significant changes (Fig. 4B).

GPOX activity did not change significantly upon Tm and PBA treatments in WT leaves (Fig. 4C), but it was generally increased in the case of all treatments in the presence of additional SA (Fig. 4C). The highest GPOX activity was recorded in the case of PBA treatments together with SA and Tm in WT plants (Fig. 4C). The lowest GPOX enzyme activities were found in NahG leaves as compared to WT control and other treatments, which showed no changes upon none of the treatments in this tomato genotype (Fig. 4C).

GST activity was increased by Tm, but PBA application did not influence it in WT leaves (Fig. 4D). SA application also generally increased GST activity itself and in all co-treatments, especially in combination with PBA (Fig. 4D). The lowest GST activities were detected in NahG plants as compared to WT, which did not change upon none of treatments (Fig. 4D).

3.5. Changes in genes expression related to glutathione metabolism under ER stress in plants with different levels of SA

After analysing the activities of key enzymes involved in glutathione metabolism, the expression of selected genes was examined under ER stress. *SlGSH1*, which plays role in the glutathione biosynthesis was decreased by Tm in WT leaves (Fig. 5A). SA treatment also decreased the expression of *SlGSH1* as compared to the control in the WT plants, and only PBA applied together with SA increased its expression (Fig. 5A).

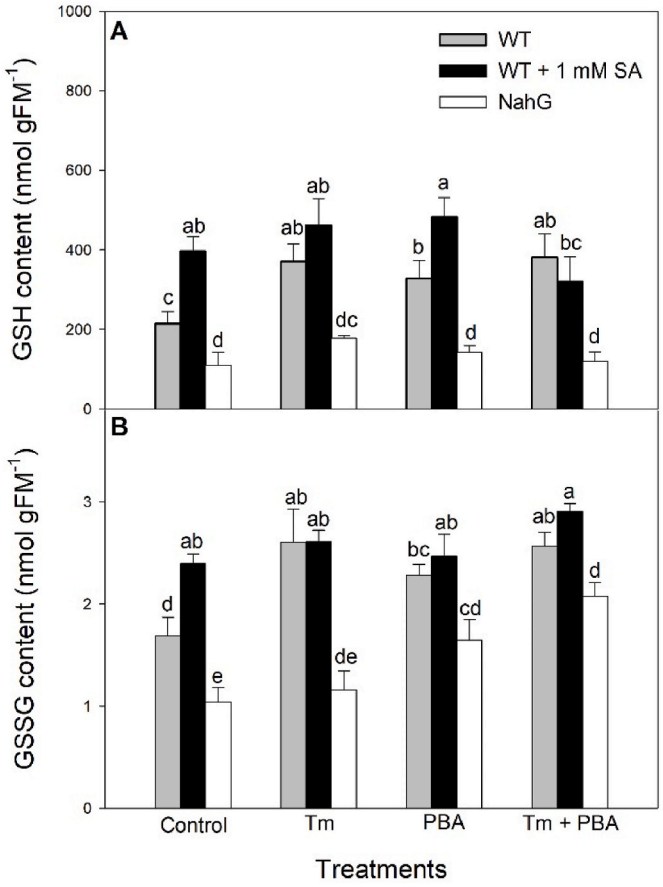

Fig. 3. Changes in the reduced glutathione (GSH; A) and oxidised glutathione (GSSG; B) contents in the leaves of wild-type (WT) and NahG tomato plants after 24 h of treatment with 5 μg ml^{-1} Tm, 1 mM PBA and 1 mM SA (mean ± SE, n = 5). Columns with different letters are significantly different from each other at P ≤ 0.05 based on the Duncan's test.

Similar changes to SA application were observed in NahG leaves, where SA deficiency decreased but PBA increased the transcript levels of *SlGSH1* (Fig. 5A).

Among the two encoding sequences of the GR genes in tomato plants, *SlGR1* did not change, but *SlGR2* expression was significantly increased by Tm in WT leaves (Fig. 5B and C). At the same time, PBA application induced the expression of both *SlGR*s and the combined Tm + PBA treatment significantly increased the accumulation of *SlGR2* transcripts (Fig. 5C). SA exposure did not alter *SlGR1* expression either alone or in combination with other ER stress-related chemicals (Fig. 5B), but in all cases increased *SlGR2* transcript levels (Fig. 5C). Similarly, *SlGR1* transcript levels did not change in NahG leaves under ER stress (Fig. 5B), but *SlGR2* was induced by Tm and PBA in this genotype, but in a lesser extent as compared to WT leaves (Fig. 5C).

SlDHAR2 expression was reduced by Tm in WT leaves and was not altered by PBA (Fig. 5D), similarly to SA supplementation (Fig. 5D). Interestingly, the expression of *SlDHAR2* was the highest in NahG leaves under control conditions as compared to WT leaves, which were suppressed by all of the chemical treatments (Fig. 5D).

Among the selected tomato sequences related to GSH catabolism, neither Tm nor PBA significantly affected the expression of *SlOXP* (Fig. 5E) and *SlGGT* (Fig. 5F) genes in WT leaves. At the same time, while SA supplementation basically decreased the expression of *SlOXP* (Fig. 5E) compared to untreated control leaves, Tm and PBA together with SA treatments increased it, which was not detected in NahG plants (Fig. 5E). In contrast, SA application basically increased the transcript levels of *SlGGT* compared to the untreated control (Fig. 5F), this increase was ameliorated by Tm but not by PBA (Fig. 5F), but the gene expression of *SlGGT* was significantly repressed and not changed by the ER stress treatments in NahG leaves (Fig. 5F).

Among the selected key GST genes in tomato, Tm treatments only increased *SlGSTT2* expression in WT leaves, similarly to PBA treatment alone (Fig. 5I). At the same time, the combined treatments of Tm and PBA resulted in significant transcript accumulation in the case of *SlGSTF2* (Fig. 5G), *SlGSTT2* (Fig. 5I) and *SlGSTT3* (Fig. 5J). SA application basically increased the expression of *SlGSTF2* (Fig. 5G) and *SlGSTT3* (Fig. 5J). SA applied together with Tm resulted in higher expression of *SlGSTT2* (Fig. 5I) and in combination with PBA in the case of *SlGSTF2* (Fig. 5G) and *SlGSTT2* (Fig. 5I). SA + Tm + PBA co-treatment resulted in significantly higher transcript accumulation of *SlGSTU5* (Fig. 5H), *SlGSTT2* (Fig. 5I) and *SlGSTT3* (Fig. 5J) in WT leaves. In NahG plants, Tm induced the expression of *SlGSTF2* (Fig. 5G) and *SlGSTT2* (Fig. 5I), but the increase in *SlGSTT2* expression was lower as compared to WT and did not change upon PBA (Fig. 5I). Transcript levels of *SlGSTU5* (Fig. 5H) and *SlGSTT2* (Fig. 5I) did not change in NahG leaves under the applied treatments.

3.6. The effect of SA on the lipid peroxidation and cell viability under ER stress

Lipid peroxidation in the leaves of WT plants increased significantly under Tm or SA exposure based on the changes in MDA content (Fig. 6A). Interestingly, there were no significant changes in MDA levels upon the combined treatments of Tm + SA in WT leaves (Fig. 6A). PBA slightly ameliorated the Tm-induced higher lipid peroxidation and significantly decreased the SA-induced elevated lipid peroxidation in WT plants (Fig. 6A). At the same time, MDA levels remained lower and unchanged in NahG leaves under ER stress (Fig. 6A).

Significant electrolyte leakage (EL) was measured in WT leaves after Tm treatment, which was slightly reduced by PBA application (Fig. 6B). At the same time, SA exposure resulted in the highest EL in WT plants, which was slightly ameliorated by Tm and especially by PBA (Fig. 6B). In NahG leaves, EL did not change significantly under ER stress (Fig. 6B).

4. Discussion

In this work, the effects of SA supplementation and endogenous SA deficiency on glutathione metabolism under ER stress in WT and NahG tomato plants were investigated using specific chemicals, the ER stress inducer Tm which inhibits N-glycosylation and the chemical chaperone PBA. Tm-induced ER stress is known to be dependent on active SA signalling (Wang et al., 2005). Our results also showed that the expression of the SA marker gene *SlPR1* induced by Tm is dependent on SA, based on the results of SA supplementation or low SA levels in NahG plants. This transgenic tomato line showed significantly lower SA levels not only under control conditions but also in response to water stress (Muñoz-Espinoza, 2015). At the same time, Tm induced higher *SlPR1* expression in tomato leaves after 24 h as compared to 1 mM SA alone. Furthermore, PBA treatments also significantly increased *SlPR1* expression, as previously reported in tomato (Czékus et al., 2022). Interestingly, both chemical treatments promoted the accumulation of not only SA but also JA and ET in leaves after 24 h (Czékus et al., 2022). However, others found only slightly increased SA level and no change in *PR1* expression in *Arabidopsis* seedlings after 24 h (Parra-Rojas et al., 2015).

Changes in expression of the molecular chaperone encoding *BiP* serve as a marker of ER stress and UPR in plants (Hayashi and Takaiwa, 2015). Treatment with Tm resulted in the highest accumulation of *SlBiP* transcripts in tomato leaves, which was reduced by the application of PBA and surprisingly by SA. It is well known that Tm induces *BiP*s expression in *Arabidopsis* in a SA-dependent manner (Wang et al., 2005; Nagashima et al., 2014), and PBA can ameliorate this increase (Czékus et al., 2022). At the same time, Hayashi and Takaiwa (2015) reported

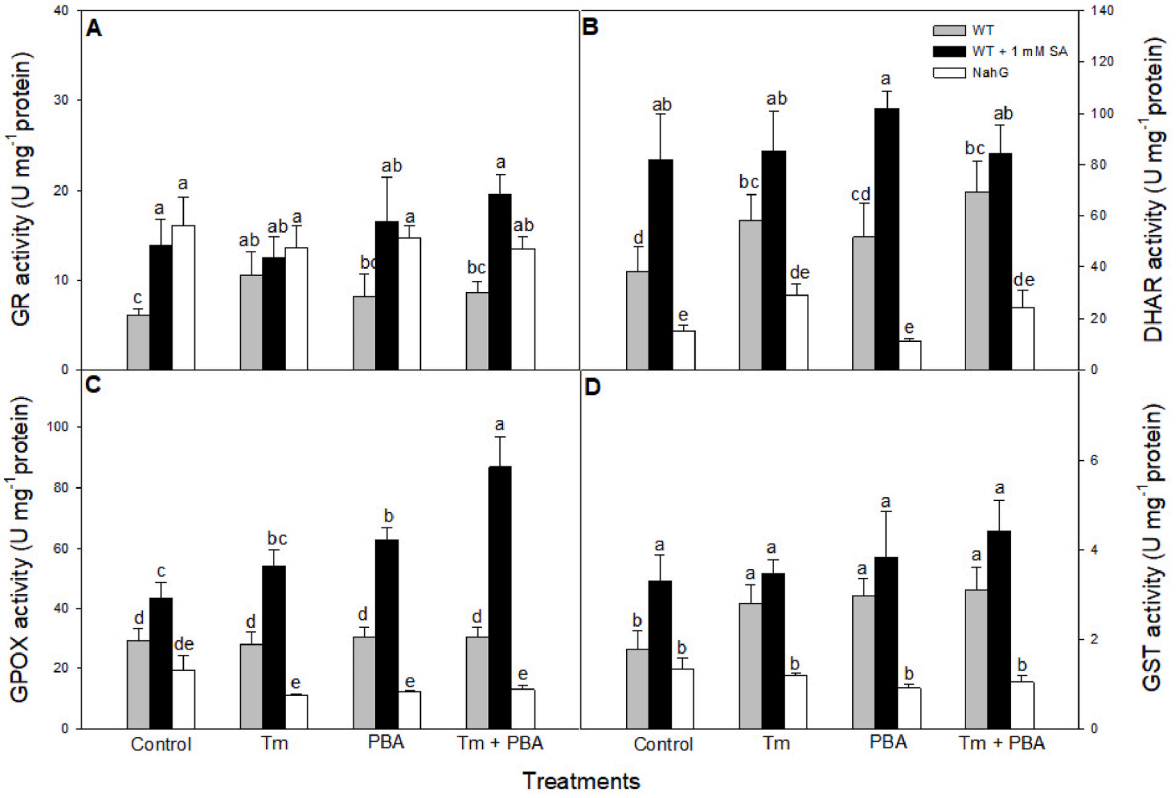

Fig. 4. Changes in the activity of glutathione reductase (GR; A), dehydroascorbate reductase (DHAR; B), glutathione peroxidase (GPOX; C), and glutathione transferase (GST; D) in the leaves of wild-type (WT) and NahG tomato plants after 24 h of treatment with 5 μg ml^{-1} Tm, 1 mM PBA and 1 mM SA (mean ± SE, n = 5). Columns with different letters are significantly different from each other at $P \leq 0.05$ based on the Duncan's test.

firstly, that exogenous SA application can override the Tm-induced UPR in *Arabidopsis*, where SA reduced the expression of *AtBiP3*, similarly to treatment with PBA (Hayashi and Takaiwa, 2015). However, the biochemical and molecular changes in the background, such as alterations in ROS metabolism, have not been elucidated up to now.

Defence responses of plants, as well as UPR are highly-dependent on the metabolism of ROS (Ozgur et al., 2015, 2018) which is regulated by SA (Poór, 2020; Saleem et al., 2021). Superoxide and H_2O_2 are among the most prominent ROS that are rapidly generated and can function as signalling components or detrimental compounds damaging cell integrity by oxidising lipids, proteins and nucleic acids (Saleem et al., 2021; Vlot et al., 2021). We measured significant superoxide and H_2O_2 production after Tm treatment in WT tomato leaves, which were not dependent on the presence of additional SA in WT or on lower SA levels in NahG plants. Ozgur et al. (2015) reported first time higher H_2O_2 levels after Tm treatment in *Arabidopsis*, which was later confirmed in tomato leaves (Czékus et al., 2020, 2022). At the same time, ROS levels were observed to depend on both active JA (Czékus et al., 2020) and ET signalling (Czékus et al., 2022) under Tm exposure. These observations can confirm that SA has little effect on Tm-induced H_2O_2 production as compared to JA or ET, but the absence of SA resulted in high superoxide accumulation under ER stress. Application of PBA together with Tm reduced this Tm-induced increase in both ROS levels, which was more effective in the case of SA supplementation and ineffective in NahG plants. It can be concluded, that the effects of PBA on ROS levels are dependent on SA, similar to JA and ET (Czékus et al., 2020, 2022). At the same time, ROS levels and their effects on the ER and on cell viability depend on antioxidant systems such as glutathione metabolism (Uzilday et al., 2017; Ozgur et al., 2018), which is regulated by SA (Poór et al., 2017; Saleem et al., 2021).

After the first report about the role of glutathione in the regulation of oxidative stress in the ER in *Saccharomyces cerevisiae* (Ponsero et al., 2017), the effects of Tm on glutathione metabolism were investigated in detail in *Arabidopsis* by Uzilday et al. (2017). At the same time, it is well known that SA, the key phytohormone in the UPR, has a significant effect on GSH levels and glutathione metabolism-related enzymes, mostly by inducing its production to promote the survival of various stresses (Görgényi Miklósné Tari et al., 2015; Kaya et al., 2020). Here, we found that both Tm and PBA promoted the accumulation of GSH in WT leaves, as well as the increase in GSSG content. Uzilday et al. (2017) also reported an increase in glutathione content in the presence of Tm in *Arabidopsis* seedlings, depending on its concentration. At the same time, until we found that SA slightly increased GSH accumulation under ER stress, the low SA concentration in NahG plants resulted in significantly lower GSH levels under Tm exposure. Similar changes were observed for GSSG, except for PBA treatments, which increased GSSG in NahG plants alone and together with Tm. Based on these results, we can conclude that Tm-induced ER stress resulted in GSH and GSSG accumulation, which is highly dependent on SA in tomato leaves. Furthermore, the effect of PBA on GSSG was less dependent on SA.

GSH/GSSG levels are regulated by several enzymes, in particular by GR and DHAR, but also by GSTs (Hasanuzzaman et al., 2019). GR and DHAR are among the key components of the Asada-Halliwell-Foyer cycle, in which H_2O_2 can be scavenged by GSH and ascorbate (Noctor et al., 2012; Hasanuzzaman et al., 2019). Under ER stress, GR activity increased significantly, as previously reported upon Tm treatment in *Arabidopsis* (Uzilday et al., 2017), but PBA reduced this Tm-induced increase in GR activity. GR is a critical enzyme under ER stress because it scavenges H_2O_2, e.g. by ERO1, and contributes to the maintenance of an optimal GSH/GSSG ratio, preventing GSH depletion. At the same time, we observed significantly higher GR activity both in the presence of additional SA and in the absence of SA using NahG tomato plants in addition to Tm treatments. These results suggest that SA participates in the regulation of GR activity, as has been reported several

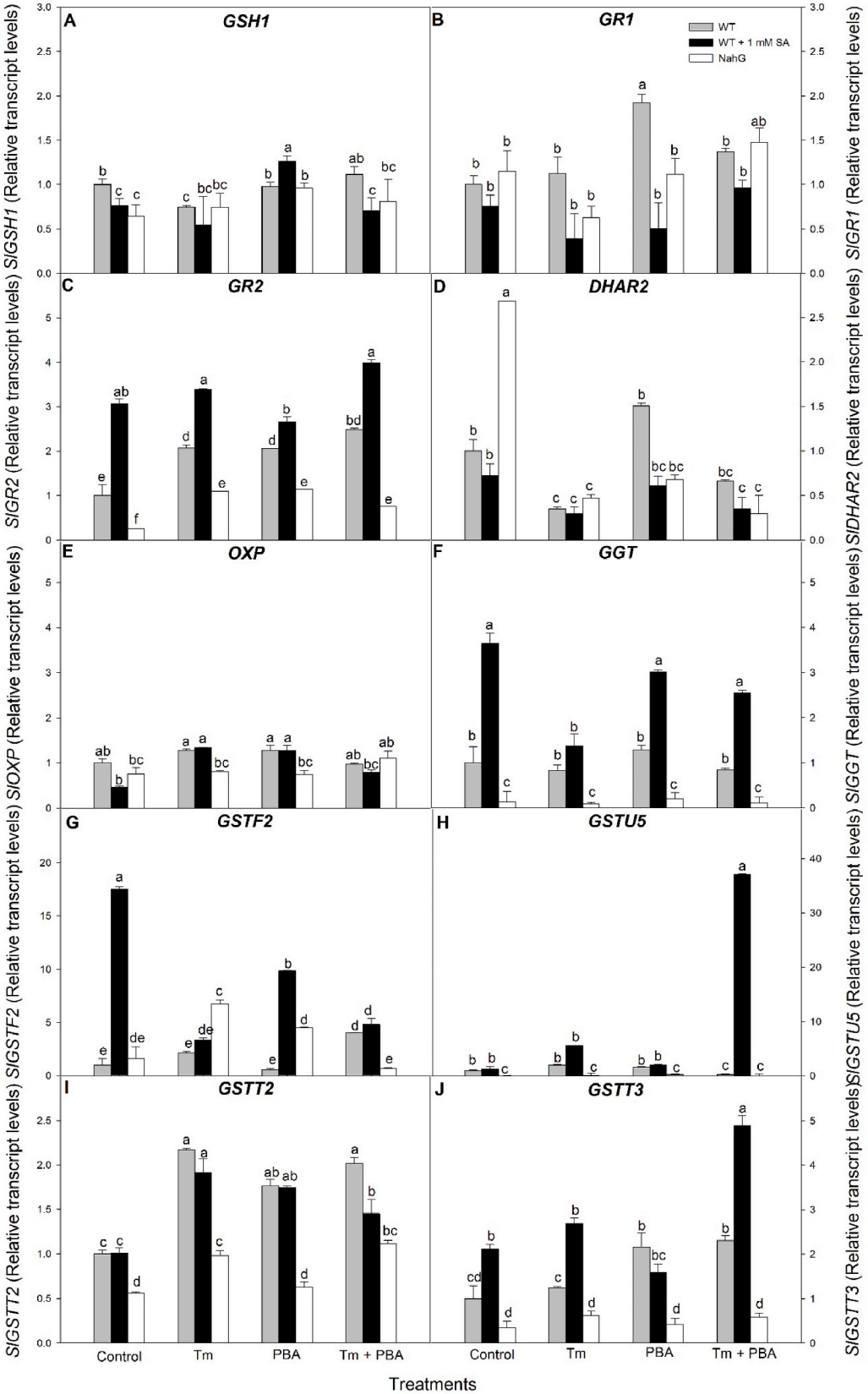

Fig. 5. Changes in the relative transcript levels of genes related to GSH biosynthesis [*SlGSH1* (A), *SlGR1* (B), *SlGR2* (C)] and consumption or degradation [*SlDHAR2* (D), *SlOXP* (E), *SlGGT* (F), *SlGSTF2* (G), *SlGSTU5* (H), *SlGSTT2* (I), and *SlGSTT3* (J)] in the leaves of wild-type (WT) and NahG tomato plants after 24 h of treatment with 5 μg ml^{-1} Tm, 1 mM PBA and 1 mM SA (mean ± SE, n = 5). Columns with different letters are significantly different from each other at $P \leq 0.05$ based on the Duncan's test.

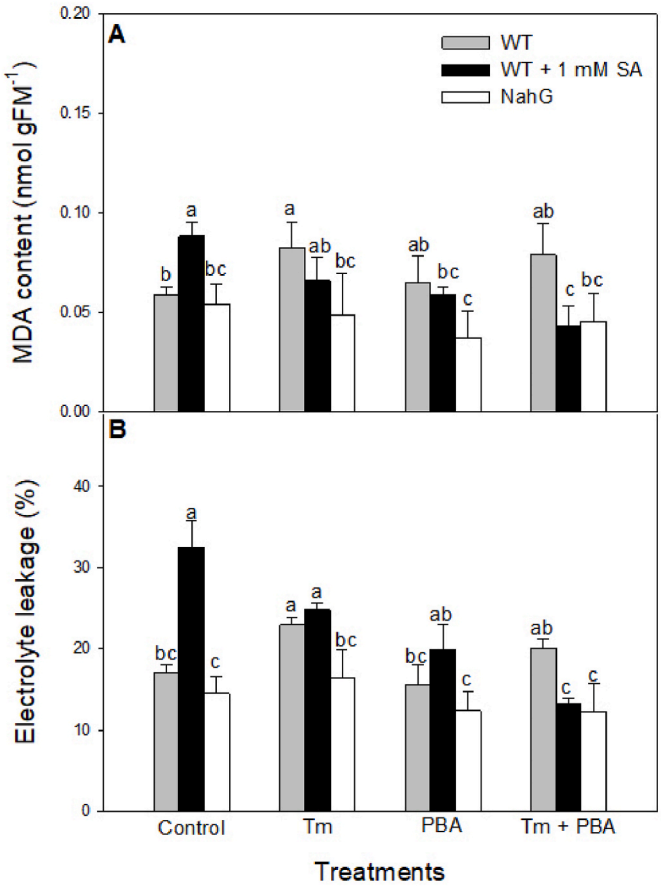

Fig. 6. Changes in the lipid peroxidation based on the malondialdehyde (MDA) content (A) and cell viability (B) based on the electrolyte leakage (EL) in the leaves of wild-type (WT) and NahG tomato plants after 24 h of treatment with 5 μg ml^{-1} Tm, 1 mM PBA and 1 mM SA (mean ± SE, n = 5). Columns with different letters are significantly different from each other at $P \leq 0.05$ based on the Duncan's test.

times (Görgényi Miklósné Tari et al., 2015; Kaya et al., 2020), but it was not investigated earlier under ER stress. Tm also increased the activity of DHAR similarly to GR. At the same time, DHAR activity was further enhanced by SA treatments as compared to Tm, but was significantly lower in NahG leaves and did not change after Tm treatment in this genotype. Although the effects of Tm and SA on DHAR activity have been demonstrated previously (Görgényi Miklósné Tari et al., 2015; Uzilday et al., 2017; Kaya et al., 2020), here we found the first time that DHAR activity is dependent on SA under ER stress. Interestingly, GPOX activity did not change after Tm or PBA treatments in the leaves of neither WT nor NahG tomatoes, as previously reported in *Arabidopsis* under ER stress (Uzilday et al., 2017), confirming the time- and organ- or genotype-dependent effects of Tm. However, SA significantly enhanced GPOX activity in WT leaves in itself or in combination with Tm and/or PBA. The effects of SA on GPOX are well-documented (Horváth et al., 2015), which is an enzyme that plays a crucial role not only in the degradation of H_2O_2, but also in the regulation of cellular redox homeostasis by maintaining the thiol/disulphide or NADPH/NADP$^+$ balance (Hasanuzzaman et al., 2019). Plant GSTs also play crucial role in the detoxification processes of cells and regulation of ROS homeostasis using GSH as co-substrate (Edwards and Dixon, 2005; Gallé et al., 2021). GST activity was not only increased by Tm, as reported in *Arabidopsis* (Uzilday et al., 2017), but also by PBA and SA in leaves of intact tomato plants. Although the effects of SA on GST induction have already been documented (Horváth et al., 2015), its role under ER stress on the regulation of GSTs is not known. Surprisingly, the activity of GSTs did not change significantly in NahG compared to WT plants or upon SA supplementation, suggesting for the first time the key role of SA in controlling GSTs under ER stress.

Related to GSH metabolism, various key genes and DNA sequences which presumably encode GSH metabolism-related genes were selected based on the literature (Islam et al., 2017; Gallé et al., 2021) and their SA-dependent expression was examined under ER stress. Surprisingly, the expression of *SlGSH1* related to glutathione biosynthesis was decreased by Tm and SA in tomato leaves, and only SA together with PBA increased slightly its transcript levels. In contrast, *GSH1* was previously found to be up-regulated by Tm in *Arabidopsis* seedlings (Uzilday et al., 2017) and by SA in tomato roots (Gallé et al., 2021), suggesting their concentration-, time- and organ-dependent effects. At the same time, the two encoding sequences of the tomato *GR* genes showed significant SA-dependent changes, especially *SlGR2* whose transcript accumulation was induced by Tm, and further increased by SA supplementation whereas it remained lower in NahG plants, suggesting the key role of SA in its induction under ER stress. Previously, both *GR*s were reported to be induced by SA in tomato roots (Gallé et al., 2021), but this is the first study which investigated the role of SA in *GR*s' induction under ER stress in leaves. Interestingly, PBA application induced the expression of both *GR*s and the combined treatments with Tm also significantly increased *SlGR2* expression suggesting the potential effect of the chemical chaperone on the *GR*s' induction. Similar to *SlGSH1*, *SlDHAR2* expression was decreased by Tm and SA in WT leaves but it was not altered by PBA or SA supplementation. However, the basal expression of *SlDHAR*2 was significantly high in NahG leaves under control conditions, which was suppressed by each treatment. In contrast, Gallé et al., 2021 reported high *SlDHAR2* expression after SA treatment in tomato roots. Based on our results, the expression of *SlOXP* and *SlGGT* was not affected by Tm in WT tomato leaves, as similarly reported in *Arabidopsis* (Uzilday et al., 2017). At the same time, our results revealed for the first time significant effects of SA on the regulation of *SlGGT*. SA supplementation significantly increased *SlGGT* transcript levels compared to the untreated control, which was ameliorated by Tm but not by PBA; in parallel, *SlGGT* was significantly repressed and did not change under ER stress in NahG leaves, suggesting the key role of SA in the regulation of apoplastic GSH catabolism regulated by GGT. Moreover, significant changes in the expression of the selected *GST*s were recorded in tomato leaves under ER stress. In higher plants, fourteen classes of GSTs can be distinguished, including tau (U), phi (F) and theta (T) (Gallé et al., 2021). In tomato, 90 GST-encoding genes with organ- and stress specificities have been identified (Islam et al., 2017). In this work, the expression of *SlGSTF2*, *SlGSTU5*, *SlGSTT2*, and *SlGSTT3* was analysed under ER stress and UPR. Based on previous data, all of them were induced by SA (Gallé et al., 2021) and upregulated under pathogen infections such as *Phytophthora infestans* and *Botrytis cinerea* in tomato leaves (Islam et al., 2017). Among these GST-encoding sequences, *SlGSTT2* showed significant expression under ER stress, which remained lower in NahG plants but its expression increased significantly in the presence of SA, suggesting its SA-dependent upregulation. At the same time, SA increased the expression of *SlGSTF2* and *SlGSTT3*, similarly as has been reported in tomato in the presence of SA (Gallé et al., 2021). The effects of PBA on the expression of *GSTs* were also observed for the first time, and a high induction of *SlGSTF2*, *SlGSTU5* and *SlGSTT3* was found when combined with Tm and/or SA, which may contribute to ameliorate the effects of ER stress.

Treatments with Tm and SA in a concentration- and time-dependent manner resulted in lipid peroxidation and thus loss of cell viability in plants (Ozgur et al., 2015; Poór et al., 2017). Here, we found that Tm induced a similar accumulation of MDA as SA, which was ameliorated by PBA as previously reported (Czékus et al., 2022), but the MDA content remained lower in NahG as compared to WT leaves, suggesting that the changes in redox regulation are induced by SA. In this genotype, ROS production as well as GSH levels and the activities of the key glutathione-related enzymes did not change under Tm-induced ER

stress. In parallel, no significant changes in EL were observed in the leaves of NahG plants after the treatments. At the same time, SA-induced EL was reduced in the case of co-treatments with Tm and especially with PBA, which may be the result of defence responses activated by both chemicals, e.g. increased GSH content and GST activity.

5. Conclusions

We can conclude that SA, as a key defence-related phytohormone, plays a role in ER stress sensing and signalling by regulating ROS and glutathione metabolism in leaves of intact tomato plants. We found that the expression of *SlBiP* was highly-dependent on SA levels upon ER stress triggered by Tm. At the same time, Tm-induced superoxide and H_2O_2 production were not dependent on SA. However, Tm-induced ER stress resulted in GSH and GSSG accumulation in the leaves of WT plants, which remained unchanged in NahG leaves, suggesting the key role of SA in this process. Furthermore, we observed that the effect of PBA on GSSG production was less dependent on the absence of SA, as increased GSSG levels were found in NahG plants when exposed to PBA. The activities of GR, DHAR and GST were increased by Tm, but only DHAR and GST were SA-dependent. GPOX activity did not change after Tm or PBA treatments in the leaves of any tomato genotypes. Among the selected genes related to glutathione metabolism, *SlGR2* and *SlGGT* are activated in an SA-dependent manner in addition to *SlGSTT2*, as their expression was lower in NahG leaves or higher in the presence of additional SA. However, the expression of *SlGSH1*, *SlGSTF2*, *SlGSTU5* and *SlGTT3* did not change under Tm treatment in WT tomato plants, whereas the relative transcript levels of *SlGSH1* and *SlDHAR2* decreased. At the same time, PBA treatment significantly increased the expression of *SlGR1*, *SlGR2*, *SlGSTT2* and *SlGSTT3*, which contributed to the amelioration of Tm-induced ER stress based on the changes in lipid peroxidation and cell viability, respectively. MDA accumulation and EL were higher in WT as compared to NahG leaves under Tm-induced ER stress in tomato leaves, further confirming the key regulatory role of SA in the redox processes.

Contribution

Conceptualization, P.P.; investigation, Z.C., D.M., P.K., K.B., M.F.L. C., and P.P.; writing—original draft preparation, P.P.; writing—review and editing, Z.C., K.B., M.F.L.C., A.G.C., and P.P.

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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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