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Role of ethylene in ER stress and the unfolded protein response in tomato (*Solanum lycopersicum* L.) plants

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ABSTRACT

The unfolded protein response (UPR) plays a significant role in the maintenance of cellular homeostasis under endoplasmic reticulum (ER) stress, which is highly dependent on the regulation of defense-related phytohormones. In this study, the role of ethylene (ET) in ER stress and UPR was investigated in the leaves of intact tomato (*Solanum lycopersicum*) plants. Exogenous application of the ET precursor 1-aminocyclopropane-1-carboxylic acid not only resulted in higher ET emission from leaves but also increased the expression of the UPR marker gene *SlBiP* and the transcript levels of the ER stress sensor *SlIRE1*, as well as the levels of *SlbZIP60*, after 24 h in tomato leaves. Using ET receptor *Never ripe* (*Nr*) mutants, a significant role of ET in tunicamycin (Tm)-induced ER stress sensing and signaling was confirmed based on the changes in the expression levels of *SlIRE1b* and *SlBiP*. Furthermore, the analysis of other defense-related phytohormones showed that the Tm-induced ET can affect positively the levels of and response to salicylic acid. Additionally, it was found that nitric oxide production and lipid peroxidation, as well as the electrolyte leakage induced by Tm, is regulated by ET, whereas the levels of H_2O_2 and proteolytic activity seemed to be independent of ET under ER stress in the leaves of tomato plants.

1. Introduction

Plants live in a complex environment in which they are constantly exposed to a wide range of stress factors. These abiotic and biotic stressors can disrupt molecular, biochemical, and physiological processes in plants and impair cellular structures, leading to growth and developmental perturbations, premature senescence, reduced fertility, and yield losses (Bari and Jones, 2009).

Under stress, the balance between the protein folding capacity and transport can be disturbed in the endoplasmic reticulum (ER), thus inducing ER stress (Nawkar et al., 2018; Pastor-Cantizano et al., 2020). The accumulation of misfolded or unfolded proteins in the lumen of the ER triggers the unfolded protein response (UPR) in cells, to mitigate ER stress. The activation of the UPR aims to save protein homeostasis by reducing the protein loading to the ER via the upregulation of various genes (e.g., those encoding chaperones) and by enhancing ER-associated protein degradation or via the induction of autophagy, which is a type of programmed cell death (PCD) (Liu and Howell, 2016). In plants, the UPR consists of two main branches, whereas a homolog of the RNA-like ER kinase (PERK) has not been identified in plants in contrast to animal systems (Pastor-Cantizano et al., 2020). The first route is regulated by the inositol-requiring enzyme 1 (IRE1), which induces the unconventional splicing of the basic leucine zipper 60 (bZIP60) transcription factor. The second branch is regulated under the control of an additional two transcription factors, bZIP28 and bZIP17. Briefly, the luminal binding protein (BiP) dissociates from IRE1 upon ER stress and is dimerized at the ER membrane. Thereafter, the RNAse function of IRE1 is activated and causes the splicing of the *bZIP60* mRNA, which is translated and upregulates UPR-related genes, thus helping protein folding and degradation (Afrin et al., 2019). bZIP28 can also be activated upon ER stress after dissociating from its basically BiP-associated form; subsequently, the basically associated BiP dissociates from bZIP28. Subsequently, bZIP28 translocates from the ER to the Golgi, where it is cleaved by the Site-2TF protease (S2P). Finally, the cleaved form of bZIP28 translocates to the nucleus and upregulates UPR-related genes by binding to the ER stress element *cis*-regulatory motifs in their promoter regions (Afrin et al., 2019). Other transcription factors related to ER stress (e.g., NAC062 and NAC089) have been identified in

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Arabidopsis (Nawkar et al., 2018). Although most of the components of the UPR have been identified in *Arabidopsis*, ER stress and the UPR have been examined only in a few cases in other crops, such as tomato, for which knowledge on the signaling components of these processes remains very limited (Park and Park, 2019).

Although the UPR is fundamental in the defense responses of plants, the potential regulatory role of defense hormones in this process remains unknown (Nawkar et al., 2018; Park and Park, 2019). Traditionally, salicylic acid (SA), jasmonic acid (JA), ethylene (ET), and abscisic acid are considered as the main defense-related or stress hormones in plants (Bari and Jones, 2009). The role of SA in ER stress and UPR is the topic that has been investigated the most in this context (Nagashima et al., 2014; Poór et al., 2019), and there is some relevant information about the role of JA in this process (Xu et al., 2019; Czékus et al., 2020). Nevertheless, the role of ET in the UPR remains mostly unclear but has been postulated as a fine-tuner of the activation of tolerance or PCD (Depaepe et al., 2021). Additionally, the role of ET may be significant in this process because perception and signaling occur at the ER (Chang, 2016).

The role of ET in the regulation of stress and defense responses is multifaceted in plants. The effects of stress-induced ET accumulation ("stress ethylene") are highly dependent on various factors, e.g., its concentration and the duration of the stress, the sensitivity of plant species and organs, and environmental factors (such as the presence or absence of light) (Poór et al., 2015; Borbély et al., 2019). The biosynthesis and action of ET have been characterized well in higher plants. The amino acid methionine is converted to *S*-adenosyl-L-methionine (SAM) by SAM-synthetase, which is a reaction that requires ATP. SAM is then converted to 1-aminocyclopropane-1-carboxylic acid (ACC) by ACC synthases (ACSs) in the cytosol, and ACC is then transformed to ET by ACC oxidase (ACO) in an oxygen-dependent reaction. Concomitantly, the levels of ACC and ET are highly regulated by transcriptional and posttranscriptional mechanisms (Chang, 2016). ET is perceived by ER membrane-localized receptors with structural similarity to bacterial two-component histidine kinases. Other downstream elements of the ET signaling, such as the negative regulator Raf-like serine/threonine kinase CONSTITUTIVE TRIPLE RESPONSE 1 (CTR1) and the positive regulator ETHYLENE INSENSITIVE 2 (EIN2), are also associated with the ER (Chang, 2016). Tomato plants possess seven ET receptors, i.e., *Sl*ETR1, *Sl*ETR2, *Sl*ETR3, *Sl*ETR4, *Sl*ETR5, *Sl*ETR6, and *Sl*ETR7, five of which were shown to bind ET with high affinity (Nascimento et al., 2021). *Sl*ETR3 is also known as *Never ripe* (*Nr*), which is the ortholog of the *Arabidopsis ETR1* receptor. *Nr* in tomato was identified as a dominant mutation in all vegetative tissues, including leaves. This mutant has functional ET biosynthesis, whereas ET signaling is blocked (Nascimento et al., 2021).

Numerous studies have demonstrated that ET production and signaling can be involved in the development of tolerance against various stress factors; however, stress-induced ET can cause autocatalytic ET synthesis, which can fortify and trigger stress-related processes and induce PCD at high concentrations (Trobacher, 2009). Oxidative stress, which comprises the rapid production and accumulation of reactive oxygen species (ROS), plays an important role in the regulation of plant defense responses and PCD. Additionally, ROS can contribute to the UPR via the modification of the protein oxidation state and the induction of the regulated or nonregulated degradation of damaged proteins (Ozgur et al., 2015). It is known that H_2O_2 and ET act synergistically in plants. ET induces H_2O_2 accumulation, which then further enhances the production of ET (Xia et al., 2015). Concomitantly, ET can also modulate ROS metabolism by regulating antioxidant enzymes (Takács et al., 2018). Furthermore, nitric oxide (NO) together with ET can promote tolerance mechanisms and initiate PCD in plants (Kolbert et al., 2019). Furthermore, the amount and timing of ET production under stress could lead to the reprogramming of defense responses or PCD mediated by SA or JA (Broekgaarden et al., 2015). It was also found that treatment with the ethylene-releasing compound ethephon (2-chloroethane phosphonic acid) resulted in a significant increase in H_2O_2 levels and NO production with parallel BiP accumulation after 24 h in a sycamore (*Acer pseudoplatanus*) cell suspension culture (Malerba et al., 2010). Additionally, it was reported that drought stress induced the expression of genes related to both ER stress signaling (e.g., *IRE1*, *bZIP60*, *bZIP28*, *bZIP17*, and *BiP*) and ET biosynthesis and signaling (e.g., *ACSs*, *ACOs*, *ETRs*, *ERSs*, and *ERFs*) after 7 days in physic nut (*Jatropha curcas*) seedlings (Zhang et al., 2015). Other researchers found that the suppression of ROS synthesis or ET perception reduced the ER stress-mediated PCD via *BiP2* in drought-stressed *Arabidopsis* roots (Mira et al., 2017). Nevertheless, the function of ET in cooperation with other defense-related phytohormones in the UPR has not been studied in detail.

In this work, the role of ET in ER stress and the UPR was investigated in intact leaves of tomato plants. Furthermore, changes in the expression of the UPR marker gene *BiP*, as well as the transcript levels of the components of the IRE1-regulated pathway, were detected after exogenous ACC treatments. The potential physiological roles of ET in ER stress sensing and the UPR was investigated in *Nr* mutant tomato plants, which are defective in ET sensing. Our experiments were focused on the role of defense-related hormones in the UPR and the ET-regulated oxidative stress and nitric oxide accumulation under ER stress.

2. Materials and methods

2.1. Plant growth conditions

Germination of wild-type (WT) and ET-receptor-mutant (*Never ripe*; *Nr*) tomato (*Solanum lycopersicum* L. cv. Ailsa Craig) seeds was conducted in the dark for 3 days. Seedlings were grown in perlite for 2 weeks and then transferred into pots and grown in hydroponic culture. Six-week-old tomato plants in hydroponic culture were used for all experiments. The nutrient solution was changed three times per week according to Czékus et al. (2020). The conditions of the controlled environment used for plant growing were as follows: photon flux density (Osram Sylvania, Danvers, MA, USA), 200 μmol m^{-2} s^{-1}; light/dark cycle, 12/12 h; day/night temperatures, 24/22 °C; and relative humidity, 55%–60%.

2.2. Treatments

Tomato plants at the five-leaf stage were treated with the ET precursor ACC at concentrations of 0.1 or 1 mM (Borbély et al., 2019) or with the ER stress-inducing agent tunicamycin (Tm) at 0.5 μg mL^{-1} via their addition into the nutrient solution (Watanabe and Lam, 2008). To estimate the direct effects of Tm on the induction of ER stress, 1 mM sodium 4-phenylbutyrate (PBA; as a widely used chemical chaperone) was also added into the nutrient solution in the presence or absence of Tm (Czékus et al., 2020). All chemicals were purchased from Sigma-Aldrich (St. Louis MO, USA). For all experiments, plants were treated at 9 A.M., with the sampling being carried out 24 h later (9 A.M. of the following day). Leaves were collected from the 3rd and 4th fully expanded leaf levels. Each experiment was repeated three times.

2.3. Detection of ET production

ET production was detected according to Poór et al. (2015) using a gas chromatograph (Hewlett-Packard, Avondale PA, USA). Specifically, 0.5 g of a leaf sample was collected into gas-tight flasks containing 0.5 mL of deionized water to provide optimal humidity to the leaves and capped with a silicone rubber stopper. ET was collected for 1 h in the dark before the increase in the wound-induced ET. After the incubation, the emitted gas with a volume of 2.5 mL was withdrawn using a gas-tight syringe and injected into the instrument. The ET emitted by plant leaves was quantified using ET standard sets.

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

RNA extraction and analysis of gene expression were performed as described by Czékus et al. (2020). The genomic DNA was eliminated by digestion with DNase I (Thermo Scientific, Waltham, MA, USA). cDNA was synthesized from single-stranded RNA by MMLV reverse transcriptase (Thermo Scientific, Waltham, MA, USA). Quantitative real-time PCR (qRT-PCR) (Piko Real-Time qPCR System, Thermo Scientific, Waltham, MA, USA) was applied to examine the relative expression of the tomato genes chosen 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 used for qRT-PCR analysis contained forward and reverse primers at 400–400 nM, 5 μL of Maxima SYBR Green qPCR Master Mix (2 ×) (Thermo Scientific, Waltham, MA, USA), 10 ng of cDNA template, and molecular-biology-grade water in a final volume of 10 μL. The cycling conditions were as follows: denaturation for 7 min at 95 °C, followed by 40 cycles of denaturation for 15 s at 95 °C and annealing/extension for 1 min at 60 °C. The software of the qRT-PCR instrument (PikoReal Software 2.2; Thermo Scientific, Waltham, MA, USA) was used to analyze the data. The expression data of each gene were calculated using reference genes and the $2^{(-\Delta\Delta Ct)}$ formula.

2.5. Measurements of SA and JA content

For measurements of SA and JA content in leaves, 1 g of leaf tissues was ground in liquid nitrogen and added to the extraction buffers. SA content was measured according to Pál et al. (2019) using high-performance liquid chromatography (Waters, Milford, MA, USA) on a reverse-phase column (Supelcosil ABZ Plus) at 25 °C and monitored with an UV/VIS detector (W474 scanning fluorescence detector, Waters, Milford, MA, USA) with excitation at 305 nm and emission at 407 nm.

JA content was determined according to Pál et al. (2019) using ultraperformance liquid chromatography (Acquity I class UPLC system; Waters, Milford, MA, USA). After the separation, mass spectrometry detection was carried out on a Waters Xevo TQXS instrument (Milford, MA, USA) equipped with a Unispray Source. For data processing, the Waters MassLynx 4.2 and TargetLynx software were used.

2.6. Determination of superoxide, hydrogen peroxide, and nitric oxide production

Leaf tissues (0.1 g) were homogenized in 1 mL of sodium phosphate buffer (100 mM, pH 7.2) containing 1 mM sodium diethyldithiocarbamate trihydrate. This mixture was first centrifuged (13,000×*g* for 15 min at 4 °C), and the supernatant (300 μL) was then added to the reaction mixture, which contained 650 μL of 100 mM sodium phosphate buffer (pH 7.2) and 50 μL of 12 mM nitroblue tetrazolium. The absorbance of the samples was determined at 540 nm before the incubation (A0) and after the 5 min incubation period (AS) using a spectrophotometer (KONTRON, Milano, Italy). Superoxide production was calculated via the following formula: $\Delta A540 = AS - A0$, and was expressed as ΔA_{540} (min^{-1} g^{-1} FM) (Czékus et al., 2020).

The production of H_2O_2 was also measured spectrophotometrically according to Takács et al. (2018). After homogenizing the leaf tissues (0.2 g) with 1 mL of trichloroacetic acid (TCA; 0.1%), samples were centrifuged (11,500×*g* at 4 °C for 20 min). The supernatant in 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). The concentration of H_2O_2 was calculated using a standard curve prepared from an H_2O_2 stock solution.

The production of NO was determined using 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM DA). Leaf disks from tomato plants were stained with 10 μM DAF-FM DA in 10 mM MES/KCl buffer (pH 6.15) under vacuum for 0.5 h in the dark at 25 °C. Subsequently, the leaf discs were rinsed twice with the buffer (MES/KCl; pH 6.15). The fluorescence intensity of the samples was monitored with a Zeiss Axiowert 200M-type fluorescence microscope (Carl Zeiss Inc.,

Table 1
Primer pairs used for qRT-PCR.

Name of tomato gene	Abbreviations used in the article	Tomato genome locus identifier	Primer pair sequences (5'−3')
ACC synthases 6	*SlACS6*	Solyc08g008100	R: 5′-AGGGTTTCCTGGATTTAGGG-3′ F: 5′-GACAACGGCATCATTGTACG-3′
ACC oxidase 4	*SlACO4*	Solyc02g081190	R: 5′-TCCAGCACCAGAGTTGATTG-3′ F: 5′-TCTCCACAGCCTTCATTGC-3′
ACC oxidase 1	*SlACO1*	Solyc07g049530	R: 5′-ATGTCCTAAGCCCGATTTGA-3′ F: 5′-CCTCCTGCGTCTGTATGAGC-3′
Allene oxide synthase	*SlAOS*	Solyc04g079730	R: 5′-CTTGGAACCGATGGACCTAA-3′ F: 5′-GGGGGAAGTCTGAAAGTATGC-3′
Allene oxide cyclase	*SlAOC*	Solyc02g085730	R: 5′-GCTATCTTCTGCCTTCCAAACT-3′ F: 5′-GTTGAGGTGCTCTGGCTCTT-3′
Basic leucine zipper 60	*SlbZIP60*	Solyc04g082890	R: 5′-TTGCTGCCGAATCTCTTTCT-3′ F: 5′-CGACTGGGAAACCTTGTGTT-3′
Chorismate mutase	*SlCM*	Solyc11g017240	R: 5′-CTGAAGAAGTGAAGGGCAAAA-3′ F: 5′-CAAGACGACGCAGGAGGTAT-3′
Ethylene response factor 1	*SlERF1*	Solyc05g051200	F: 5′-GGAACATTTGATACTGCTGAAGA-3′ R: 5′-AGAGACCAAGGACCCCTCAT-3
Inositol-requiring enzyme 1a	*SlIRE1a*	Solyc02g082470	R: 5′-CGCTTTACATCACCAGGACA-3′ F: 5′-CAGCAGCCCCTATTTCAGC-3′
Inositol-requiring enzyme 1b	*SlIRE1b*	Solyc04g082620	R: 5′-CCAGTCCTCTATTGCCTCTCA-3′ F: 5′-CCTGACCTAAATGCCCAGAT-3′
Isochorismate synthase	*SlICS*	Solyc06g071030	R: 5′-TGACCGAGGAATGTATGCTG-3′ F: 5′-CCAAGACCCTTTTCAACCAA-3′
Lipoxygenase 4	*SlLOX4*	Solyc03g122340	R: 5′-TGGCAGCACATAGGCAGTTA-3′ F: 5′-AGCGACCAGGAGTAAAGCAA-3′
Luminal binding protein	*SlBiP*	Solyc08g082820	R: 5′-TCAGAAAGACAATGGGACCTG-3′ F: 5′-GCTTCCACCAACAAGAACAAT-3′
Pathogenesis-related protein 1	*SlPR1*	Solyc01g106620	F: 5′-CATCCCGAGCACAAAACTATG-3′ R: 5′-CCCCAGCACCAGAATGAAT-3′
Phenylalanine ammonia-lyase 5	*SlPAL5*	Solyc09g007910	R: 5′-CGAGCAACACAACCAAGATG-3′ F: 5′-TCCTCCAAATGCCTCAAGTC-3′

Jena, Germany) equipped with a high-resolution digital camera (Axiocam HR, Carl Zeiss Inc., Jena, Germany). The AXIOVISION REL. 4.8 software (Carl Zeiss Inc., Munich, Germany) was used for data evaluation (Czékus et al., 2020). All chemicals were from Sigma-Aldrich (St. Louis MO, USA).

2.7. Measurement of malondialdehyde (MDA) content in leaves

Leaf samples (0.1 g) were homogenized in 1 mL of 0.1% TCA and 0.1 mL of 4% butylated hydroxytoluene. After centrifugation (11,500×*g* for 20 min at 4 °C), 0.5 mL of the supernatant was added to 2 mL of 0.5% thiobarbituric acid dissolved in 20% TCA. Samples were then incubated at 98 °C for 30 min and then cooled on ice. The absorbance was determined spectrophotometrically at 532 and 600 nm (KONTRON, Milano, Italy). MDA content was quantified on the basis of the extinction coefficient of 155 mM^{-1} cm^{-1} (Czékus et al., 2020). All chemicals were from Sigma-Aldrich (St. Louis MO, USA).

2.8. Measurement of electrolyte leakage (EL)

EL was determined according to Poór et al. (2015). Three leaf disks with a diameter of 1 cm were placed in 20 mL of ultrapure distilled water and incubated for 2 h at 25 °C in the dark. For measurements, a conductivity meter (HANNA Instruments, Woonsocket, Rhode Island, USA) was used. After the incubation, EL was determined for the first time (C1); subsequently, after incubation of the samples at 95 °C for 40 min and cooling down, the total conductivity (C2) was determined. Finally, the relative EL [(C1/C2) × 100] (%) was calculated.

2.9. Determination of protease activity in leaves

Leaves (0.25 g) were homogenized in 1 mL of ice-cold 50 mM sodium acetate buffer (pH 6.1). The samples were centrifuged (11,500×*g*, 4 °C, 10 min), and the protein content of the supernatants was determined. The protein extract (50 μL) was added to the mixture of 0.3 mL of 1% azocasein (w/v dissolved in 0.1 N NaOH) and 0.65 mL of potassium phosphate buffer (pH 5.5). Samples were incubated at 37 °C for 2 h in the dark, and the reaction was stopped by the addition of 0.3 mL of 10% (w/v) TCA. Samples were incubated on ice for 20 min and then centrifuged at 15,500×*g* at 4 °C for 10 min. The supernatants were measured spectrophotometrically at 440 nm (KONTRON, Milano, Italy). One arbitrary unit of activity is equal to the protein quantity that is necessary to increase the absorbance by 0.01 at 440 nm (Czékus et al., 2020). All chemicals were from Sigma-Aldrich (St. Louis MO, USA).

2.10. Statistical analysis

Results demonstrate means ± S.E. (n = 3). Statistical analysis was assigned using Sigma Plot 11.0 software (Systat Software Inc., Erkrath, Germany). Results were analyzed with Duncan's multiple range test by the analysis of variance where in the case of $P < 0.05$, differences were considered to be significant.

3. Results

3.1. Effects of exogenous ET precursor

Because many plant hormones, such as ET, have pivotal importance in the plant defense responses that are activated under diverse environmental stress conditions, our work focused on the role of ET in ER stress and the UPR. First, to assess the effects of ET on the regulation of ER stress and the UPR, WT tomato plants were treated exogenously with ACC as a potent inducer of ET production. We checked the efficiency of different concentrations of ACC on ET generation in leaves. Both 0.1 and 1 mM ACC treatments increased ET accumulation significantly, but the latter concentration was more efficient (Fig. 1).

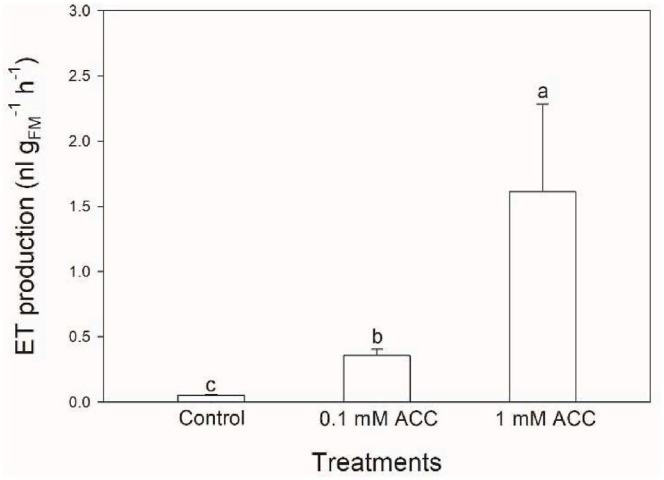

Fig. 1. Changes in the ethylene (ET) production of leaves after treatment with 0.1 or 1 mM 1-aminocyclopropane-1-carboxylic acid (ACC) in wild-type (WT) tomato plants. Results are represented as means ± SE, n = 3. Significant differences between data are signed with different letters in the case of $p < 0.05$.

After providing evidence that ACC treatment can be used as an efficient ET generator in leaves, the effects of ET on the expression of various ER stress marker genes were monitored. Exogenous ACC treatment not only increased the relative transcript accumulation of the ER stress signaling components *SlIRE1a* (Fig. 2A) and *SlIRE1b* (Fig. 2B) but also upregulated *SlbZIP60* (Fig. 2C) in a concentration-dependent manner. UPR signaling was also activated by ACC based on the increased expression of the *SlBiP* gene but only at the 1 mM concentration of ACC (Fig. 2D).

3.2. ET-dependent expression of ER stress and UPR genes

Based on these observations, we were interested in the possible role of ET in the mediation of ER stress and the UPR. For this purpose, we conducted experiments both in WT and in the ET-insensitive *Never ripe* (*Nr*) mutant tomato plants. First, the induction of the IRE1-mediated pathway of ER stress signaling was investigated after Tm treatment in plants of both genotypes. Despite the observation that the relative transcript levels of *SlIRE1a* and *SlIRE1b* were basically lower in *Nr* mutants compared with WT plants, their expression pattern showed a similar tendency after Tm treatment, which significantly elevated their expression in plants of both genotypes. Concomitantly, the expression of *SlIRE1b* was decreased in *Nr* compared with WT leaves after Tm exposure (Fig. 3). The increase in both IRE1 coding sequences was significantly decreased by the application of the chemical chaperone PBA (Fig. 3A and B). Additionally, significant *SlbZIP60* transcript accumulation was detected after Tm treatment in plants of both genotypes. Despite the observation that PBA upregulated *SlbZIP60*, it also effectively suppressed the increase in the accumulation of the *SlbZIP60* transcript induced by Tm treatment when the two chemicals were applied together (Fig. 3C). Expression of the UPR marker gene *SlBiP* was significantly induced by Tm in both tomato plant genotypes; however, it was significantly lower in *Nr* compared with WT leaves (Fig. 3D). Furthermore, this elevation in *SlBiP* expression was considerably mitigated by PBA treatment, thus confirming the specificity of Tm to induce ER stress (Fig. 3D).

3.3. Role of ET in the defense-related phytohormone network under ER stress

To unravel the specific ET-dependent role of various plant hormones in the ER stress response, quantitative real-time PCR analysis of various genes involved in the biosynthesis of ET, JA, and SA was conducted in

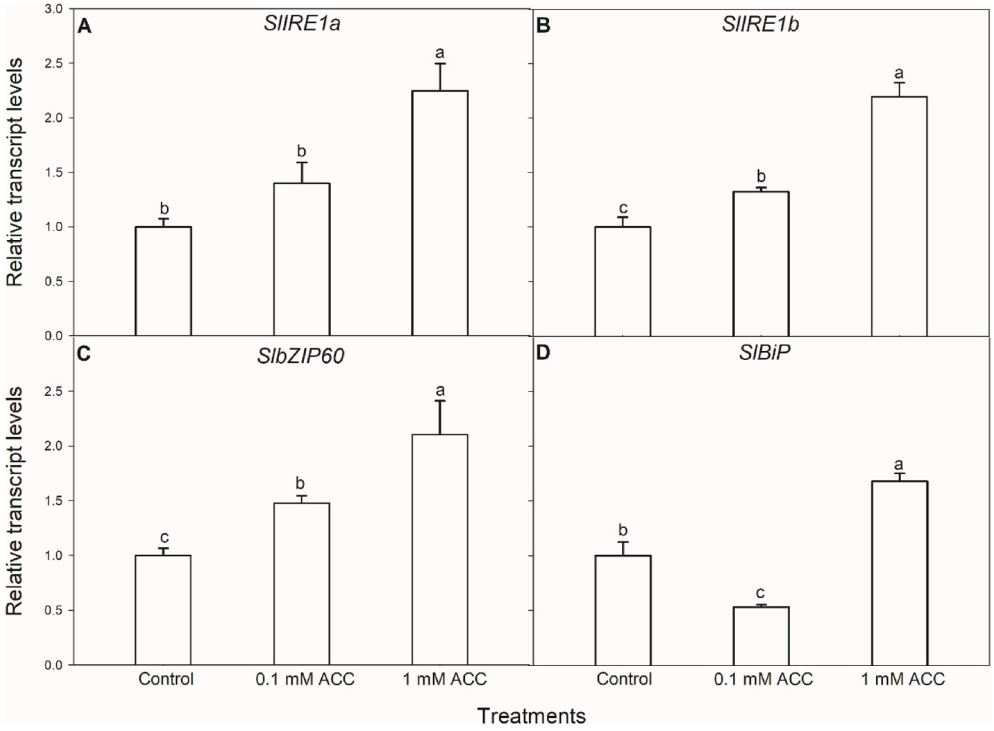

Fig. 2. Changes in the relative transcript levels of *SlIRE1a* (A), *SlIRE1b* (B), *SlbZIP60* (C) and *SlBiP* (D) after treatment with 0.1 or 1 mM 1-aminocyclopropane-1-carboxylic acid (ACC) in the leaves of wild-type (WT) tomato plants. Means ± SE, n = 3. Significant differences between data are signed with different letters in the case of $p < 0.05$.

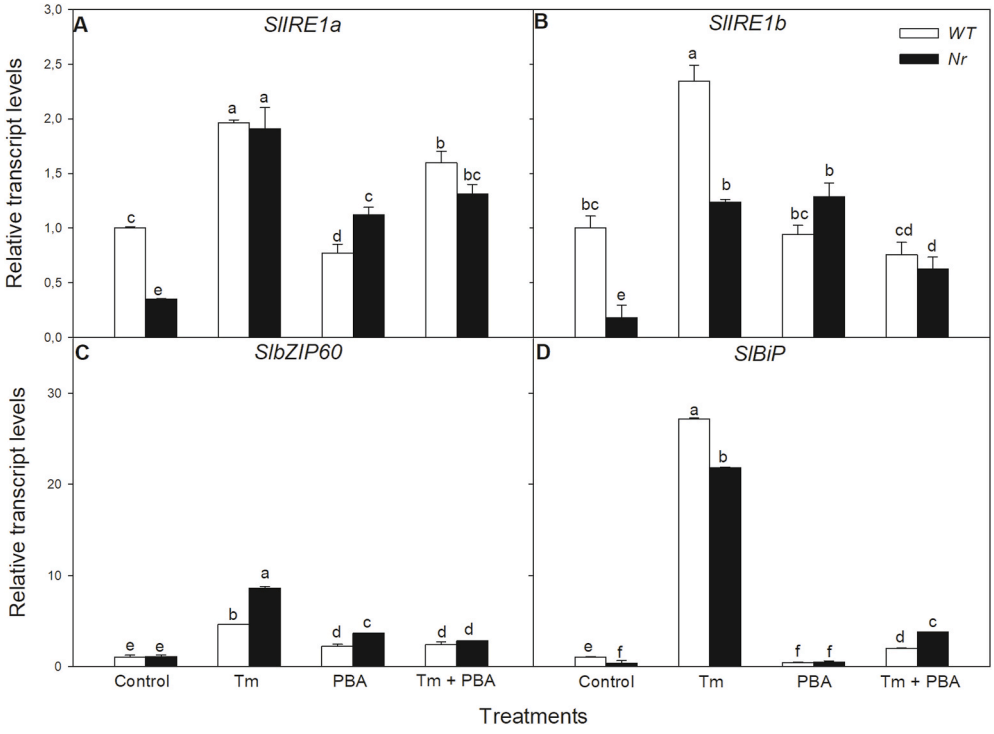

Fig. 3. Changes in the relative transcript levels of *SlIRE1a* (A), *SlIRE1b* (B), *SlbZIP60* (C) and *SlBiP* (D) after treatment with tunicamycin (Tm; 5 μg mL^{-1}) and 4-phenylbutyrate (PBA; 1 mM) either alone or in combination (Tm + PBA) in the leaves of wild-type (WT) and ET receptor mutant *Never ripe* (*Nr*) tomato plants. Means ± SE, n = 3. Significant differences between data are signed with different letters in the case of $p < 0.05$.

WT and *Nr* tomato plants. The expression of the ET biosynthesis-related *SlACS6* gene was significantly increased only in WT plants upon Tm treatment; moreover, the application of the chemical chaperone PBA did not attenuate it (Fig. 4A). In contrast to *SlACS6*, the accumulation of the *SlACO4* and *SlACO1* transcripts was significantly elevated after Tm treatment and significantly reduced by PBA, thus confirming the direct

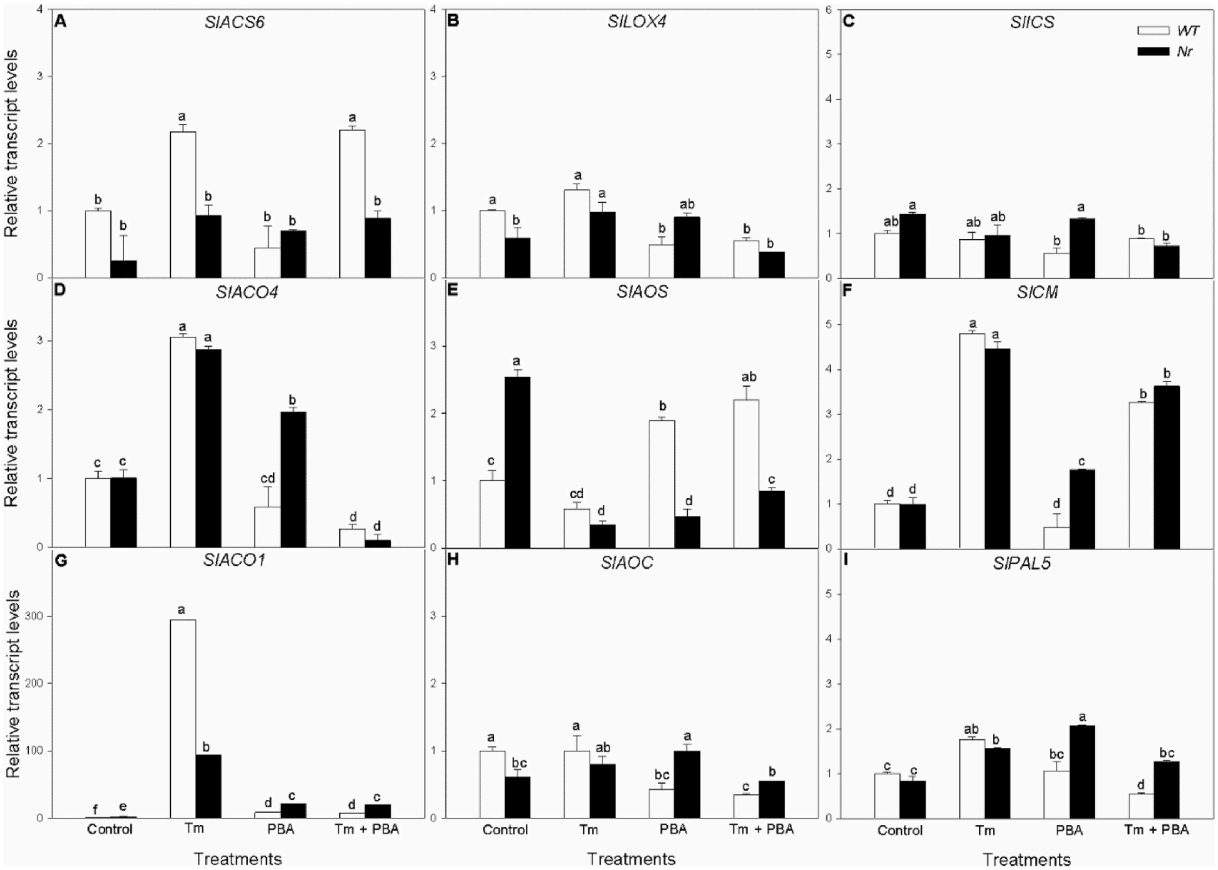

Fig. 4. Changes in the relative transcript levels of *SlACS6* (A), *SlLOX4* (B), *SlICS* (C), *SlACO4* (D), *SlAOS* (E), *SlCM* (F), *SlACO1* (G), *SlAOC* (H) and *SlPAL5* (I) after treatment with tunicamycin (Tm; 5 μg mL^{-1}) and 4-phenylbutyrate (PBA; 1 mM) either alone or in combination (Tm + PBA) in the leaves of wild-type (WT) and ET receptor mutant *Never ripe* (*Nr*) tomato plants. Means ± SE, n = 3. Significant differences between data are signed with different letters in the case of $p < 0.05$.

role of Tm in inducing ET biosynthesis at the transcriptional level. A similar tendency was observable for *SlACO4* and *SlACO1* expression in WT and *Nr* plants; however, the induction of *SlACO1* was more pronounced in the presence of active ET signaling (Fig. 4D, G).

Contrary to that observed for ET, the biosynthesis of JA was not induced under ER stress triggered by Tm at the transcriptional level. A remarkable change in the accumulation of *SlLOX4* alone was recorded upon Tm in *Nr* mutants; however, it was not detectable in WT plants (Fig. 4B). Moreover, *SlAOC* and *SlAOS* were not upregulated after Tm treatment in the examined tomato genotypes (Fig. 4E, H). Interestingly, PBA elevated the relative transcript accumulation of *SlAOS* in WT plants, whereas treatment with both Tm and PBA reduced it significantly in mutants lacking active ET signaling (Fig. 4E).

Based on our observations regarding the relative transcript accumulation of specific genes related to SA biosynthesis, it seems that ER stress-triggered SA accumulation is primarily induced through the phenylalanine ammonia lyase (PAL) rather than the isochorismate (IC) pathway. In turn, the expression of *SlICS* was unaffected by Tm in WT and *Nr* tomato leaves (Fig. 4C), whereas the transcript level of *SlCM* and *SlPAL5* was significantly increased upon Tm treatment compared with control plants in both genotypes. Furthermore, PBA suppressed the upregulation of these genes, thus providing further evidence that ER stress induced by Tm is the main cause of SA biosynthesis-related transcript accumulation (Fig. 4F, I).

Changes in the levels of the main hormones involved in defense responses under ER stress, i.e., ET, JA, and free SA, were also assessed. Tm significantly elevated the ET emission in both tomato genotypes; by contrast, the ET level was basically higher in *Nr* mutant compared with WT plants. Finally, PBA did not decrease the Tm-induced ET levels (Fig. 5A).

Besides ET, a significant accumulation of JA was observable upon Tm in WT and *Nr* tomato leaves, although PBA did not modify these changes significantly (Fig. 5B).

Interestingly, a drastic increase in the level of free SA was observed after Tm exposure in both genotypes, albeit to a lesser extent in *Nr* compared with WT leaves. Additionally, PBA reduced the TM-induced SA accumulation in *Nr* plants (Fig. 5C).

The dependence of the induction of specific hormonal response genes on active ET signaling was also monitored. The expression of the ET/JA-responsive *SlERF1* gene was significantly increased upon Tm and suppressed by PBA in WT plants, whereas in the ET-insensitive *Nr* mutants, its relative transcript abundance was basically lower and unaffected by Tm but increased by PBA treatment (Fig. 6A).

In accordance with the accumulation of free SA triggered by Tm, the expression of the SA-mediated signaling marker gene *SlPR1* was also significantly increased by Tm in WT as well as in *Nr* plants, although *SlPR1* exhibited a lower level in *Nr* leaves. The application of the chemical chaperone PBA significantly reduced the Tm-induced expression of *SlPR1* in both genotypes (Fig. 6B).

3.4. Fine regulatory role of ET in the decision to die or survive under ER stress

The ET-mediated oxidative stress under ER stress was estimated by determining the production of $O_2^{\bullet-}$ and H_2O_2 in the leaves of WT and *Nr* plants. Accumulation of $O_2^{\bullet-}$ was significantly elevated by Tm and suppressed by PBA both in WT and *Nr* leaves (Fig. 7A). A similar trend in the level of H_2O_2 was observed; however, the accumulation in H_2O_2 after Tm exposure was more pronounced in the leaves of WT vs. *Nr* plants (Fig. 7B). The trend of NO production was also similar to the

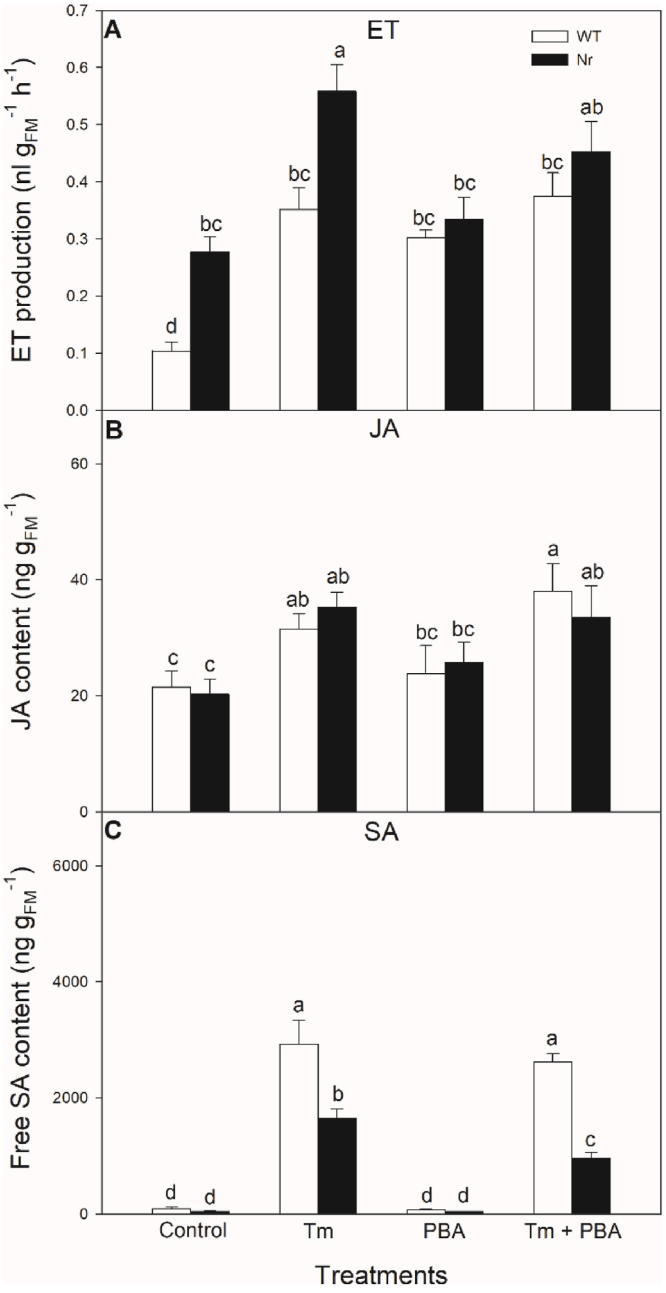

Fig. 5. Changes in ethylene (ET; A), jasmonic acid (JA; B) and free salicylic acid (SA; C) levels after treatment with tunicamycin (Tm; 5 μg mL^{-1}) and 4-phenylbutyrate (PBA; 1 mM) either alone or in combination (Tm + PBA) in the leaves of wild-type (WT) and ET receptor mutant *Never ripe* (*Nr*) tomato plants. Means ± SE, n = 3. Significant differences between data are signed with different letters in the case of $p < 0.05$.

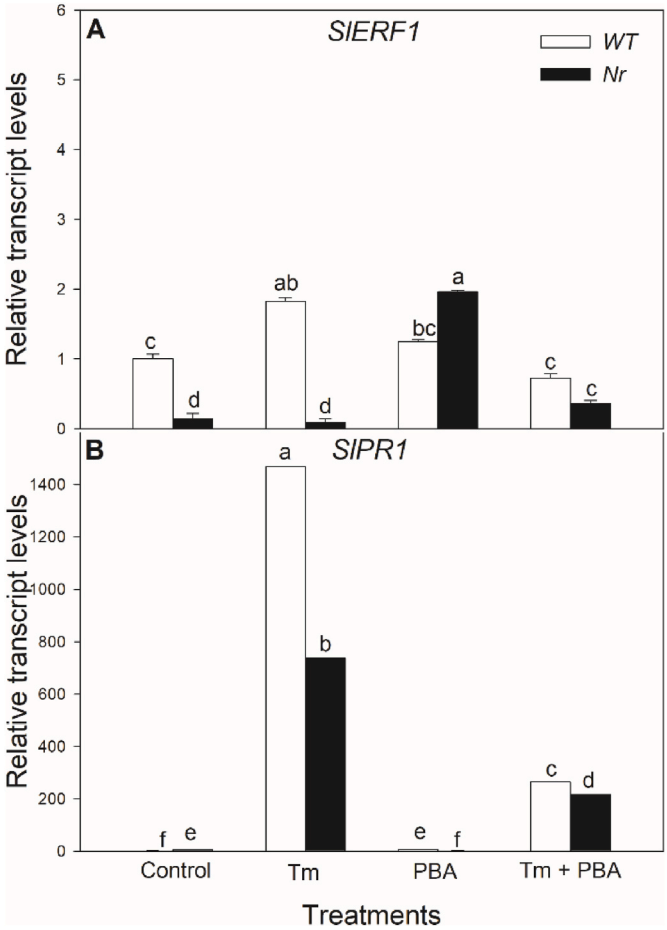

Fig. 6. Changes in the relative transcript levels of *SlERF1* (A) and *SlPR1* (B) after treatment with tunicamycin (Tm; 5 μg mL^{-1}) and 4-phenylbutyrate (PBA; 1 mM) either alone or in combination (Tm + PBA) in the leaves of wild-type (WT) and ET receptor mutant *Never ripe* (*Nr*) tomato plants. Means ± SE, n = 3. Significant differences between data are signed with different letters in the case of $p < 0.05$.

changes observed in ROS levels in the WT leaves, although Tm did not elevate NO production in *Nr* leaves (Fig. 7C). Concomitantly, treatment with PBA resulted in significant NO generation in *Nr* leaves (Fig. 7C).

Oxidative damage was also investigated on the basis of the changes in lipid peroxidation in both tomato genotypes. For this purpose, the MDA content of the leaves was determined after treatment with Tm. Interestingly, the level of MDA was significantly increased by Tm in WT tomato plants, but it was unaffected in the *Nr* mutant plants. In turn, treatment with PBA did not decrease the Tm-induced elevation in MDA levels in WT plants (Fig. 8A).

The proteolytic activity was also significantly increased upon Tm treatment in the leaves of WT plants, whereas only a slight elevation was observed in the ET-insensitive *Nr* mutants. Additionally, PBA did not significantly affect the Tm-induced increase in proteolytic activity (Fig. 8B).

Finally, the induction of cell death by Tm was determined on the basis of the level of electrolyte leakage, which was significantly increased in WT plants upon Tm treatment; however, EL remained unchanged in *Nr* mutants after PBA exposure (Fig. 8C).

4. Discussion

4.1. Exogenous ET producer induced the expression of the ER stress marker gene SlBiP

Even though the role of specific phytohormones related to the plant defense response, especially SA, has been thoroughly studied in ER stress signaling and the UPR (Nawkar et al., 2019; Park and Park, 2019; Poór et al., 2019), the role of other hormones, such as ET, in the UPR remain mostly unclear. In this study, we investigated the potential role of ET in ER stress and the UPR using intact tomato plants. The molecular and physiological effects of the ER stress-inducing agent Tm and the chemical chaperone PBA, as well as the changes in ET-dependent signaling components, were examined in WT and ET-receptor-mutant *Nr* plants.

ET plays a crucial role in the regulation of plant responses against various abiotic and biotic stressors (Trobacher, 2009). Production of ET within 24 h in the injured tissues or in the presence of abiotic stress

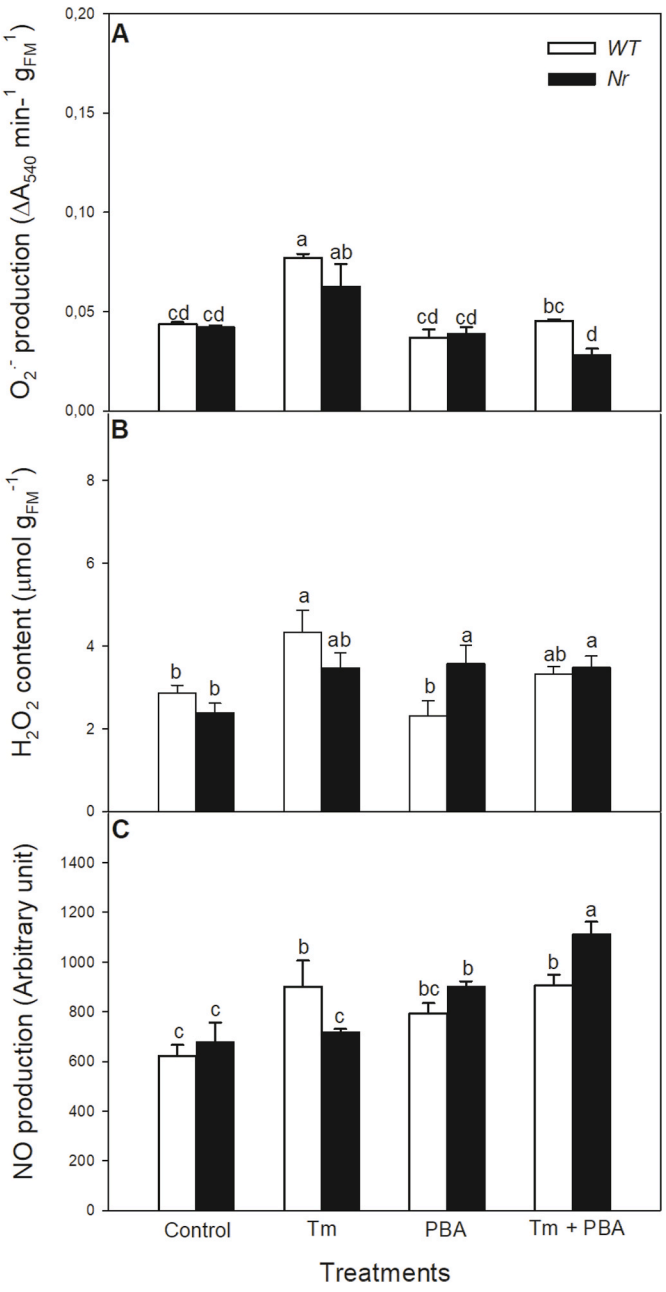

Fig. 7. Changes in the $O_2^{\cdot-}$ (A), H_2O_2 (B) and NO (C) levels after treatment with tunicamycin (Tm; 5 µg mL^{-1}) and 4-phenylbutyrate (PBA; 1 mM) either alone or in combination (Tm + PBA) in the leaves of wild-type (WT) and ET receptor mutant *Never ripe* (*Nr*) tomato plants. Means ± SE, n = 3. Significant differences between data are signed with different letters in the case of $p < 0.05$.

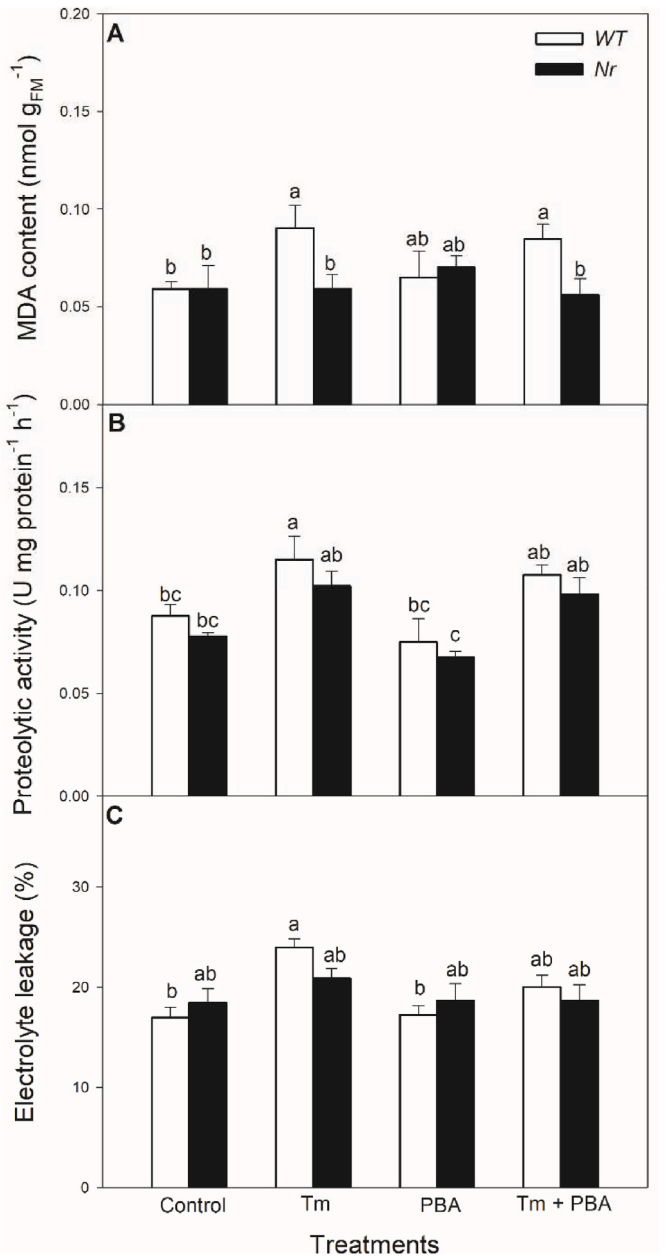

Fig. 8. Changes in the malondialdehyde (MDA) content (A), proteolytic activity (B) and electrolyte leakage (C) after treatment with tunicamycin (Tm; 5 µg mL^{-1}) and 4-phenylbutyrate (PBA; 1 mM) either alone or in combination (Tm + PBA) in the leaves of wild-type (WT) and ET receptor mutant *Never ripe* (*Nr*) tomato plants. Means ± SE, n = 3. Significant differences between data are signed with different letters in the case of $p < 0.05$.

stimuli (e.g., salt) could be vital for the regulation of tolerance responses in various plant species (Poór et al., 2015). In the case of plant–microbe interactions, ET regulates the defenses against the necrotrophic fungus *Botrytis cinerea* based on the detection of its accumulation within the first 24 h after the infection of tobacco with this pathogen (Chagué et al., 2006). Additionally, ET contributes to the preinvasion immunity of *Arabidopsis* through the regulation of bacterial flagellin (flg22)-triggered ROS production within 24 h (Mersmann et al., 2010). Based on these results, the 24 h time point after the exogenous ET precursor ACC, as well as ER stress-modulating treatments via the rooting system were selected to investigate the role of ET in the UPR in the leaves of intact plants in greater detail. The role of ET in the modulation of ROS and sugar metabolism during the induction of senescence and PCD has been studied well (Hoeberichts et al., 2007); however, the knowledge about its function in the alleviation of toxic stress effects and ER stress is far from complete (Depaepe et al., 2021). It is well known that both SA and JA play a role in the rapid coordination of the expression of defense-related genes, signaling components, and chaperones, which are required to ensure the optimal folding capacity, modification, and transport of proteins under stress conditions (Moreno et al., 2012; Nawkar et al., 2018). Concomitantly, it is also known that ET often modulates the defense signaling pathway induced by SA or JA (Broekgaarden et al., 2015). Previously, it was found that exogenous treatments with JA or SA significantly increased the expression of *BiP* genes in various plant species (Nagashima et al., 2014; Xu et al., 2019; Czékus et al., 2020). The BiP family of molecular chaperones are key

components in the processes of protein folding, assembly, translocation, and degradation (Gupta and Tuteja, 2011). In the case of treatment with the ET-releasing compound ethephon, significant BiP accumulation and ROS production were observed after 24 h in a sycamore (*Acer pseudoplatanus*) cell suspension culture (Malerba et al., 2010). Based on our results, the effects of the ET precursor ACC depended on its concentration based on the alteration in the expression of the ER stress marker gene *SlBiP*. Although treatment with both ACC concentrations applied via the rooting medium induced ACC concentration-dependent ET emission in the leaves of intact tomato plants after 24 h, the higher concentration (1 mM ACC) alone triggered a significant accumulation of *SlBiP* transcripts. Different effects of various ACC concentrations on ROS metabolism in tomato leaves and roots were observed previously (Borbély et al., 2019). Concomitantly, exogenous treatment with both ACC concentrations elevated the expression of the coding sequences of the ER stress sensor IRE1 and bZIP60 in a concentration-dependent manner in tomato leaves, suggesting that ET not only plays a role in the UPR but has also a significant effect on ER stress signaling components.

4.2. Expression of SlBiP was lower in Nr leaves, suggesting a role for ET in the UPR

These results provide evidence that exogenous ACC and the generated ET can induce ER stress and the UPR in tomato leaves. The potential role of ET in these processes was further analyzed using WT and ET-receptor-mutant (*Nr*) tomato plants. To test the potential role of ET in the UPR, plants were treated via the rooting medium with Tm, which is an inhibitor of the N-glycosylation of secreted glycoproteins, and with the chemical chaperone PBA, which are widely used to modulate ER stress in plants under laboratory conditions (Liu and Howell, 2016). It was observed that Tm significantly induced the expression of *SlBiP* in both tomato genotypes, albeit to a lower extent in *Nr* leaves, suggesting a role for ET in the UPR. The application of PBA together with Tm suppressed the accumulation of the *SlBiP* transcripts in both tomato genotypes, confirming that Tm induced the UPR. Other authors also found that 1 mM PBA was able to decrease the Tm-induced expression of BiP (Watanabe and Lam, 2008). The expression of *SlIRE1a* and *SlIRE1b* changed similarly to BiP: treatment with Tm induced the elevation of their transcript levels and the application of PBA downregulated them in the leaves. Interestingly, the transcript levels of both ER stress sensor genes were basically lower in *Nr* plants, suggesting a role for ET in the regulation of IRE and ER stress sensing, which was also confirmed by the downregulation of *SlIRE1b* and *SlBiP*. IRE1a and IRE1b have distinct functions in *Arabidopsis* under ER stress. IRE1a plays role in bZIP60 splicing under pathogen infection, whereas IRE1b is required for bZIP60 processing under Tm-induced ER stress (Moreno et al., 2012). We found that Tm significantly induced the expression of *SlbZIP60* in both tomato genotypes, albeit to a greater extent in *Nr* leaves. Concomitantly, PBA decreased the Tm-induced accumulation of the *SlbZIP60* transcript. Similarly, an increase in the levels of the *SlbZIP60* transcript was found after Tm treatment, heat stress, or tobacco rattle virus infection in tomato (Kaur and Kandoth, 2021). Moreover, the expression of *SlbZIP60* was slightly increased in the absence of active JA signaling in *jai1* tomato leaves (Czékus et al., 2020).

4.3. Active ET signaling contributed to SA accumulation and signaling under ER stress

The potential interactions between ET and other defense-related hormones, such as JA and SA, were further investigated in both tomato genotypes after treatment with the ER stress-modulating agents. First, the changes in the expression of the main marker genes related to hormone biosynthesis in tomato were examined. Treatment with Tm significantly induced the expression of all ET-related (*SlACS6*, *SlACO4*, and *SlACO1*) and several SA-related (*SlCM* and *SlPAL5*) biosynthetic genes after 24 h in the leaves of plants of both genotypes. Concomitantly, the expression of *SlACS6* and *SlACO1* was decreased in *Nr* compared with WT leaves. However, the levels of ET and SA, as well as the expression of their response genes (*SlERF1* and *SlPR1*), were also elevated in plants of both genotypes upon Tm exposure, whereas the levels of SA and the expression of *PR1* were lower in *Nr* compared with WT leaves, suggesting a role for ET in SA accumulation and signaling under ER stress and in the modulation of SA-mediated ER stress and UPR based on the expression of *SlBiP*. Although SA plays a pivotal role in ER stress (Poór et al., 2019), its interaction with ET remains unclear (Depaepe et al., 2021). The relationship between the two hormones can be both synergistic and antagonistic. SA can inhibit ET synthesis (Takács et al., 2018) but can also promote ET accumulation in tomato under salt stress (Gharbi et al., 2016). Moreover, ET can induce the accumulation of SA under *Xanthomonas campestris* infection in tomato (O'Donnell et al., 2001). It is well known that SA induced the ER capacity to promote the production, folding, and transport of defense proteins, such as PR1 (Poór et al., 2019). Furthermore, SA enhanced the expression of BiPs in *Arabidopsis* (Moreno et al., 2012; Nagashima et al., 2014). However, other authors found that Tm only slightly elevated SA levels and did not change the expression of *PR1* in *Arabidopsis* seedlings after 24 h (Parra-Rojas et al., 2015). Besides SA, Tm induced the accumulation of JA, which was not dependent on the active ET signaling at this time point in tomato. Interestingly, the expression of *SlLOX4* alone was increased upon Tm treatment in *Nr* mutants compared with WT plants; this gene was found to be highly expressed in response to methyl-jasmonate exposure or wounding (Upadhyay and Mattoo, 2018). Increasing evidence demonstrates the role of JA in the UPR (Xu et al., 2019; Czékus et al., 2020); however, the interaction between JA and ET in this process remains unclear. Nevertheless, JA and ET act mostly synergistically under pathogen infection, to regulate defense responses via ERF1, for instance, which can prevent disease progression in the case of *Botrytis cinerea* and *Fusarium oxysporum* infection (Lorenzo et al., 2003; Berrocal-Lobo and Molina, 2004). Surprisingly, the application of PBA did not alleviate the Tm-induced upregulation of ET/JA and SA levels, as well as the expression of several hormone biosynthesis-related genes (*SlACS6*, *SlAOS*, and *SlCM*) in the two tomato genotypes, suggesting that other pathways are involved in the molecular regulation of ER stress.

4.4. Nitric oxide production and lipid peroxidation were mediated by ET under ER stress

The defense responses of plants, as well as ER stress signaling, are regulated by the production and metabolism of ROS and NO (Ozgur et al., 2015; Poór et al., 2019). Among ROS, the superoxide anion ($O_2^{\cdot -}$) and hydrogen peroxide (H_2O_2) are most prominent, which form rapidly and can be harmful to cell integrity (by targeting lipids, proteins, and nucleic acids), as strongly oxidizing compounds rapidly initiate PCD together with NO at high concentrations. Moreover, H_2O_2 is the most stable ROS and can diffuse, thus acting as a signaling molecule at lower concentrations and mediating the defense responses of plants by modulating antioxidant enzymes (Xia et al., 2015; Takács et al., 2018). There is a close interaction between ET and ROS/NO metabolism (Poór et al., 2015; Borbély et al., 2019); in fact, the ET–ROS/NO interplay can be the decision point between PCD vs. survival depending on the duration and severity of the stress (Kolbert et al., 2019; Depape et al., 2021). We found that exogenous treatment with Tm induced significant superoxide and H_2O_2 production in tomato leaves and that these tendencies were less pronounced in *Nr* compared with WT leaves. Other researchers also found that Tm increased H_2O_2 production in *Arabidopsis* leaves (Ozgur et al., 2015). The application of PBA together with Tm decreased the Tm-induced increase in superoxide and H_2O_2 content in WT leaves, whereas PBA increased H_2O_2 levels in *Nr* leaves in an unknown manner. Additionally, a significantly lower NO production was observed in *Nr* leaves, which, together with the decreased superoxide

production, may contribute to survival after Tm-induced ER stress.

The accumulation of ROS upon Tm treatment or the presence of other stress factors led to lipid and protein oxidation, which can also induce the UPR (Liu and Howell, 2016). Previously, it was found that the application of Tm resulted in higher lipid peroxidation in *Arabidopsis* (Ozgur et al., 2015). Treatment with Tm also induced lipid peroxidation based on the measurements of MDA content in WT tomato leaves, which was moderated (albeit not significantly) by PBA at this time point. Concomitantly, the MDA content was significantly lower in *Nr* leaves under Tm exposure, suggesting that active ET signaling contributed to the regulation of lipid peroxidation. This process may be dependent on superoxide production, which can trigger the formation of additional reactive ROS (OH and $^1O_2$), thus contributing to the peroxidation of membrane lipids (Xia et al., 2015; Takács et al., 2018). The degradation of oxidized proteins is controlled by the action of the 26S proteasome or by other proteases (Nyström, 2005). Tm elevated the total proteolytic activity in both genotypes after 24 h; therefore, it did not afford ET-dependent regulation at this time point. However, the proteolytic activity was slightly lower in *Nr* leaves. A higher activity of proteases is an important part of PCD initiation, as well as of ER stress and immune responses in plants (Stael et al., 2019). Elevated levels of both MDA and proteolytic activity contributed to the higher degree of loss of cell viability based on the measurement of EL. In turn, EL was not changed significantly in *Nr* leaves upon the application of ER stress modulators, suggesting that Tm-induced ET plays a role in the initiation of PCD based on changes in SA, ROS, MDA, and the cell death marker EL.

5. Conclusion and future prospects

We concluded that ET, as a fine-regulator of plant defense responses, plays a role in ER stress sensing and signaling in intact tomato leaves. Exogenous application of the ET precursor ACC not only resulted in higher ET emission from leaves but also upregulated the UPR marker gene *SlBiP* and the transcript levels of the ER stress sensor *SlIRE1*, as well as the levels of *SlbZIP60* after 24 h. Using ET receptor *Nr* mutants, the positive role of ET in Tm-induced ER stress sensing and signaling was confirmed by the changes in the expression levels of *SlIRE1b* and *SlBiP*. Based on the analysis of other defense-related phytohormones, Tm-induced ET can affect positively the levels and response of SA. We found that NO production and lipid peroxidation, as well as EL induced by Tm, were regulated by ET, whereas levels of H_2O_2 and proteolytic activity seemed to be independent of ET under ER stress in the leaves of intact tomato plants at this time point.

Data availability

Tomato genes originated from the National Centre for Biotechnology Information (NCBI; https://www.ncbi.nlm.nih.gov/) and Sol Genomics Network (SGN; http://solgenomics.net/) databases. The authors affirm that all data necessary for confirming the conclusions of the article are present within the article and figures.

Author contributions

Conceptualization, P.P.; investigation, Z.C., G.SZ. and P.P.; writing—original draft preparation, Z.C. and P.P.; writing—review and editing, Z.C., G.SZ., I.T. M.I.R.K. and P.P.

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.

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