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NO is involved in JA- and H_2O_2-mediated ALA-induced oxidative stress tolerance at low temperatures in tomato

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ABSTRACT

Low temperature is a limiting factor in tomato production during early spring and winter in North China. Plants perceive low temperature through activation of cold-sensitive signaling pathways, which up-regulate cold-responsive gene expression and increase plant cold tolerance. Many studies reported that 5-aminolevulinic acid (ALA) protect plants against environmental stresses. We showed that ALA pretreatment enhanced cold-triggered oxidative stress tolerance in tomato via hydrogen peroxide (H_2O_2) signaling and subsequent cross-talk with redox signals. Here, we investigated whether ALA induced the jasmonic acid (JA) and nitric oxide (NO) signaling in response to cold stress in tomato, and evaluated the relationships between JA, NO, and H_2O_2. Tomato plants were pretreated with inhibitors of JA synthesis [salicylhydroxamic acid (SHAM) and diethyldithiocarbamic acid (DIECA)] or NO synthesis [tungstate and NG-nitro-L-arginine methyl ester (L-NAME)] as well as scavengers of NO [2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO)] or H_2O_2 [dimethylthiourea (DMTU)]. Then, these plants were treated with exogenous ALA, JA, or H_2O_2. Finally, plants were grown under normal or low temperature conditions. The results showed that ALA dramatically elevated JA levels under normal-and low-temperature conditions. Exogenous JA and H_2O_2 dramatically increased superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and glutathione reductase (GR) activities and reduced membrane lipid peroxidation. The JA synthesis inhibitors SHAM and DIECA did not significantly affect membrane lipid damage and SOD, CAT, and GR activities, compared with cold-treated plants alone. Whereas ALA significantly attenuated the inhibition effects of SHAM and DIECA. In contrast, JA and H_2O_2 mitigated the DMTU-, SHAM-, and DIECA-mediated reduction in antioxidation. ALA, JA, and H_2O_2 up-regulated *nitrate reductase* (*NR*) and *nitric oxide synthase* (*NOS*) transcript levels and NR and NOS activities, thereby triggering the NO bust. cPTIO, tungstate and L-NAME weakened JA-mediated, and essentially abolished H_2O_2-mediated antioxidase activity and mitigated membrane lipid damage. These results indicate that ALA induced H_2O_2 and JA displayed independent but synergistic roles in regulating tomato antioxidation. NO may act downstream of H_2O_2 along with JA to regulate antioxidant enzyme gene expression and increase tomato cold tolerance. In conclusion, NO is a downstream signal of H_2O_2 which cooperated with JA, mediated ALA-regulated oxidative stress tolerance under low temperatures in tomato.

1. Introduction

Plants are frequently subjected to adverse environmental conditions that affect growth and development, including biotic (pathogens and herbivores) and abiotic stresses [extreme temperatures (Li et al., 2016b), salt (Li et al., 2015), drought (Wang et al., 2016b), hypoxia (Gao et al., 2011), and heavy metals (Li et al., 2016c)]. Low temperatures are sensed by plants and increase cytoplasmic calcium in response, which is subsequently perceived by calcium sensors (Boudsocq and Sheen, 2013). This cytoplasmic signal can cross-talk with jasmonic

Abbreviations: ALA, 5-aminolevulinic acid; APX, ascorbate peroxidase; CAT, catalase; cPTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide; DAF-FM DA, diaminofluorescein-FM diacetate; DIECA, diethyldithiocarbamic acid; DMTU, dimethylthiourea; GR, glutathione reductase; H_2O2, hydrogen peroxide; JA, jasmonic acid; L-NAME, NG-nitro-L-arg methyl ester; LT, low temperature; LTA, low temperature plus 5- aminolevulinic acid; NO, nitric oxide; NOS, nitric oxide synthase; NR, nitrate reductase; REC, relative electrical conductivity; SHAM, salicylhydroxamic acid; SOD, superoxide dismutase

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acid (JA) and reactive oxygen species (ROS) signaling pathways, which trigger nitric oxide (NO) signaling, then through C-repeat binding factor (CBF)-dependent or CBF-independent pathways and subsequently regulate cold-responsive gene expression to improve plant antioxidation pathways (Zhao et al., 2017; Eremina et al., 2016; Zhu, 2016). However, sustained and/or severe low temperature ultimately causes excessive ROS accumulation, which causes DNA and protein damage, lipid peroxidation, and physiological and metabolic disorders, ultimately resulting in reduced growth and vigor (Nahar et al., 2015; Hu et al., 2017).

JA and NO have crucial roles in plant stress responses. JA is a crucial hormone signaling molecule that increases cold stress responses and cold tolerance by repressing plant growth (Eremina et al., 2016). JA treatment induces the expression of CBFs and CBF-regulated genes under chilling conditions and increases plant freezing tolerance (Hu et al., 2013; Wang et al., 2016a). JA also enhances cold-acclimation-induced freezing tolerance by modulating the biosynthesis of secondary metabolites via CBF-independent pathways (Hu et al., 2013). NO is a critical signaling molecule giving responses under biotic and abiotic stresses (Domingos et al., 2015; Tian et al., 2007; Xie et al., 2013, 2014). NO could activate downstream signaling molecules, such as abscisic acid and mitogen-activated protein kinases (MPKs, Lv et al., 2018, 2017), cold-responsive gene expression (Puyaubert and Baudouin, 2014), and proline accumulation (Zhao et al., 2009), to adapt chilling stress. Also, NO could improve the plant stress tolerance via upregulating antioxidase genes expression and the activities of enzymes (Cui et al., 2011).

Most studies of plant NO production examine nitric oxide synthase (NOS)-like enzymes (Gupta et al., 2011) and/or nitrate reductase (NR) (Chen et al., 2016; Wilson et al., 2008; Zhao et al., 2009). NOS converts L-arginine to L-citrulline and NO in animals (Wendehenne et al., 2001). Several orthologous NOS-like enzymes have been identified in plants (Gupta et al., 2011), although they have not been extensively characterized. NR-dependent release of NO in plants can be triggered by hormones (Bright et al., 2006), hydrogen peroxide (H_2O_2) (Sun et al., 2018), and elicitors (Sun et al., 2014; Reda et al., 2018). H_2O_2 is a type of ROS (Reczek and Chandel, 2015; Mittler, 2017), which has a key role in plant cold stress resistance. Zhou et al. (2012) reported that the apoplast H_2O_2 was crucial for tomato cold acclimation-induced cold tolerance. Zhang et al. (2016) showed that H_2O_2 triggered both transcriptional regulation and antioxidation reactions in rice, in response to cold stress. H_2O_2 was also involved in brassinosteroids enhanced tomato cold tolerance by improving the ratio of reduced 2-Cys Prx and antioxidase activities (Xia et al., 2018). Previous study illustrated that H_2O_2 may interact with NO, to increase tomato cold response and tolerance by regulating ABA levels (Lv et al., 2018). Reports showed that H_2O_2 may act upstream of NO, to activate MAPK and enhance the antioxidation in maize under water stress (Zhang et al., 2007) and wheat under aluminum stress (Sun et al., 2018). Although H_2O_2 could trigger NO production, NO may, in turn, prevent excess ROS accumulation by blocking NADPH oxidase activity via *S*-nitrosylation (Domingos et al., 2015). In contrast, treatment of tobacco leaves with NO donors triggered H_2O_2 production, but H_2O_2 treatment did not trigger NO production (Pasqualini et al., 2009). These results suggest a complex relationship between H_2O_2 and NO during plant stress responses. However, the relationships among NO, H_2O_2, and JA were dependent or independent in plant chilling stress response and tolerance were still need further research.

Protected cultivation of tomatoes does not prevent low-temperature damage (8–15 °C) during the early spring and winter in North China, which severely limits tomato growth and yield. Thus, it is essential to improve the chilling tolerance of tomato. The key precursor of all synthesized porphyrins, 5-aminolevulinic acid (ALA), is regarded as a potential plant growth regulator that may enhance plant's cold tolerance (Balestrasse et al., 2010; Korkmaz et al., 2010; Wang et al., 2004). Our previous studies showed that ALA pretreatment triggered an initial H_2O_2 signaling and subsequent cross-talk with AsA/GSH signaling, which improved plant antioxidation capacity (Liu et al., 2018). In the ALA→H_2O_2 signaling→antioxidation pathway, we speculated ALA may directly or/and indirectly induce NO production which involved in H_2O_2 regulated antioxidant pathways, or/ and may affect JA cross-talk signaling, increasing oxidative stress tolerance at low temperatures. Here, we try to explore the roles of JA and NO in ALA-induced cold tolerance in tomato and examine the relationships between JA, NO, and H_2O_2.

2. Materials and methods

2.1. Plant culture and treatment

Tomato cv. Jinpeng No. 1 (cold- sensitive) was used in this experiment. Plants were cultivated as described in our previous report (Liu et al., 2018). Plants were used for experiments when the fifth true leaves were completely expanded. Thirty plants were analyzed for each treatment.

We used four treatments to examine JA levels in tomato leaves pretreated with distilled water or 25 mg L^{-1} ALA (Sigma Aldrich, St. Louis, MO, USA) (Liu et al., 2018) under normal conditions [25 °C/18 °C (day/night), control and ALA] or low-temperature conditions [15 °C/8 °C (day/night), distilled water control (LT) and ALA (LTA)]. After 12 h, the control and ALA-treated plants under 25 °C/18 °C were maintained in the same growth conditions, whereas the LT and LTA plants were transferred to the conditions of 15 °C/8 °C, with the same light and humidity as the control plants. After 24 h, the fifth leaves of all plants were collected for JA analysis.

To investigate the role of JA in ALA-induced antioxidant activity, all the tomato leaves were pretreated with 200 μM salicylhydroxamic acid (SHAM) or 100 μM diethyldithiocarbamic acid (DIECA), which suppresses JA biosynthesis by inhibiting lipoxygenase (LOX, Nahar et al., 2011; Yuan et al., 2017). The leaves were treated with 25 mg L^{-1} ALA or 100 μM JA (Nahar et al., 2011; Yuan et al., 2017) after 8 h, and then subjected to normal or low-temperature conditions after 12 h. After 24 h, the fifth leaves were harvested to analyze malondialdehyde (MDA) content, relative electrical conductivity (REC), and antioxidase activities.

To analyze the relationship between H_2O_2 and JA, all the tomato leaves were pretreated with 200 μM SHAM or 100 μM DIECA and 5 mM dimethylthiourea (DMTU, scavenges H_2O_2 and O_2–, Liu et al., 2018). After 8 h, the leaves were treated with 5 mM H_2O_2 (Liu et al., 2018) or 100 μM JA. After 12 h, plants were maintained at normal temperature or transferred to low-temperature conditions as described above. After 24 h, the fifth leaves were harvested to analyze MDA content, REC, and antioxidase activities.

To define the role of NO in tomato cold tolerance, we measured NO production induced by ALA, JA, and H_2O_2. Plants were pretreated with distilled water, 25 mg L^{-1} ALA, 100 μM JA, or 5 mM H_2O_2. After 12 h, plants were maintained at normal temperature or transferred to low-temperature conditions [15 °C/8 °C (day/night)]. The NO content was monitored at different time points. To study the effects of NO in H_2O_2- and JA-induced oxidative stress tolerance, all the tomato leaves were pretreated with 200 μM 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO, an NO scavenger), 200 μM sodium tungstate (tungstate, an NR inhibitor), or 200 μM NG-nitro-L-arginine methylester (L-NAME, a specific NO synthase inhibitor, Cui et al., 2011; Diao et al., 2017; Zhang et al., 2007). After 8 h, plants were sprayed with 100 μM JA or 5 mM H_2O_2. After 12 h, plants were maintained at normal temperature or subjected to low-temperature conditions. The fifth leaves were harvested after 24 h to measure MDA content, REC, and antioxidase gene expression and activity.

Finally, we examined the effects of sodium nitroprusside (SNP, NO donor), cPTIO, tungstate, and L-NAME on H_2O_2 production. All the tomato leaves were pretreated with 200 μM SNP (Cui et al., 2011;

Zhang et al., 2007), 200 μM cPTIO, 200 μM tungstate, or 200 μM L-NAME. After 12 h, the plants were maintained under normal conditions or transferred to low-temperature conditions. After 24 h, the H_2O_2 contents were measured. Three biological replicates were performed for each experiment.

2.2. JA measurement

JA levels were measured using HPLC-MS/MS (Zoonbio Biotechnology Co., Ltd) according to a previously published method (You et al., 2016).

2.3. Evaluation of cold tolerance

REC was measured according to the published method of Zhou and Leul (1998). The MDA content was measured according to the method of Hodges et al. (1999). F_v/F_m was analyzed according to the method of Perez-Bueno et al. (2015) using the Open FluorCam FC 800-O and Fluorcam7 software (PSI, Brno, Czech Republic).

2.4. Measurements of endogenous H_2O_2 levels

The H_2O_2 levels were estimated according to the method of Willekens et al. (1997). Sample absorbance was measured at 412 nm.

2.5. Antioxidant enzyme assays

Superoxide dismutase (SOD; EC 1.15.1.1) activity was calculated by defining the amount of enzyme needed to inhibit photochemical reduction of nitro blue tetrazolium by 50% as one unit of SOD activity. This was monitored at 560 nm (Giannopolitis and Ries, 1977). All other antioxidase activities assays were performed as described by Noctor et al. (2016). Catalase (CAT, EC 1.11.1.6) activity was measured by monitoring the reduction of H_2O_2 at 240 nm for 2 min. Dehydroascorbate reductase (DHAR, EC 1.8.5.1) activity was determined by monitoring the changes of A_{265} in ascorbate levels for 3 min. Glutathione reductase (GR, EC 1.6.4.2) activity was determined via monitoring the decrease of A_{340} in NADPH levels for 3 min. Ascorbate peroxidase (APX, EC 1.11.1.11) activity was measured by monitoring the decrease of ascorbate at 290 nm for 2 min. Monodehydroascorbate reductase (MDHAR, EC 1.6.5.4) activity was determined by monitoring the decrease of NADPH at 340 nm for 3 min.

2.6. Determination of endogenous NO levels and NOS and NR activities

NO accumulation was visualized using diaminofluorescein-FM diacetate (DAF-FM DA, Beyotime Institute of Biotechnology) according to the method of Sun et al. (2014) with slight modifications. Briefly, leaf sections (5 × 5 mm) were washed for 10 min in 20 mM HEPES-NaOH buffer (pH7.4), and then loaded with 10 μM DAF-FM DA for 30 min at 25°C in dark. The sample was washed with 20 mM HEPES-NaOH buffer (pH7.4) to remove excess probe. Then, the samples were visualized using a laser scanning confocal microscope (FV1000 MPE, Olympus, Tokyo, Japan) with an excitation wavelength of 488 nm and an emission wavelength of 525 nm. Fluorescence intensities were analyzed using Image *J* software (NIH, Bethesda, MD, USA). NO content was measured by performing a colorimetric assay with Griess reagent (Sigma Aldrich, USA) according to the method of Lv et al. (2018). Leaf samples (0.3 *g*) were homogenized in 1.5 mL glacial acetic acid (pH 3.6) in an ice bath and centrifuged at 10,000*g* for 15 min. The reaction contained 1 ml of supernatant and 1 ml of Griess reagent, which was mixed and allowed to stand at 25°C for 30 min. The samples were then analyzed spectrophotometrically at 560 nm.

NOS and NR activities were measured with a colorimetric assay kit (Nanjing Jiancheng Bioengineering Institute, Jiangsu, China). Leaf samples (0.5 g) were homogenized in 1.5 mL of 0.1 M HEPES-KOH buffer (pH7.4), containing 5 mM dithiothreitol, 0.1% Triton X-100, 1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 5 mM Na_2MoO_4, 10% (v/v) glycerol, 1 mM leupeptin, 1% polyvinylpyrrolidone, and 20 mM FAD. After centrifuging at 10,000*g* for 30 min at 4°C, the supernatant was used to determine NR and NOS activities according to the manufacturer's instructions.

2.7. RNA extraction and gene expression analyses

Total RNA was extracted from tomato leaves with a Plant RNA Kit (OmegaBio-Tek, Doraville, GA, USA) according to the manufacturer's recommendations. Reverse transcription was performed using a PrimeScript TM RT Reagent Kit (Takara, Shiga, Japan) according to the manufacturer's instructions. Gene-specific primers were listed in Table S1. For qRT-PCR analysis, we used Takara TB Green™ *Premix Ex Taq*™ II (Takara, Shiga, Japan) in 20 μL reactions. PCR analysis was performed with an ABI StepOne Plus Real-Time PCR System (Applied Biosystems, Carlsbad, USA). We used two different reference genes, *actin7* and *GAPDH* (Vandesompele et al., 2002), and relative gene expression levels were calculated as described by Livak and Schmittgen (2001). Three biological replicates were performed for each experiment.

2.8. Statistical analysis

Statistical analysis of the bioassays was performed with SAS software version 8.0 (SAS Institute, Cary, NC, USA) using Tukey's test at a level of $P < 0.05$.

3. Results

3.1. JA had a key role in mitigating oxidative stress but was not essential in ALA-induced oxidative stress tolerance under low temperatures

JA is a crucial hormone that regulates plant cold tolerance (Eremina et al., 2016).

Therefore, we evaluated whether ALA treatment affected JA levels in tomato under normal- and low-temperature conditions (Fig. 1). The results showed that exogenous ALA dramatically elevated JA levels by 304% under normal-temperature conditions and by 101% under low-temperature conditions compared with plants without ALA treatment. Low-temperature treatment also triggered JA production compared with control plants.

Low-temperature conditions dramatically increased the MDA content and REC by 79% and 63%, respectively (Fig. 2), compared with control plants under normal-temperature conditions.

Under low temperature, treatment with exogenous JA and ALA

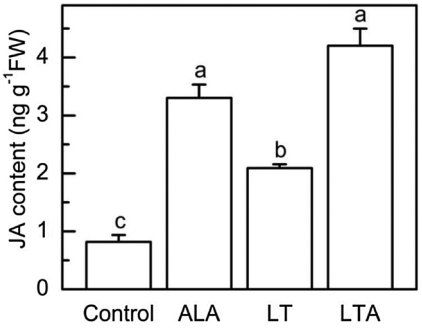

Fig. 1. ALA-induced JA accumulation in tomato leaves at low temperatures. Tomato leaves were treated with distilled water or 25 mg L^{-1} ALA and then exposed to normal temperatures (control and ALA) or low temperatures (LT and LTA) after 12 h. After 24 h, the JA content was measured in fifth leaves. Data are expressed as the mean ± standard error of three independent biological replicates. Different letters indicate significant differences of $P < 0.05$ according to Tukey's test.

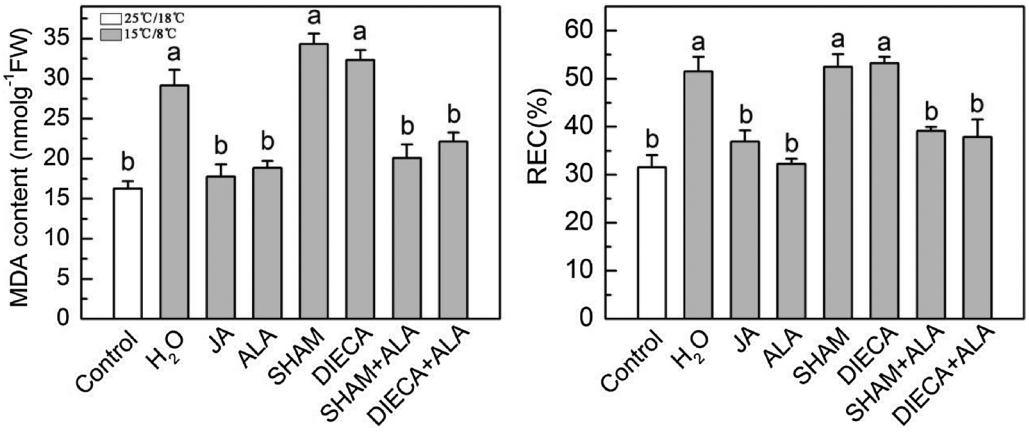

Fig. 2. Effects of the JA synthesis inhibitors SHAM and DIECA on ALA-mitigated membrane lipid peroxidation in tomato leaves at low temperatures. Tomato leaves were pretreated with SHAM (200 μM) or DIECA (100 μM). After 8 h, leaves were treated with distilled water, JA (100 μM) or ALA (25 mg L^{-1}). After 12 h, plants were maintained at normal temperatures (control, sprayed with distilled water, the same in the following figures) or transferred to low-temperature conditions (the H_2O treatment was the plants sprayed with distilled water and then exposed to low temperature, the same in the following figures). MDA contents and REC were measured in fifth leaves after 24 h. Data are expressed as the mean ± standard error of three independent biological replicates. Different letters indicate significant differences at $P < 0.05$.

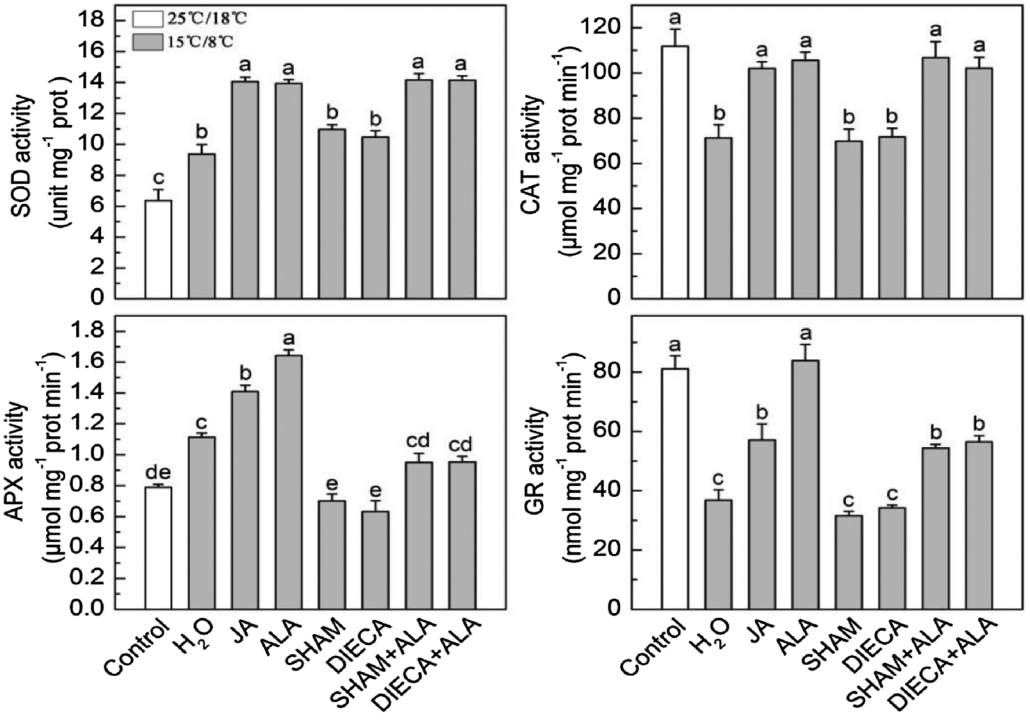

Fig. 3. Effects of JA synthesis inhibitors SHAM and DIECA on ALA-induced antioxidant enzyme activities. Tomato leaves were treated with SHAM (200 μM) or DIECA (100 μM). After 8 h, leaves were treated with distilled water, JA (100 μM) or ALA (25 mg L^{-1}). After 12 h, plants were maintained at normal temperatures (control) or transferred to low-temperature conditions. SOD, CAT, APX, and GR activities were measured in fifth leaves after 24 h. Data are expressed as the mean ± standard error of three independent biological replicates. Different letters indicate significant differences at $P < 0.05$.

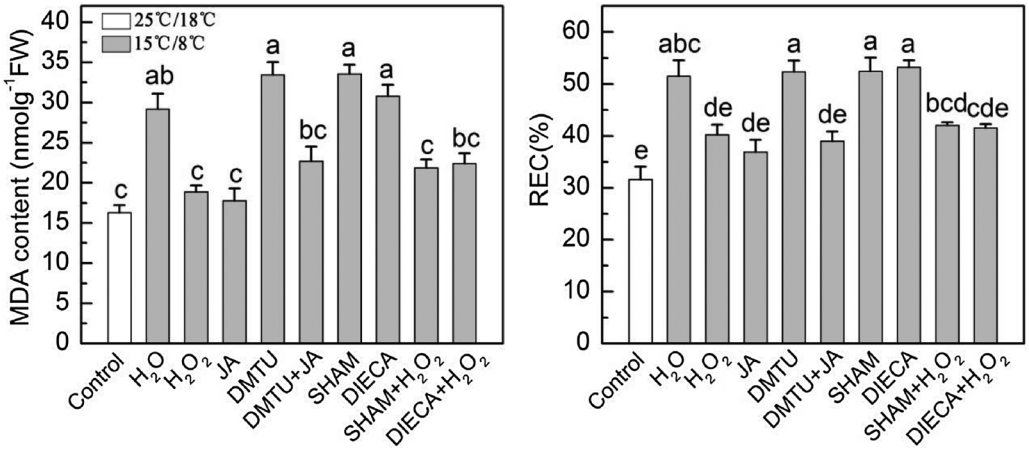

Fig. 4. Cross-talk between JA and H_2O_2 reduces membrane lipid peroxidation in tomato leaves at low temperatures. Tomato leaves were treated with DMTU (5 mM), SHAM (200 μM),or DIECA (100 μM). After 8 h, leaves were treated with distilled water, JA (100 μM) or H_2O_2 (5 mM). After 12 h, plants were maintained at normal temperatures (control) or transferred to low-temperature conditions. MDA contents and REC were measured in fifth leaves after 24 h. Data are expressed as the mean ± standard error of three independent biological replicates. Different letters indicate significant differences at $P < 0.05$.

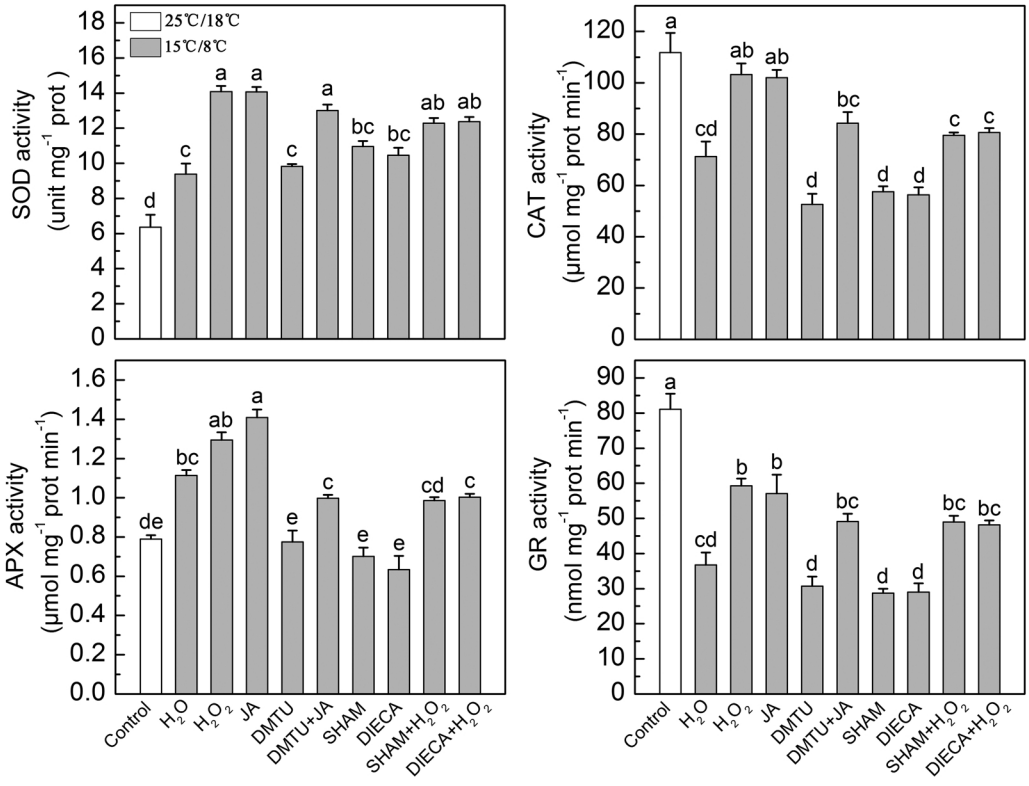

Fig. 5. Cross-talk between JA and H_2O_2 increased antioxidant enzyme activities in tomato leaves at low temperatures. Tomato leaves were treated with DMTU (5 mM), SHAM (200 μM), and DIECA (100 μM). After 8 h, leaves were treated with distilled water, JA (100 μM) or H_2O_2 (5 mM). After 12 h, plants were maintained at normal temperatures (control) or transferred to low-temperature conditions. SOD, CAT, APX, and GR activities were measured in fifth leaves after 24 h. Data are expressed as the mean ± standard error of three independent biological replicates. Different letters indicate significant differences at $P < 0.05$.

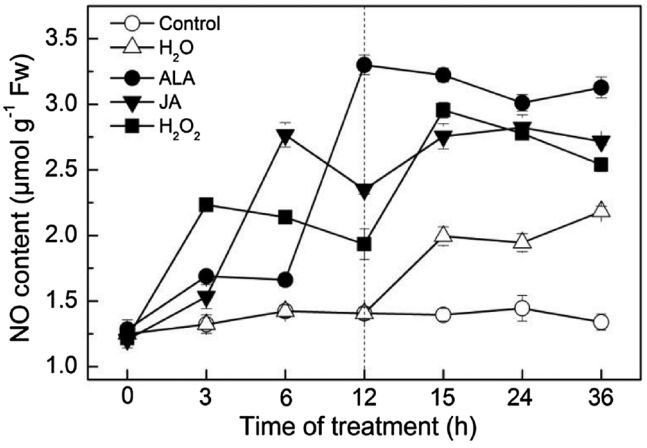

Fig. 6. Changes in endogenous NO levels induced by ALA, JA, or H_2O_2 in tomato leaves. Tomato leaves were treated with ALA (25 mg L^{-1}), JA (100 μM), or H_2O_2 (5 mM). The NO content was measured starting at 0 h. After 12 h, plants were maintained at normal temperatures (control) or transferred to low-temperature conditions. Data are expressed as the mean ± standard error of three independent biological replicates.

reduced the MDA content by 39% and 35%, respectively, and reduced REC by 28% and 37%, respectively, compared with plants subjected to low temperature alone. Inhibition of endogenous JA using SHAM and DIECA did not significantly affect MDA content and REC compared with plants subjected to low temperature alone. Treatment with ALA in addition to SHAM and DIECA markedly reduced membrane lipid damage to the same levels observed in JA- and ALA-treated plants.

Low temperature significantly increased SOD and APX activities, whereas CAT and GR activities declined under low-temperature conditions compared with control plants (Fig. 3).

JA and ALA treatment significantly enhanced SOD, CAT, APX, and GR activities, whereas SHAM and DIECA treatment significantly reduced APX activity without affecting other antioxidase activities compared with plants subjected to low temperature alone. Treatment with SHAM or DIECA plus ALA dramatically elevated antioxidase activities compared with plants treated with SHAM or DIECA alone (Fig. 3).

3.2. JA cooperated with H_2O_2 to mitigate oxidative stress at low temperatures

We previously showed that H_2O_2 participated in ALA-regulated redox balance to improve antioxidant capacity in tomato (Liu et al., 2018).

Here, we analyzed the relationship between JA and H_2O_2 in enhancing tomato cold tolerance. The results showed that cold treatment plus H_2O_2 and JA reduced MDA content and REC compared with cold treatment alone (Fig. 4). Eliminating endogenous H_2O_2 with DMTU and inhibiting JA with SHAM or DIECA resulted in slightly higher membrane lipid damage than that in untreated cold-stressed plants. Addition of exogenous JA or H_2O_2 to DMTU-, SHAM-, and DIECA-treated plants dramatically reduced the MDA content and REC, which were essentially the same as the levels observed in H_2O_2- or JA-treated plants (Fig.4).

Treatment with H_2O_2 or JA significantly elevated SOD, CAT, APX, and GR activities (Fig. 5). DMTU, SHAM, or DIECA treatment inhibited APX activity, slightly reduced CAT and GR activities, and did not affect SOD activity compared with cold treatment alone. Addition of JA after treating with DMTU significantly improved the antioxidase enzyme activities, whereas H_2O_2 treatment after SHAM or DIECA substantially elevated CAT, APX, and GR activities (Fig. 5) compared with DMTU-, SHAM-, or DIECA-treated plants.

3.3. ALA, JA, and H_2O_2 triggered endogenous NO production

NO is an endogenous signaling molecule in plant defense responses (Neill et al., 2008). The results showed that addition of exogenous ALA, JA, or H_2O_2 substantially elevated endogenous NO levels after 12 h (Fig. 6).

Under normal-temperature conditions, the maximum NO bursts

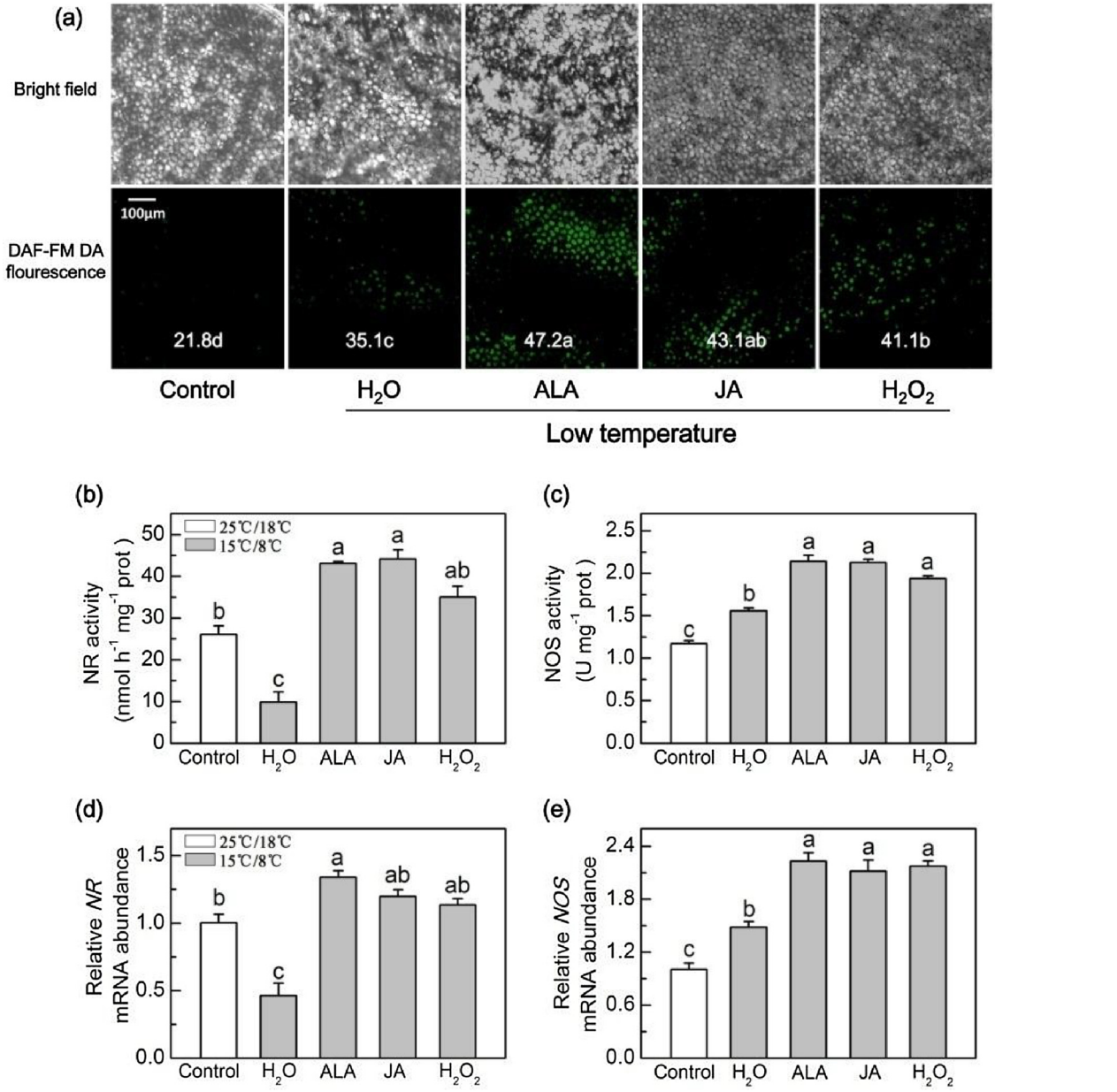

Fig. 7. Endogenous NO synthesis is induced by ALA, JA, or H_2O_2 in tomato leaves. Tomato leaves were treated with distilled water, ALA (25 mg L^{-1}), JA (100 μM), or H_2O_2 (5 mM). After 12 h, plants were maintained at normal temperatures (control) or transferred to low-temperature conditions. The NO contents, activities and genes expression of NOS and NR were measured in fifth leaves after 24 h. (a) Fluorescence detection of NO using DAF-FM DA staining and a confocal microscope. Bar, 100 μm. (b) NR activity. (c) NOS activity. (d) *NR* transcription level and (e) *NOS* transcription level (the gene transcription levels in control plants were normalized to 1). Data are expressed as the mean ± standard error of three independent biological replicates. Different letters indicate significant differences at $P < 0.05$.

occurred at 12, 6, and 3 h with ALA, JA, and H_2O_2 treatment, respectively. Low temperature significantly enhanced NO production compared with control plants.

Under low-temperature conditions, JA and H_2O_2 treatment resulted in higher NO levels, whereas ALA treatment did not significantly affect NO content compared with these treatments under normal temperatures at 12 h (Fig. 6). After 36 h, NO levels in ALA-, JA-, and H_2O_2-treated plants were increased by 135%, 68%, and 38%, respectively, compared with cold treatment alone.

We examined the NO levels using DAF-FM DA, and the NOS and NR enzymes activities and genes expression in tomato leaves which pretreated with ALA, JA and H_2O_2 for 12 h, and then grow under normal- and low-temperature conditions for 24 h (Fig. 7). DAF-FM DA caused the same trends in NO levels as described above. ALA-, JA-, and H_2O_2-treated plants enhanced the NO levels by 34%, 23%, and 17%, respectively, compared with plants subjected to low temperature alone (Fig. 7a). Compared with control plants, low temperature significantly increased NOS activity and gene expression but reduced NR activity and gene expression (Fig. 7b–e). Treatment with ALA, JA, or H_2O_2 increased NOS activity by 37%, 36%, and 33%, respectively, and increased NR activity by 337%, 348%, and 256%, respectively, compared with cold treatment alone.

3.4. NO was involved in JA- and H_2O_2-induced oxidative stress tolerance under low temperatures

Treatment with the NO scavenger cPTIO or the NO synthesis inhibitor tungstate or L-NAME followed by JA treatment did not significantly affect MDA content and REC, compared with JA treatment alone (Fig. 8a). Treatment with cPTIO, tungstate, or L-NAME followed

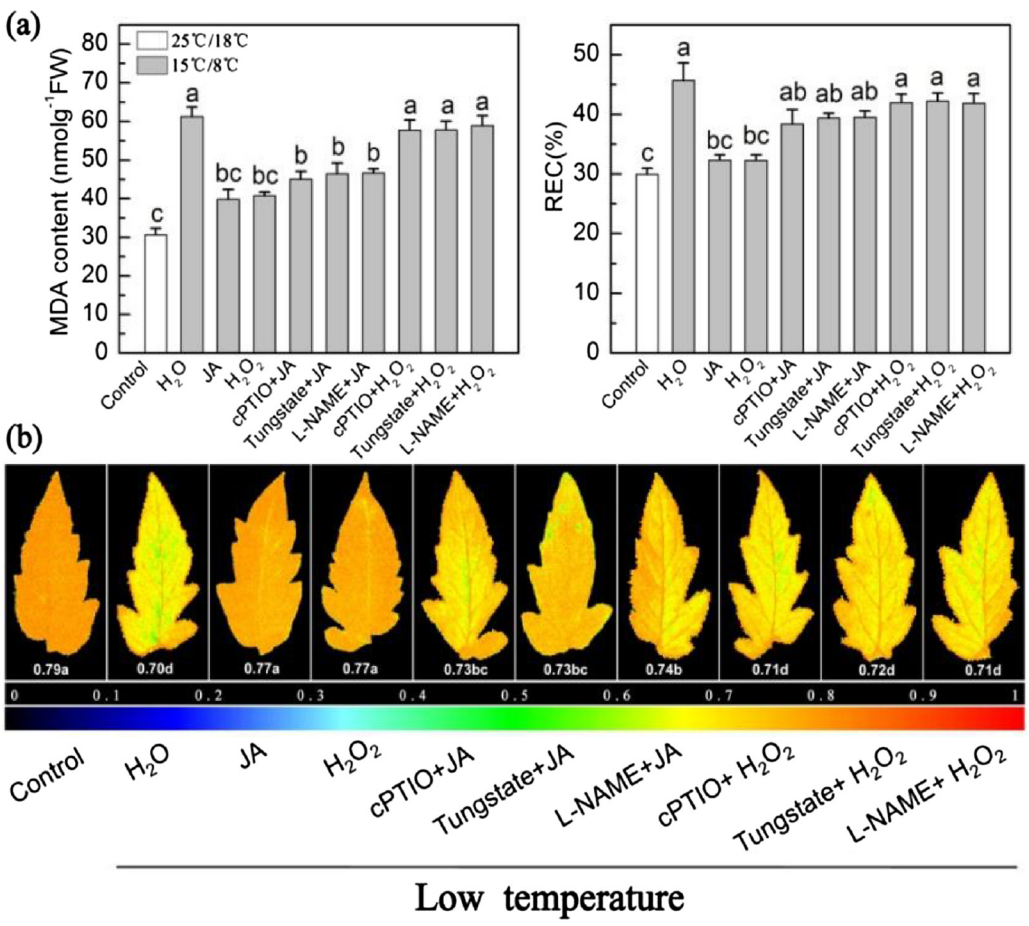

Fig. 8. NO is involved in JA- and H_2O_2-regulated membrane lipid peroxidation and F_v/F_m in tomato leaves at low temperatures. Tomato leaves were treated with cPTIO (200 μM), tungstate (200 μM), or L-NAME (200 μM). After 8 h, leaves were treated with distilled water, JA (100 μM) or H_2O_2 (5 mM). After 12 h, plants were maintained at normal temperatures (control) or transferred to low-temperature conditions. (a) MDA contents and REC, and (b) F_v/F_m were measured in fifth leaves after 24 h. Data are expressed as the mean ± standard error of three independent biological replicates. Different letters indicate significant differences at $P < 0.05$.

by H_2O_2 did not mitigate membrane damage at low temperatures compared with H_2O_2 treatment alone (Fig. 8a). Low temperature largely reduced F_v/F_m compared with control plants, whereas treatment with JA and H_2O_2 at low temperatures reversed this trend (Fig. 8b). JA treatment of NO-inhibited plants dramatically elevated F_v/F_m, but it was still lower than in JA-treated plants, compared with cold treatment alone. However, elimination of NO essentially abolished the H_2O_2-mediated increase in F_v/F_m compared with H_2O_2 application alone at low temperatures.

JA treatment of NO-inhibited plants did not significantly affect CAT, APX, and GR activities but did reduce SOD activity compared with JA treatment alone (Fig. 9b). H_2O_2 treatment of NO-inhibited plants significantly decreased antioxidant enzyme activities, in contrast to H_2O_2 treatment alone. The expression of antioxidant enzyme genes showed the same trends as the enzyme activities (Fig. 9).

Low temperature significantly up-regulated *Cu/ZnSOD* and *APX5* expression but down-regulated *CAT1* and *GR1* expression compared with control plants (Fig. 9a). Treatment of cold-stressed plants with JA and H_2O_2 substantially up-regulated these genes compared with cold treatment alone. JA or H_2O_2 treatment of NO-inhibited cold-stressed plants substantially reduced the expression of these genes compared with JA and H_2O_2 treatments alone. H_2O_2 treatment of NO-inhibited plants essentially abolished H_2O_2-mediated increases in antioxidant enzyme gene expression compared with cold treatment alone. In addition, treatment with SNP, cPTIO, tungstate, or L-NAME did not significantly affect H_2O_2 content compared with cold treatment alone (Supplementary Fig.S1).

4. Discussion

Low temperature is a primary factor that limits tomato production (Barrero-Gil et al., 2016; Duan et al., 2012). Plants sense low temperatures and transmit that information to downstream pathways using several signaling molecules, including calcium, ROS, hormones, NO, and MPK and CBF transcription factors, which activate the expression of cold-responsive (COR) genes (Zhu, 2016; Lv et al., 2017). Exogenous plant growth regulators have been widely used to improve cold tolerance in crop plants. Our previous study showed that ALA induced H_2O_2 signal, which subsequently increased tomato cold tolerance (Liu et al., 2018). In the present study, exogenous ALA induced endogenesis JA levels (Fig. 1). JA is a crucial hormone that regulates many physiological processes, including stomatal development (Han et al., 2018), plant growth (Yang et al., 2012), glucosinolate accumulation (Guo et al., 2013), and stress responses (Kazan, 2015; Yuan et al., 2017; Yang et al., 2017; Du et al., 2017). Exogenous JA increased tomato cold tolerance by enhancing antioxidase activities (Figs. 2 and 3), consistent with previous studies of freezing tolerance in Arabidopsis (Hu et al., 2017, 2013), cold stress resistance in rice (Du et al., 2013), and cold responses in tomato (Wang et al., 2016a). However, inhibition of endogenous JA by SHAM and DIECA did not significantly affect plant cold tolerance. These results suggest that endogenous JA was crucial for cold tolerance, but it was not the only signaling pathway involved in tomato perception of and defense against cold stress. Others signaling molecule also may play crucial roles in ALA-mediated enhancement of tomato oxidation stress resistance at low temperatures, such as H_2O_2 (Liu et al., 2018), NO (Diao et al., 2017; Zhao et al., 2009), and GSH (Noctor et al., 2012; Suzuki et al., 2012; Li et al., 2016a).

Previous studies illustrated that H_2O_2 may activate JA biosynthesis, which was a systemic signal to respond to plant biotic and abiotic stress (Wasternack et al., 2006; Han et al., 2013), then, increased the plant oxidative tolerance (Yuan et al., 2017). Some studies reported that NO may act downstream of H_2O_2 (Sun et al., 2018) and JA (Wang and Wu,

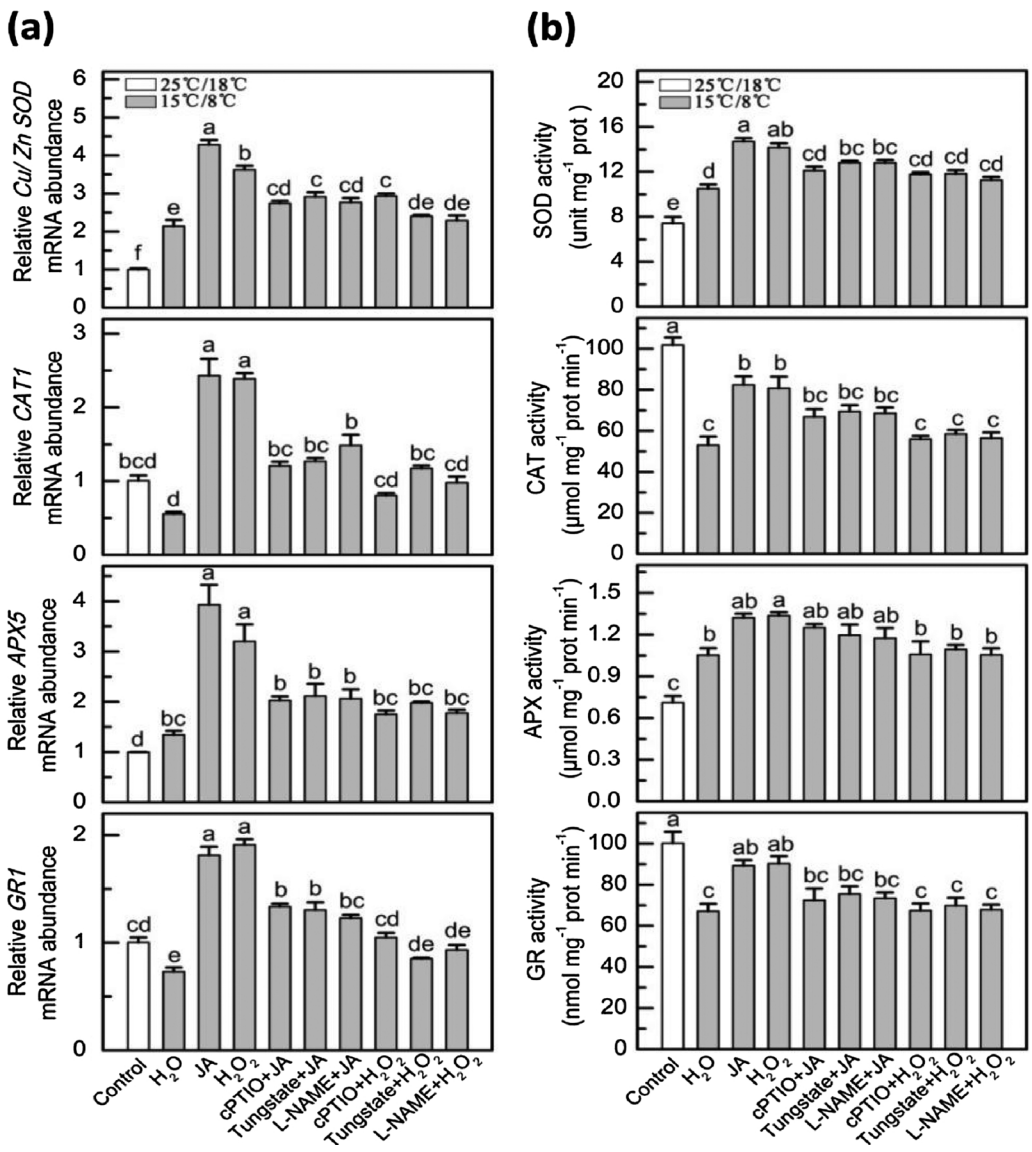

Fig. 9. NO is involved in JA- and H_2O_2-regulated antioxidant enzymes gene expression and activity in tomato leaves at low temperatures. Tomato leaves were treated with cPTIO (200 μM), tungstate (200 μM), or L-NAME (200 μM). After 8 h, leaves were treated with distilled water, JA (100 μM) or H_2O_2 (5 mM). After 12 h, plants were maintained at normal temperatures (control) or transferred to low-temperature conditions. *Cu/Zn SOD*, *CAT*, *APX*, and *GR* expression levels and activities were measured in fifth leaves after 24 h. (a) Relative antioxidase transcriptional levels (the levels in control plants were normalized to 1). (b) Antioxidase activities. Data are expressed as the mean ± standard error of three independent biological replicates. Different letters indicate significant differences at $P < 0.05$.

2005) in signaling pathways mediating stress-tolerance responses. Our study suggests that JA may interact with H_2O_2 to regulate cold-induced oxidative stress in tomatoes, rather than a simple linear relationship with H_2O_2 inducing JA or vice versa (Figs. 4 and 5). But the exact mechanisms still need further research.

NO is an important signaling molecule in plant abiotic stress tolerance (Sun et al., 2018). Although NO can be synthesized by at least seven enzymatic and non-enzymatic pathways (Gupta et al., 2011). There are two main synthetic pathways in plants, including a NOS-like-dependent pathway (Guo et al., 2003) and an NR-dependent pathway (Desikan et al., 2002). Many plant growth regulators and external factors can trigger NO production. Fu et al. (2015) reported that NOS triggered NO-mediated ALA-induced oxidation resistance in *Elymus nutans* Griseb leaves under chilling stress. Methyl jasmonate treatment activates NOS and induces NO burst in plant cells (Wang and Wu, 2005). Pretreatment of wheat seedlings with H_2O_2 donor (glucose/glucose oxidase) triggered NR-induced NO production under aluminum stress (Sun et al., 2018). In the present study, low temperature induced NO accumulation in tomato via elevated *NOS* expression and activity, but not via NR which may be inhibited by low temperatures (Fig. 7). However, fed with ALA, JA, and H_2O_2 could eliminate low temperature weakened-NR activity, and trigger endogenous NO production (Figs. 6 and 7). The NO burst in ALA treated plants was later than JA- and H_2O_2-treated plants (Fig. 6). And, ALA could dramatically enhance endogenesis H_2O_2 (Liu et al., 2018) and JA levels (Fig.1). Taken together, we speculated that ALA may directly trigger NO production or first induce JA and H_2O_2, which then promoted NO production. Some studies reported that plant stress responses generated NO before H_2O_2, whereas other studies reported that H_2O_2 mediated NO production via NOS (Cui et al., 2011). In addition, NO and H_2O_2 bursts may also occur in parallel or within the same time frame (Qiao et al., 2014). The present results suggest that NO levels may not affect endogenous H_2O_2 content (Supplementary Fig. S1). Meanwhile, our results showed that eliminating endogenous NO, essentially abolished H_2O_2-mediated, while only attenuated JA-mediated tomato oxidative resistance (Figs. 8 and 9). These results indicated that NO might act downstream of H_2O_2 to regulate antioxidase gene expression. However, JA regulated tomato oxidation resistance only slightly depend on NO, and it may function coordinately with H_2O_2→NO signal pathway. The precise relationships between ALA-induced JA, H_2O_2, and NO, and their interactions with other signals and protein kinases (e.g., MPKs) through CBF-dependent or CBF-independent pathways in enhancing cold tolerance of tomato requires further studies. Whether ALA has a receptor, like hormones, or other primary signals (such as calcium) responded to ALA's effect, also is an interesting question needed further explore.

Based on presented data in this study, we propose a model about the

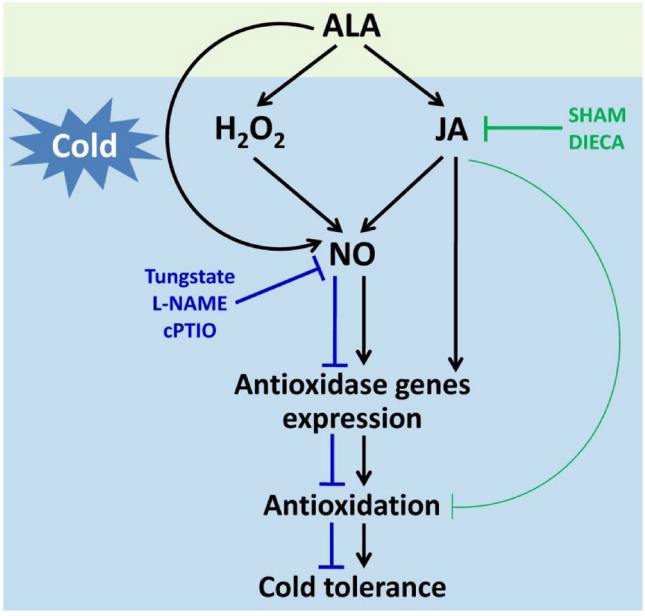

Fig. 10. Proposed model for NO involvement in JA- and H_2O_2-mediated ALA-induced oxidative stress tolerance at low temperatures in tomato. The bold line represent the positive regulation or a major inhibition, and the thin line show a partial inhibition.

relationships of JA, H_2O_2 and NO, involved in ALA induced plant cold response and tolerance (Fig. 10). Pretreatment of tomato leaves with ALA elevated endogenous JA, H_2O_2, and NO levels. When pretreated plants exposed to low temperature, JA, and H_2O_2 all up-regulated *NR* and *NOS* transcription levels, which then elevated NR and NOS activities and triggered the NO bust. NO may act downstream of H_2O_2, and up-regulate antioxidase gene expression and activity, thereby mitigating membrane lipid damage in tomato plants. Reduction of NO levels by inhibiting NO synthesis with tungstate and L-NAME, or scavenging NO with cPTIO, down-regulated the antioxidase genes expression and activities, and depressed the tomato cold tolerance. Reduction of JA levels by inhibiting JA synthesis with SHAM and DIECA only slightly weakened, but did not eliminate the plant cold tolerance. So, ALA induced JA may cooperate with but does not completely depend on H_2O_2→NO signal pathway to increase antioxidase gene expression and activities of tomatoes at low temperature.

Authors contribution

T.L., X.H., and J.L. designed the experiments and wrote the manuscript. T.L., and J.X. performed the experiments. T.L., and X.H. analyzed the data. All authors have read and approved the final version of the manuscript.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.envexpbot.2018.10.020.

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