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Combined effect of ethylene- and salicylic acid-signaling insensitive mutation on *Arabidopsis* response to low temperature

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Abstract

The roles of ethylene (ET) or salicylic acid (SA) in plant response to low temperature (LT, 5 °C) have been implicated. However, the combined effect of ET- and SA-signaling on plant growth and metabolism under LT remains to be evaluated. In this study, we comparatively analyzed the response of *Arabidopsis ethylene insensitive* (*ein*) 2-*1* (an ET insensitive mutant), *nonexprressor of pathogenesis relative* (*npr*)*1-1* (an SA insensitive mutant) and double mutant *ein2-1/npr1-1* plants to LT. The results show that a LT of 5 °C induced plant growth retardation to a less degree in *ein2-1*, an intermediate degree in *npr1-1*, but a much larger in *ein2-1/npr1-1* compared to the wild-type (WT) plants. The LT susceptibility of the *ein2-1/npr1-1* plants was correlated to a lower net photosynthetic rate and proline content, and a higher content of H_2O_2 and malondialdehyde and electrolyte leakage relative to the WT plants. Lower activities of superoxide dismutase, peroxidase, and catalase, as well as a lower glutathione content and a ratio of its reduced form to its oxidized form were also observed in the double mutant plants as compared with the WT plants. However, at normal conditions (23 °C), all the tested physiological and biochemical parameters were comparable between the *ein2-1/npr1-1* and WT plants, and plant growth was even better in the double mutant than in the WT plants. On the contrary, most of the above-mentioned parameters were advantageous in the *ein2-1* and *npr1-1* plants over the WT plants under the LT conditions. These data suggest that a parallel function or physiological redundancy of nonexpressor of pathogenesis relative 1 and ethylene insensitive 2 existed in the *Arabidopsis* plant response to the LT. On the other hand, an interaction between ET- and SA-signaling occurred during this process.

Additional key words: catalase, chilling, cross talk, lipid peroxidation, peroxidase, photosynthesis, superoxide dismutase.

Introduction

Chilling temperatures, defined as low but not subzero temperatures, are one of the major limitations to productivity and geographical distribution of many plant species by affecting all phases of plant growth and development and various physiological and biochemical processes such as photosynthesis (Ruelland *et al*. 2009), uptake of water and nutrients, cell-membrane integrity (for a review see Miura and Tada 2014), and so on. Plant adaptation to a low temperature (LT) involves a complex cross-talk between different regulatory levels. Among them phytohormones including ethylene (ET) and salicylic acid (SA) have been studied intensively. For instance, chilling treatment dramatically increases plant endogenous SA content (Dong *et al*. 2014). A temporal application of moderate concentrations of SA efficiently enhances plant tolerance to chilling or freezing, whereas high concentrations or continual application of SA significantly decreases plant tolerance to LT (Miura and Tada 2014). In addition, SA-accumulating *Arabidopsis* mutants *cpr1* (*constitutive expresser of pathogenesis related genes 1*), *acd6* (*accelerated cell death 6*), and *siz1* [*SAP* (scaffold attachment factor) and *Miz*1 (Msx2-interacting zinc finger)] exhibit a higher sensitivity to LT than wild type (WT) plants (Scott *et al*. 2004, Miura and Ohta 2010), whereas a SA-deficient mutant *eds5* (*enhanced disease susceptibility 5*) or a *nahG* (salicylate

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Abbreviations: CAT - catalase; *ein2-1* - *ethylene insensitive 2-1*; ET - ethylene; GSH - reduced glutathione; GSSG - oxidized glutathione; JA - jasmonic acid; LT - low temperature; MDA - malondialdehyde; MS - Murashige and Skoog; *npr1-1* - *nonexpressor of pathogenesis relative 1*; P_N - net photosynthetic rate; POD - peroxidase; ROS - reactive oxygen species; SA - salicylic acid; SOD - superoxide dismutase; WT - wild type.

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hydroxylase gene) transgenic line display an improved LT tolerance (Scott *et al*. 2004). The reduction of SA content in *acd6* and *siz1* plants by nahG activity restores LT sensitivity (Miura and Ohta 2010). Sensitivity to LT in SA-accumulating mutants and at high concentration or continual application experiments is likely related to over-production of reactive oxygen species (ROS) (Miura and Tada 2014). In the case of ET, its increased content enhanced *Arabidopsis* freezing susceptibility, whereas a decreased ET biosynthesis or an ET signaling blockage enhanced plant freezing tolerance (Shi *et al*. 2012). Likewise, an increasing evidence has demonstrated that the ET-involved plant response to LT conditions is associated with a balance between oxidative stress and antioxidative defense response (*e.g.*, Wu *et al*. 2008, Shi *et al*. 2012, 2014).

Plant growth, development, and response to exogenous and endogenous stimuli are under a tight regulation by phytohormones, synergistic or antagonistic interactions. However, the evidence for cross talk between SA and ET comes mainly from plant responses to biotic stresses (*e.g*., pathogen or insect attack). The synergistic or antagonistic action between SA and jasmonic acid (JA)/ET constitutes one of the most important defense systems in plant response to biotic stress (Spoel and Dong 2008). Many pathogen resistant genes, such as defensin gene (*PDF1.2*), regulated by both ET and JA are suppressed by SA in a *nonexpressor of pathogenesis relative 1* (NPR1)-dependent manner (Koornneef *et al*. 2008). Besides a direct protective effect on the plant response to biotic attack, ET has also been shown to participate in regulation of SA-mediated biological functions. For instance, exogenous ET enhances expression of SA-responsive marker gene *pathogenesis relative 1* (*PR1*) in a nonexpressor of pathogenesis relative (NPR1)-depenent manner. On the other hand, exogenous ET can relieve the dependency of SA-JA cross talk on NPR1 function, namely that ET can recover SA-mediated suppression of a JA-responsive gene in the *npr1* mutant in an NPR1-independent manner. The latter role requires ethylene insensitive 2 **(**EIN2) function and is thus regulated by the ET signaling pathway (Leon-Reyes *et al*. 2009). Regarding the regulatory role of ET, the most likely explanation is that ET signaling allocates more monomeric NPR1, an active form of NPR1 generated from SA-mediated reduction of an NPR1 oligomer (an inactive form, linked through intermolecular disulfide bridges) in the cytosol, into the nucleus where it activates expression of SA-responsive genes such as *PR1*. This certainly decreases NPR1 accumulation in the cytoplasm, which may affect SA-JA cross talk, because this process is controlled by the cytosol NPR1. However, this can be compensated by ET-mediated SA-JA cross talk in an NPR-1 independent manner (Leon-Reyes *et al*. 2009). Rather limited information is available about cross talk between SA and ET in plant response to abiotic stress. Winter rye plants accumulate antifreeze proteins, such as glucanases (PR2), chitinases (PR11), and thaumatin-like proteins (PR5), during cold acclimation. These proteins are also induced by exogenous SA or ET (Yu *et al*. 2001) suggesting that a cross talk between ET and SA may occur in the cold acclimation mechanism. In a previous study, we observed that the inability to perceive both ET and SA lead to a more sensitive plant phenotype under Al stress, which is associated with a decreased antioxidant capacity and increased oxidative stress, but not with Al uptake (Zhang *et al*. 2014).

Although much attention has been focused on the contribution of either ET or SA to plant response to LT, studies on their interaction are necessary to improve our understanding on the complex role of phytohormones. This can be accomplished by the use of mutants. Therefore, in this study, we compared the performances of mutants *ein2-1* and *npr1-1*, and a double mutant *ein2-1/npr1-1* relative to WT plants under LT conditions. Arabidopsis *ein2-1* plants display insensitivity to exogenous or endogenous ET at morphological, physiological, and molecular levels (Ecker 1995) and have been intensively employed to dissect the biological functions of ET signaling in plant growth and development such as flowering, fruiting, senescence, as well as in response to biotic and abiotic stresses. NPR1 is an important transducer of SA signaling in plants. *Arabidopsis* mutant *npr1-1* plants lost SA-mediated *PR1* expression and systemic acquired resistance to pathogens (Cao *et al*. 1994, 1997) and have been repeatedly used to assess the biological functions of SA signaling in plant response to a wide range of abiotic stresses.

Materials and methods

Arabidopsis thaliana L. ecotype Col-0, and its ET insensitive mutant *ein2-1* (Guzman and Ecker 1990), SA insensitive mutant *npr1-1* (Cao *et al*. 1994), and double mutant *ein2-1/npr1-1* were offered by Prof. Dong (Duke University, Durham, USA), and Prof. Pieterse (the Utrecht University, Utrecht, The Netherlands). The *ein2-1/npr1-1* double mutant was created by crossing homozygous *npr1-1* and *ein2-1* plants and screened. Seeds were immersed in 0.1 % (m/v) agarose solution and cold-treated at 4 °C for 2 d, then sown in a self-made device containing a half strength Murashige and Skoog (1/2 MS) solution with pH 6.2 according to a previously described method (Hao *et al*. 2012). Ten days after germination at day/night temperatures of 23/18 °C, a 14-h photoperiod, an irradiance of 100 μmol m^{-2} s^{-1} provided by 36-W fluorescent tubes, and a 70 % humidity, the seedlings were thinned and the plants with identical growth were left. For LT treatment, the plants were

shifted to controlled environment chambers (*Senxin X 280*, Shanghai, China) at a constant air temperature of 5 °C and a photoperiod and irradiance similar to that of the control plants. The control plants were kept at the 23 °C chamber. For biochemical analysis, plant samples were collected from fully expanded leaves of either control plants (23 °C, 25 d) or LT treated plants (5 °C for 60 d) and frozen in liquid N_2. Based on the growth stage-based phenotypic analysis described by Boyes *et al*. (2001), plants grown at 23 °C for 25 d and 5 °C for 60 d are at the same growth stage under the present experimental conditions.

Six plants of each genotype grown at 5 or 23 °C were collected at different intervals. Whole plants were dried at 80 °C for 48 h to determine changes in dry mass. Net photosynthetic rate (P_N) was measured using a portable photosynthetic system (*LI-6200, LI-COR,* Lincoln, NE, USA) with a leaf chamber specific for *Arabidopsis* (*LI-6400-17*) at an irradiance of 150 μmol m^{-2} s^{-1}, a CO_2 concentration of 370 μmol mol^{-1} and a temperature of 23 °C. Proline content in leaves was detected by the method of Bates *et al*. (1973). Hydrogen peroxide (H_2O_2) was measured as described by He *et al*. (2014). Malondialdehyde (MDA) content in leaves was quantified according to the method of Shalata and Tal (1998). Briefly, leaf samples (0.2 g) were ground with 1 cm^3 of 50 mM phosphate buffer solution (pH 7.8) and centrifuged at 10 000 *g* and 4 °C for 15 min. The reaction mixture containing 0.7 cm^3 of supernatant and 1.3 cm^3 of 0.5 % thiobarbituric acid in 20 % trichloroacetic acid was incubated at 100 °C in water bath for 20 min and then cooled quickly in an ice bath. The reaction mixture was centrifuged at 10 000 *g* and 4 °C for 5 min. The absorbance of supernatant was measured at 532, 600, and 450 nm. The content of MDA was calculated as $06.45 \times (A_{532} - A_{600}) - 0.56 \times A_{450}$. Electrolyte leakage was analyzed using a conductivity meter (*SA29-DDB-11A, Midwest Group,* Beijing, China) by the method used previously (Hao *et al*. 2012).

For antioxidative enzyme determination, fresh leaves were harvested and homogenized in liquid nitrogen using a mortar and pestle. The powder was well mixed with 5 cm^3 of an ice cold extract solution consisting of 50 mM phosphate-buffered saline (pH 7.8), 0.1 mM EDTA, 1 % (v/v) *Triton X-100*, and 4 % (m/v) polyvinylpyrrolidone, then incubated on ice for 10 min, and centrifuged at 12 000 *g* and 4 °C for 15 min. The supernatant was used to analyze enzyme activities. Superoxide dismutase (SOD; EC 1.15.1.1) activity was measured by the photochemical method (Beyer and Fridovich 1987). A reaction mixture was established as described previously (Guo *et al*. 2014). One unit of SOD was defined as the amount of the enzyme required to inhibit the photoreduction of nitroblue tetrazolium by 50 %. Catalase (CAT; EC 1.11.1.6) activity was determined based on decomposition of H_2O_2 at 240 nm for 3 min as described by Aebi (1983); for details see Guo *et al*. (2014). A decrease of 0.01 absorbance at 240 nm per minute was defined as one unit of CAT. Peroxidase (POD; EC 1.11.1.7) activity was measured according to Hemeda and Klein (1990) in a reaction mixture described previously (Guo *et al*. 2014). An increase of 0.01 absorbance at 470 nm per minute was defined as one unit of POD. For all these measurements, a spectrophotometer *Spectronic 20* (*Bausch and Lomb*, Wilmington, MA, USA) was used. For expression of specific activity, protein content in the crude enzyme extract was determined by the method of Bradford (1976) using bovine serum albumin as standard. For isoenzyme analysis, the crude enzyme extract was separated by native polyacrylamide gel electrophoresis and stained on the gel as described by Hao *et al*. (2012). Content of the reduced form of glutathione (GSH) was analyzed according to the method of Griffith and Meister (1979). The oxidized form of glutathione (GSSG) was calculated from a difference between the amount of glutathione in 1,4-dithiothreitol (DTT)-treated samples and DTT-non-treated samples.

Each experiment was carried out six times. Data were analyzed by one-way analysis of variance (*ANOVA*) and means were compared using Duncan's multiple-range test at $\alpha = 0.05$.

Results and discussion

The LT conditions resulted in growth retardation of all the tested plants, to a greater extent in the *ein2-1/npr1-1* plants after 20 d, and to a less extent in the *ein2-1* and *npr1-1* plants after 30 d. This difference became more apparent with an increase in LT-treatment (Fig. 1). The differential responses were also fully reflected in the leaf phenotypes of representative plants exposed to the LT for 60 d (Fig. 2*A*). In the normal conditions (23 °C), the *ein2-1* and *ein2-1/npr1-1* plants grew better than did the WT plants already 20 d after germination (Fig. 1 and 2*B*), and the differences were increasingly pronounced after 40 d (Fig. 2*C*). Growth of the 23 °C *npr1-1* plants was always comparable to the WT plants (Fig. 1 and 2*B*,*C*).

Arabidopsis ET insensitive mutants, such as *etr1-1*, *ein4-1*, *ein2-5*, and *ein3-1*, have been well demonstrated to confer improved tolerance to short-term freezing stress (-5 °C for 1 h, Shi *et al*. 2012). In the present study, we found that the *ein2-1* plants exhibited an enhanced tolerance to the long-term chilling treatment. Together with the observation that a better growth occurred in the *ein2-1* plants under the normal temperature, it can be concluded that ET signaling negatively regulated *Arabidopsis* plant growth under either the LT or the normal temperatures. In the case of SA, a deficiency or

signaling blockage has been shown to improve *Arabidopsis* plant tolerance to LT (Scott *et al*. 2004), whereas a high accumulation of SA caused by mutation potentiated plant sensitivity to LT (Scott *et al*. 2004, Miura and Ohta 2010). These findings together with our observation in the present study imply that endogenous SA content and/or signaling play a negative regulatory role at least in *Arabidopsis* response to LT. Nevertheless, the similar growth of the *npr1-1* and WT plants at 23 °C demonstrates that SA signaling did not affect plant growth under the normal temperature. More interestingly, in the present study, we as the first found that the genetic combination of the two tolerant parents (*ein2-1* and *npr1-1*) created a more sensitive progeny (*ein2-1/npr1-1*) to the LT. This implies an existence of a cross talk between ET- and SA-signaling during plant exposure to the LT. Further, we attempted to explore possible cross nodes in physiological and biochemical parameters.

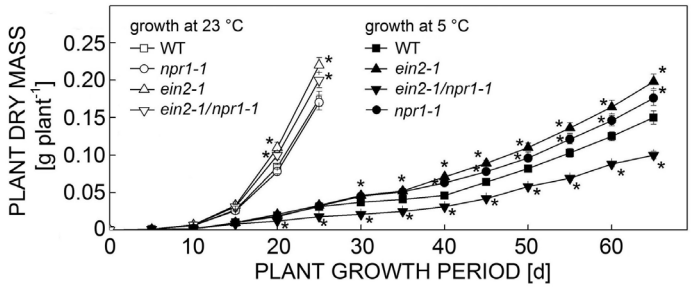

Fig. 1. The effect of a low temperature of 5 °C on plant dry mass. WT - wild type, *npr1-1* - nonexpressor of pathogenesis relative 1 mutant, *ein2-1* - ethylene insensitive 2-1 mutant, *ein2-1*/*npr1-1* - double mutant. Values are expressed as means ± SDs (n = 6, * - significant differences at $P < 0.05$ from WT plants).

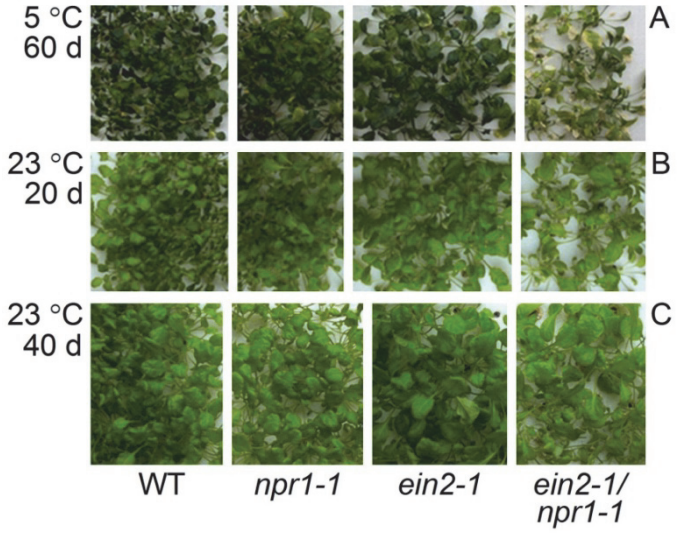

Fig. 2. Representative pictures showing the tested plant phenotypes. WT - wild type, *npr1-1* - nonexpressor of pathogenesis relative 1 mutant, *ein2-1* - ethylene insensitive 2-1 mutant, *ein2-1*/*npr1-1* - double mutant.

Net photosynthetic rate has long been recognized as one of the most chilling-sensitive processes. Therefore, we analyzed the effect of ET- and SA-signaling blockage on P_N response to the LT. As expected, the LT decreased P_N of all tested genotypes with a greater degree occurred in the *ein2-1/npr1-1* plants and a less but similar degree in the WT, *npr1-1*, and *ein2-1* plants (Fig. 3*A*). A LT-caused inhibition of *Arabidopsis* P_N has been reported in previous studies (*e.g*., Savitch *et al*. 2001). Because fixation of CO_2 is very limited by LT, photoinhibition occurs even under a relatively low irradiance (Takahashi and Murata 2008), which induces ROS generation, in turn causing destruction of the photosynthetic apparatus. However, *Arabidopsis* is a relatively cold-insensitive species able to grow to maturity at 5 °C (Scott *et al*. 2004). This might be at least associated with a mechanism that *Arabidopsis* photosynthesis can partially recover during LT acclimation (Savitch *et al*. 2001). In the present study, we also noticed that a moderate P_N activity was maintained in all the tested genotypes after the long-term LT treatment, and this may determine a certain plant growth.

The outcome of plant response to LT is dependent on the regulatory role of phytohormones alone or in combination. For instance, gibberellin and JA have antagonistic effects on plant growth at LT (Wingler

2015). Under LT, some photosynthesis-related processes are regulated by ET or SA, but little information is available about a combined effect of ET and SA. In considerations of the comparable P_N among the tested genotypes at 23 °C (Fig. 3*A*), the present study suggests that a parallel function of NPR1 and EIN2 existed to maintain a moderate P_N activity at the LT, probably by regulating photosynthesis-related protein expression and post-translational modification because these processes are sensitive to LT, and are regulated by phytohormones (Janmohammadi *et al*. 2015, Wingler 2015). A further study, especially at a molecular level, will provide an insight into the regulation mechanism.

Free proline content increased upon the LT treatment (Fig. 3*B*), and the specific accumulation patterns indicated an involvement of this compound in plant response to the LT and also implied an interaction between ET- and SA-signaling. A large body of evidence has documented that proline content is regulated by content and/or signaling ET or SA, positively or negatively, in plant responses to stresses (*e.g*., Hao *et al*. 2012, Khan *et al*. 2014). Free proline plays an important role in plant growth and development as well as in a response to various stresses including LT due to its function not only as osmoprotectant but also as free radical scavenger. A previous study showed that free proline content is much higher in chilling-tolerant *Arabidopsis* genotypes than in WT plants (Tamirisa *et al*.

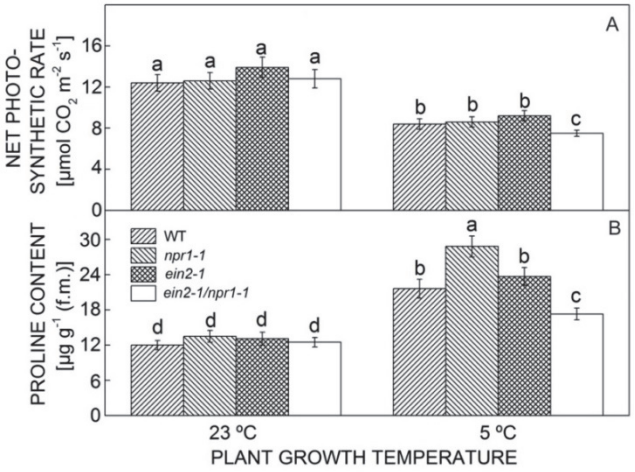

Fig. 3. The effect of a low temperature of 5 °C on plant net photosynthetic rate (*A*) and proline content (*B*) in a wild type (WT) and in *npr1-1* (nonexpressor of pathogenesis relative 1) mutant, *ein2-1* (ethylene insensitive 2-1) mutant, and *ein2-1*/*npr1-1* double mutant. Means ± SDs (n = 6). The *bars* with different lower-case letters indicate significant differences at $P < 0.05$.

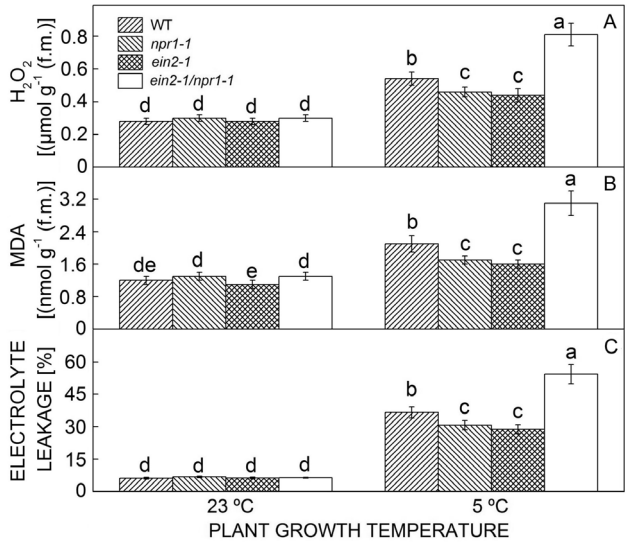

Fig. 4. Low temperature (5 °C)-induced oxidative stress in a wild type (WT) and in *npr1-1* (nonexpressor of pathogenesis relative 1) mutant, *ein2-1* (ethylene insensitive 2-1) mutant, and *ein2-1*/*npr1-1* double mutant characterized by H_2O_2 content (*A*), malondialdehyde (MDA) content (*B*), and electrolyte leakage (*C*). Means ± SDs (n = 6). The *bars* with different lower-case letters indicate significant differences at $P < 0.05$.

2014). However, proline accumulation under heat stress decreases *Arabidopsis* thermotolerance (Lv *et al*. 2011). This may be interpreted by different responsive mechanisms of plants exposed to low and high temperatures (Yamori *et al*. 2014).

Oxidative damage is a general consequence caused by stresses, and antioxidative defense is the main mechanism by which plants cope with stressing conditions. Therefore, in the present study, we focused on oxidative-antioxidative contest. The LT induced an obvious oxidative stress to all the tested plants as indicated by H_2O_2 accumulation, MDA production, and electrolyte leakage (Fig. 4*A*-*C*). The differences among the tested genotypes suggest that they were the factors responsible for the LT-induced plant growth inhibition. Activities of SOD increased by 53, 30, and 21 %, respectively, in the *npr1-1*, *ein2-1*, and WT plants relative to their respective 23 °C plants (Fig. 5*A*). However, no significant difference was detected in the double mutant. Activities of POD decreased by the LT to a higher extent in the *ein2-1/npr1-1* and WT plants than in the *ein2-1* and *npr1-1* plants (Fig. 5*B*). Although the LT elevated CAT activities, the fluctuations were narrow in the tested genotypes except for the WT plants, which had an increase of about 38 % relative to its 23° C parallel (Fig. 5*C*). The isoenzyme profiles of these three antioxidative enzymes provide interesting information as indicated by the band numbers and intensities (Fig. 6). More specifically, three bands of SOD isoenzymes were detected in the collection of the tested genotypes; they were marked I, II, and III, respectively (Fig. 6). Band III was the major one, and the strongest staining was found in the *ein2-1* plants followed by the *npr1-1*, WT, and double mutant plants at both 23 °C and 5 °C. Bands I and II were much weaker than band III, but they significantly differed among the tested genotypes. For instance, band I was present in *npr1-1* and *ein2-1* plants, but absent in WT and *ein2-1/npr1-1* plants, whereas band II was observed in *npr1-1* and *ein2-1/npr1-1* plants, but not in WT and *ein2-1* plants (Fig. 6). At least four POD bands could be detected and were marked I - IV (Fig. 6). Notably, band IV was much stronger in the *ein2-1* plants than in the other tested plants regardless of the plant growth temperatures suggesting that ET signaling negatively regulated POD expression. However, intensity was restored in the *ein2-1/npr1-1* plants. Together with a comparable level in the *npr1* and WT plants, it was proposed that the expression of POD band IV was suppressed by both NPR1-dependent and NPR1-independent mechanisms of EIN2 function. This is similar to the situation of ET signaling-involved regulation in SA-mediated biological functions as described in the introduction. Band I of POD appeared mainly in the *ein2-1* plants and band III in the WT plants. Band II was sensitive to the LT, by which it almost disappeared in all the 5 °C plants. Although CAT has many isoenzymes *in planta*, we detected only one band under the present experimental conditions. The band intensity of CAT was positively related with that CAT activity measured by spectrophotometer.

There have been many researches addressing activity changes of antioxidative enzymes in plant response to LT, among which most of the evidence supports a notion that increased enzyme activities confer an improved

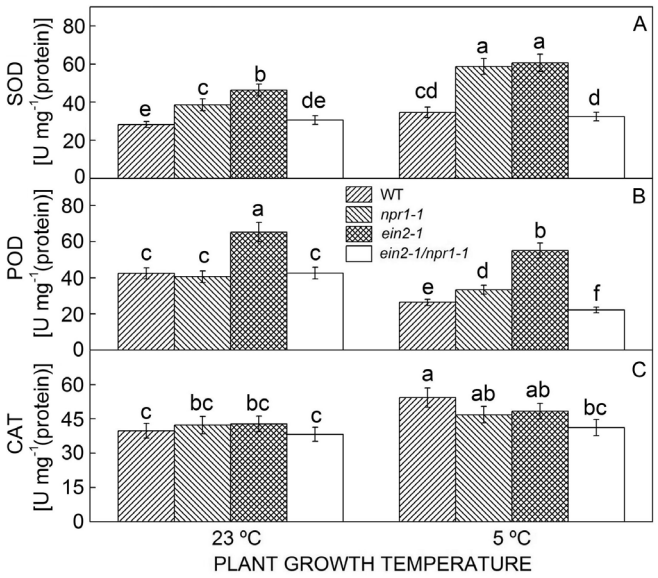

Fig. 5. The effect of a low temperature of 5 °C on activities of superoxide dismutase (SOD, *A*), peroxidase (POD, *B*), and catalase (CAT, *C*) in a wild type (WT) and in *npr1-1* (nonexpressor of pathogenesis relative 1) mutant, *ein2-1* (ethylene insensitive 2-1) mutant, and *ein2-1*/*npr1-1* double mutant. Means ± SDs (n = 6). The *bars* with different lower-case letters indicate significant differences at $P < 0.05$.

tolerance to LT (*e.g*., Tamirisa *et al*. 2014). This is true in our study. Previous researches indicated various links between LT-induced oxidative stress and a regulatory role of ET or SA (*e.g*., Scott *et al*. 2004, Shi *et al*. 2012). However, it was rarely reported that the combination of ET- and SA-signaling affects the LT-induced oxidative stress. The present study clearly shows that the signaling blockage in both ET and SA dramatically promoted the LT-induced oxidative stress, which was at least partially related to the decreased activities of SOD, POD, and CAT in the double mutant plants. However, the plant genome includes over 150 genes that encode different ROS-scavenging enzymes and most of them are temperature responsive (Suzuki and Mittler 2006). Therefore, future studies should include more antioxidative enzymes.

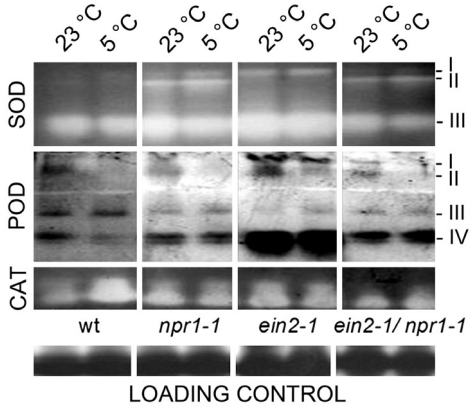

Fig. 6. The isoenzyme profiles of superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT) in a wild type (WT) and in *npr1-1* (nonexpressor of pathogenesis relative 1) mutant, *ein2-1* (ethylene insensitive 2-1) mutant, and *ein2-1*/*npr1-1* double mutant as affected by a low temperature of 5 ºC. Crude enzyme extracts were separated by PAGE (10 μg of protein per lane) and stained for enzyme activities. The loading amount was monitored by Coomassie brilliant blue G-250 staining.

Glutathione exists in both the reduced form (GSH) and the oxidized form (glutathione disulphide; GSSG), and its influence on cellular redox status depends on both GSH content and GSH/GSSG ratio (Schaffer and Buettner 2001). Our data show that the LT treatment decreased the total glutathione content most pronouncedly in the double mutant plants followed by the WT plants, whereas an obvious mitigation occurred in the *ein2-1* and *npr1-1* plants (Fig. 7*A*-*C*). This is well correlated with the other above-mentioned parameters, as well as with the plant phenotypes. These data also show that the simultaneous blockage of ET-signaling and SA-signaling not only affected the redox status of glutathione pool, but also lowered glutathione biosynthesis. As non-enzymatic antioxidant, glutathione plays a pivotal role not only as an important cellular redox buffer, due to scavenging ROS produced by LT (Zhao *et al*. 2015), but also as a signaling molecule involved in the plant defense response such as promoting nuclear accumulation of NPR1 (Kovacs *et al*. 2015). The latter is something like the situation of ET signaling-promoted NPR1 allocation in the nucleus (as described in the introduction). The ratio of GSH/GSSG has been generally recognized as biomarker of cellular redox conditions. Many biological events, such as NPR-dependent processes, occur only in reducing conditions (Kovacs *et al*. 2015). This imply that the LT sensitivity in the *ein2/npr1* plants was related to the oxidative conditions as indicated by the lower ratio of GSH/GSSG (Fig. 7*B*). Many studies have demonstrated that

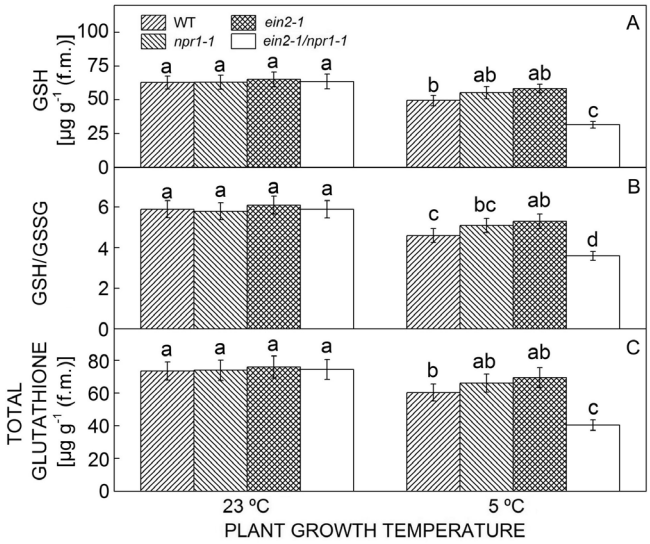

Fig. 7. The effect of a low temperature of 5 ºC on content of reduced glutathione (GSH, *A*), ratio of glutathione reduced/oxidized forms (GSH/GSSG, *B*), and total GSH content (*C*) in a wild type (WT) and in *npr1-1* (nonexpressor of pathogenesis relative 1) mutant, *ein2-1* (ethylene insensitive 2-1) mutant, and *ein2-1*/*npr1-1* double mutant. Means ± SDs (n = 6). The *bars* with different lower-case letters indicate significant differences at $P < 0.05$.

glutathione biosynthesis and signaling rely on SA function in an NPR1-dependent manner as well as on ET functions (*e.g*., Iqbal *et al*. 2013, Ghanta *et al*. 2014). Thus, glutathione-related metabolism might be a cross regulatory node by ET and SA in plant response to LT.

An early study showed that *npr1* and *ein2* plants exhibited a typical hypersensitive reaction (HR) to *P. maculicola* ES4326/*avrRpt2*, which prevented the pathogen from spreading beyond the site of inoculation. However, the double mutant *ein2/npr1* plants exhibited disease symptoms spreading beyond the site of inoculation, suggesting that EIN2 and NPR1 may have parallel functions (Clarke *et al*. 2000). We previously observed that the *ein2-1* and *npr1-1* plants have a significant improvement in Al^{3+} tolerance, but the *ein2/npr1* plants exhibit a more sensitive phenotype (Zhang *et al*. 2014). Taken together, these data suggest that the parallel functions of EIN2 and NPR1 might commonly exist in plant response to environmental stimuli.

In summary, based on the performance of the *ein2-1*, *npr1-1*, and *ein2/npr1* plants relative to the WT plants, this study demonstrates that parallel functions of EIN2 and NPR1 existed during the*Arabidopsis* response to the LT. On the other hand, cross talk between ET- and SA-signaling occurred in this process. This was reflected by the LT-responsive patterns in plant growth and by some metabolism processes such as photosynthesis, proline accumulation, oxidative stress, and antioxidative defense among the tested genotypes. However, in view of the unknown molecular mechanism, it is worth further study to reveal this interaction especially at the molecular level.

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