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RESEARCH PAPER

Roles of NIA/NR/NOA1-dependent nitric oxide production and HY1 expression in the modulation of *Arabidopsis* salt tolerance

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Abstract

Despite substantial evidence on the separate roles of *Arabidopsis* nitric oxide-associated 1 (NOA1)-associated nitric oxide (NO) production and haem oxygenase 1 (HY1) expression in salt tolerance, their integrative signalling pathway remains largely unknown. To fill this knowledge gap, the interaction network among nitrate reductase (NIA/NR)- and NOA1-dependent NO production and HY1 expression was studied at the genetic and molecular levels. Upon salinity stress, the majority of NO production was attributed to NIA/NR/NOA1. Further evidence confirmed that HY1 mutant *hy1-100*, *nia1/2/noa1*, and *nia1/2/noa1/hy1-100* mutants exhibited progressive salt hypersensitivity, all of which were significantly rescued by three NO-releasing compounds. The salinity-tolerant phenotype and the stronger NO production in gain-of-function mutant of HY1 were also blocked by the NO synthetic inhibitor and scavenger. Although NO- or HY1-deficient mutants showed a compensatory mode of upregulation of *HY1* or slightly increased NO production, respectively, during 2 d of salt treatment, downregulation of ZAT10/12-mediated antioxidant gene expression (*cAPX1/2* and *FSD1*) was observed after 7 d of treatment. The hypersensitive phenotypes and stress-related genes expression profiles were differentially rescued or blocked by the application of NO- (in particular) or carbon monoxide (CO)-releasing compounds, showing a synergistic mode. Similar reciprocal responses were observed in the *nia1/2/noa1/hy1-100* quadruple mutant, with the NO-releasing compounds exhibit the maximal rescuing responses. Overall, the findings present the combination of compensatory and synergistic modes, linking NIA/NR/NOA1-dependent NO production and *HY1* expression in the modulation of plant salt tolerance.

Key words: *Arabidopsis*, haem oxygenase-1 (HY1), NIA/NR, nitric oxide, NOA1, salt tolerance.

Introduction

Salinity stress is a major factor that significantly inhibits plant growth and decreases crop productivity (Zhu, 2000, 2001; Chinnusamy *et al.*, 2004). Typically, it imposes ionic toxicity, osmotic stress, and even oxidative damage on plant cells. In response to salinity stress, plants that have successfully survived should reestablish, sustain, or enhance: (a) ion and osmotic homeostasis; (b) stress damage control and repair, or reactive oxygen species (ROS) detoxification; and (c) growth control. It has also been claimed that homeostasis and detoxification signalling could not only lead to salinity tolerance, but also negatively regulate the growth inhibition response (Mittler, 2002; Zhu, 2002; Krasensky and Jonak, 2012; Liu *et al.*, 2012; Xie *et al.*, 2012a).

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Nitric oxide (NO), a redox-active gaseous molecule, plays a key signalling role in a broad spectrum of physiological and development processes throughout the whole plant life cycle (Delledonne, 2005; Gupta *et al.*, 2011). For example, ample evidence demonstrated that NO could act as a signal molecule mediating responses to abiotic stresses, including salt stress (Uchida *et al.*, 2002; Zhao *et al.*, 2004, 2007), drought and dehydration (García-Mata and Lamattina, 2001; Lozano-Juste and León, 2010), and cold acclimation and freezing tolerance (Zhao *et al.*, 2009).

In animals, NO is synthesized via the enzyme NO synthase (NOS) (Dulak and Józkowicz, 2003). Although NOS-like activity has been detected widely in plants, and inhibitors of mammalian NOS inhibit NO generation in plants, no archetypal NOS-encoding gene(s) have been isolated so far in higher plants (Crawford *et al.*, 2006; Zemojtel *et al.*, 2006; Van Ree *et al.*, 2011). *Arabidopsis* nitric oxide associated 1 (NOA1), originally reported to encode a protein with a NOS activity, has been shown to be a GTPase, which plays a role in binding RNA/ribosomes (Sudhamsu *et al.*, 2008). A relationship of NOA1-mediated NO production and plant responses against salinity stress (Zhao *et al.*, 2007) and pathogens (Mandal *et al.*, 2012) has been reported, although the reduced accumulation of NO in *noa1* plants might be due to their inability to accumulate the sucrose reserve (Van Ree *et al.*, 2011). The production of NO in plants can also be generated by nitrate reductase (NR), the critical enzyme responsible for nitrate assimilation (Rockel *et al.*, 2002; Zhao *et al.*, 2009; Gupta *et al.*, 2011). In *Arabidopsis*, NR is encoded by two genes, *NIA1* and *NIA2* (Yamasaki and Sakihama, 2000). The involvement of NR-mediated NO production in stomatal closure and cold acclimation has been demonstrated genetically (Bright *et al.*, 2006; Zhao *et al.*, 2009). The mitochondrial electron transport chain and the non-enzymic pathway in the apoplast are also involved in NO production (Bethke *et al.*, 2004; Gupta *et al.*, 2005, 2011; Planchet *et al.*, 2005).

Recently, plant haem oxygenase (HO; EC 1.14.99.3), the rate-limiting enzyme responsible for the breakdown of haem into another gaseous molecule (CO), iron, and bilirubin, has received overwhelming research attention (Shekhawat and Verma, 2010). In *Arabidopsis*, a small family of HOs with four members is classified into two subfamilies. The inducible HO1 subfamily includes HY1, HO3, and HO4, while HO2 is the only member of the HO2 subfamily (Gisk *et al.*, 2010). Although HO1/CO was firstly described as a signalling system in animals (Bilban *et al.*, 2008), new and exciting physiological roles as a bioregulator of plant developmental processes as well as environmental adaptation have been reported (Shekhawat and Verma, 2010; Bose *et al.*, 2013). Close links among HO1/CO and auxin (Cao *et al.*, 2011), glutathione (Han *et al.*, 2008), NO (Noriega *et al.*, 2007; Xuan *et al.*, 2008), hydrogen peroxide (H_2O_2) and other ROS (Yannarelli *et al.*, 2006; Chen *et al.*, 2009; Wu *et al.*, 2011b; Xie *et al.*, 2011a; Xu *et al.*, 2012; Bose *et al.*, 2013), Ca^{2+} (Wu *et al.*, 2011a), or hydrogen sulfide (Lin *et al.*, 2012) pathways have also been suggested.

In plants, both NO and HO1/CO are integral components of osmotic signalling (Liu *et al.*, 2010). Our data have shown that HO1/CO might confer an increased tolerance to salinity stress in wheat seedling roots, which is partially mediated by the NO signal (Xie *et al.*, 2008), and further suggested that *Arabidopsis* HY1 plays an important role in salt acclimation signalling and requires the participation of RbohD-derived ROS peak II (Xie *et al.*, 2011b; Bose *et al.*, 2013). However, the genetic evidence for the cross talk between NO and HY1 in plant responses against salinity stress remains elusive.

Although there are some debates on the nature of AtNOA1 participating in NO biosynthesis (Crawford *et al.*, 2006; Guo, 2006; Zemojtel *et al.*, 2006), in most cases, *Atnoa1* mutants are valuable materials for studying the physiological functions of NO (Zhao *et al.*, 2007; Van Ree *et al.*, 2011). Meanwhile, Lozano-Juste and León (2010) have reported that the germination of *nia1/2/noa* mutant was hypersensitive to exogenous abscisic acid, salt, and osmotic stress (in particular). However, the corresponding detailed mechanism, such as the crosstalk with other signalling components and related transduction cascade, remains to be elucidated. The present study addressed the functions of both NO and HY1 signalling pathways upon salinity stress in *Arabidopsis* by generating a quadruple mutant in which both NR/NOA1 and HY1 that are severely impaired. The observed physiological responses of *Arabidopsis* plants were linked with complex sets of information provided by a combination of genetic and molecular analysis. Most importantly, corresponding elucidation of both NO- and HY1-deficient mutant plants led to identify a cross-talk signalling, including the compensatory and synergistic models of NIA/NR/ NOA1-dependent NO and HY1 expression, in the modulation of salt tolerance in *Arabidopsis*.

Materials and methods

Chemicals

All chemicals were purchased from Sigma-Aldrich (St Louis, MO, USA) unless otherwise stated. The chemicals used for treatments were sodium nitroprusside (SNP), diethylamine NONOate (NONOate), S-nitrosoglutathione (GSNO), carbon monoxide-releasing molecule-2 (CORM-2), N^G-nitro-L-arginine methyl ester hydrochloride (L-NAME), tungstate, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide potassium salt (cPTIO) (Ederli *et al.*, 2006; Zhao *et al.*, 2009; Chen *et al.*, 2010; García-Arnandis *et al.*, 2011; Desmard *et al.*, 2012; Xie *et al.*, 2012b). The concentrations used in this study were determined in pilot experiments from which maximal induced responses were obtained.

Additionally, the Old SNP/NONOate/CORM-2 solutions were used as the negative controls by maintaining the separated solution of SNP/NONOate/CORM-2 (50 μM each) for at least 10 d in the light in a specific open tube to eliminate the entire NO or CO, respectively (Tossi *et al.*, 2009).

Plant materials and growth conditions

Arabidopsis thaliana hy1-100 (CS236, Col-0), *hy1* (CS67, L*er*), *noa1* (CS6511, Col-0), and *nia1/2* (CS2356, Col-0) mutants were obtained from the *Arabidopsis* Biological Resource Center (http://www.arabidopsis.org/abrc), and the homogenous *HY1* overexpression line *35S:HY1-4* was previously constructed (Xie *et al.*, 2011b). The L*er* ecotype seeds were provided by Dr Chuanyou Li (Institute

of Genetics and Developmental Biology, Chinese Academy of Sciences, China).

Seeds were surface-sterilized for 20 min and washed three times with sterile water, then cultured in Petri dishes on solid Murashige and Skoog (MS) medium (pH 5.8) with 1% sucrose. For culturing NR-related mutants, the nitrogen in the MS medium included 1 mM NH_4^+ and 1.94 mM NO_3^- [Zhao *et al.*, 2009; preliminary experiments also showed the performance of the wild-type and the *nia1/2* mutant is clearly associated with the exogenous N supplement, although both NH_4^+ and NO_3^- are essential, NR mutants could grow better with NH_4^+ (Supplementary Fig. S1, available at *JXB* online)]. Plates containing seeds were kept at 4 °C for 2 d, and then transferred into a growth chamber with a 16/8 light/dark cycle (23/18 °C) and 120 µmol m^{-2} s^{-1} irradiation.

Generation of the triple-mutant line nia1/2/noa1 *and quadruple mutant line* nia1/2/noa1/hy1-100

The triple-mutant line *nia1/2/noa1* and the quadruple mutant line *nia1/2/noa1/hy1-100* were obtained by crossing mutant lines *nia1/2* with *noa1*, and then *nia1/2/noa1* with *hy1-100*. F1 plants were self-pollinated and the resulting F_2 individuals were genotyped using PCR primers (Supplementary Table S1) specific for the presence of the mutations (Supplementary Fig. S2). Homozygous *hy1*-related mutants were further identified by Western blot analysis (Supplementary Fig. S3) and the corresponding phenotypes (including yellow cotyledons, etc) were observed (Supplementary Figs. S4 and S5).

Salt-tolerance assay and phenotype analysis

For salt-tolerance analysis, 5-day-old seedlings of each genotype were cultured in MS medium with or without various chemical pretreatments for the indicated times, followed by 150 mM NaCl or other treatments as indicated. Alternatively, stratified seeds of each genotype were sowed in the MS medium with or without 150 mM NaCl in the presence or absence of L-NAME, tungstate, or cPTIO for the indicated times. The phenotypes, including the primary root growth, fresh weight, chlorophyll content, and germination rate, were then observed at the indicated times (Lichtenthaler, 1987; Xie *et al.*, 2011b, 2012b). Meanwhile, representative images were photographed.

Real-time quantitative reverse-transcription PCR

Total RNA was isolated using the Trizol reagent (Invitrogen, Gaithersburg, MD, USA) according to the manufacturer's instructions. Real-time quantitative reverse-transcription (RT) PCR were performed using a Mastercycler ep® realplex real-time PCR system (Eppendorf, Hamburg, Germany) with SYBR® Premix Ex Taq™ (TaKaRa Bio, China) according to the manufacturer's instructions. Using specific primers (Supplementary Table S2), relative expression levels of corresponding genes are presented as values relative to corresponding control samples at the indicated times or under the indicated conditions, after normalization to *actin2/7* (At3g18780) transcript levels.

Confocal determination of NO production

Endogenous NO level was assayed by confocal microscopy using a fairly specific NO fluorescent probe 4-amino-5-methyl-amino-2',7'-di-fluorofluorescein diacetate (DAF-FM DA). *Arabidopsis* seedlings were collected at the indicated times and loaded with 10 µM DAF-FM DA for 20 min before washing in 20 mM HEPES buffer (pH 7.8) three times for 5 min each and then analysed using a TCS-SP2 laser scanning confocal microscope (LSCM; Leica, excitation at 488 nm, emission at 500–530 nm). All manipulations were performed at 25 ± 1 °C. Pictures were taken based on 20 overlapping confocal planes of 2 µm each using the Leica software package. Each genotype had 6 replicates per experiment. Data are presented as relative units of pixel intensities via region of interest analysis, provided by the Leica software (Sieberer *et al.*, 2009; Liesche and Schulz, 2012) or as the means of fluorescence intensity relative to that of the wild-type under the control condition (0 h).

NO detection by electron paramagnetic resonance

Arabidopsis seedlings were homogenized and incubated in buffered solution (50 mM Hepes, 1 mM DTT, 1 mM $MgCl_2$, pH 7.6). After centrifugation at 12,000 *g* for 2 min, the supernatant was added to 300 µl of freshly made Fe(II)$(DETC)_2$ solution (2 M $Na_2S_2O_4$, 3.3 mM DETC, 3.3 mM $FeSO_4$, 33 g l^{-1} BSA), incubated for 2 min at room temperature and frozen in liquid nitrogen until analysis (Huang *et al.*, 2004; Sun *et al.*, 2012). Electron paramagnetic resonance (EPR) measurements were performed on a A300 spectrometer (Bruker Instrument, Germany) under the following conditions: microwave frequency, 9.854 GHz; microwave power, 63.496 mW; modulation amplitude 4.00 G; modulation frequency, 100 kHz.

Statistical analysis

Data are shown as means ± standard error (SE) of at least three independent experimental replications. The analysis of variance (ANOVA), multiple comparison, and t-test were also used for data analysis. In particular, the data for different genotype samples upon different treatments were analysed using a general linear model of a completely randomized design with the treatments as one fixed factor. Differences in the phenotypic indicators among various genotypes were analysed by a nested ANOVA, with genotypes nested within different treatments [model: phenotypic indicators = replication + treatment + sample (treatment)], followed by a multiple comparison. A threshold of $P < 0.05$ was taken to claim significance. All procedures were programmed to run in a SAS version 9.1 environment (SAS Institute, Cary, NC, USA).

Results

NIA1/2/NOA1-dependent NO production mediates Arabidopsis *salt tolerance*

To clarify how NO is synthesized in *Arabidopsis*, this study crossed *nia1/2* double mutants with the *noa1* mutant and found plants with homozygous mutations in the three genes by performing PCR-based genotyping of F_2 progeny (Supplementary Fig. S2). As expected (Lozano-Juste and León, 2010), stratified *nia1/2*, *noa1*, and *nia1/2/noa1* mutants could fully germinate in normal growth conditions. It was further observed that the *nia1/2/noa1* mutant was more sensitive to salinity stress than its parental lines, as measured by the responses of germination rate, and survival rate of germinated seeds (Supplementary Figs. S6 and S7). These results extended the observation reported by Zhao *et al.* (2004) and Lozano-Juste and León (2010).

To verify the possibility that *NIA1/2* and *NOA1* were closely associated with NO production, the responses of endogenous NO levels in *Arabidopsis* roots were examined by using a fairly specific NO-fluorescent probe (DAF-FM DA). As expected, compared with the wild-type, the basal or NaCl-induced DAF-FM-associated fluorescence levels were gradually reduced in the roots of *noa1*, *nia1/2*, and even *nia1/2/noa1* plants (Fig. 1A and Supplementary Fig. S8). It

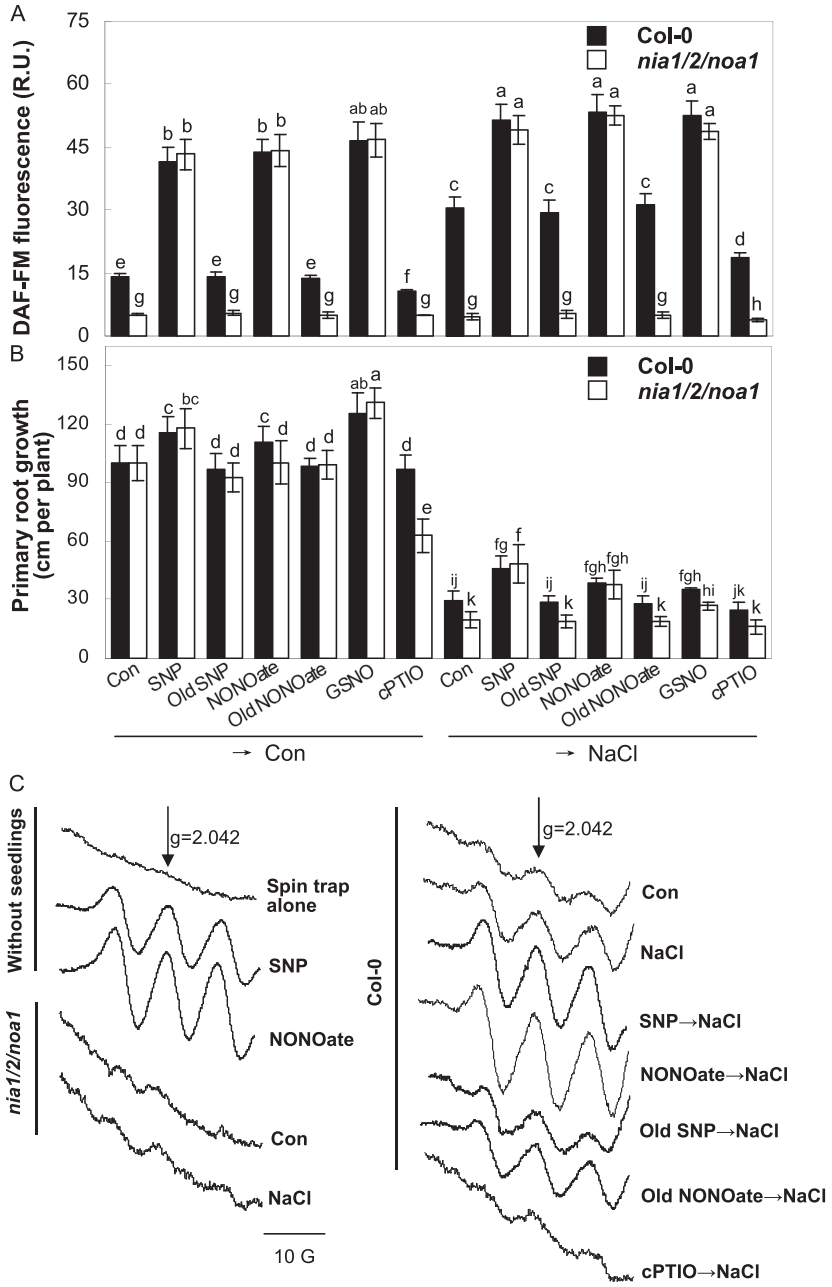

Fig. 1. NIA1/2/NOA1-dependent NO production and *Arabidopsis* salt tolerance. (A) DAF-FM fluorescence in wild-type and *nia1/2/noa1* mutant seedling roots detected by LSCM after preincubation in the absence or presence of 1 μM SNP, Old SNP, NONOate, Old NONOate, or GSNO, or 200 μM cPTIO for 1 h and then treatment with or without 150 mM NaCl for another 1 h. (B) Primary root growth of wild-type and *nia1/2/noa1* mutant seedlings after preincubation as indicated in Fig. 1A for 5 d and then treatment with or without 150 mM NaCl for 7 d. (C) NO content determined by electron paramagnetic resonance. After preincubation in the absence or presence of 10 μM SNP, Old SNP, NONOate, Old NONOate, or 400 μM cPTIO for 1 h, wild-type and *nia1/2/noa1* mutant seedlings were treated with or without 150 mM NaCl for another 1 h. A spin trap containing 10 μM SNP or 10 μM NONOate was used as positive controls. Signals were recorded at identical settings. Data are mean ± SE of three independent experiments. Means were compared by a multiple comparison on the basis of the nested ANOVA, with genotypes nested within different treatments [model: phenotypic indicators = replication + treatment + sample (treatment)]. Bars with different letters denote a significant difference according to multiple comparison ($P < 0.05$).

was further suggested that the detected fluorescence was specifically associated with the NO production because: (1) three types of NO-releasing compounds, namely SNP, NONOate, and GSNO, could obviously induce DAF-FM-associated fluorescence in both the wild-type and *nia1/2/noa1*; (2) Old SNP and Old NONOate, two degradation products of NO-releasing compounds, had no such inducible effects on the fluorescence intensity; and (3) the induced fluorescence detected in NaCl-treated roots was significantly reduced in the presence of (cPTIO), an NO scavenger (Fig. 1A).

It has been proposed that DAF might measure other reactive species in addition to NO (Mur *et al.*, 2011). To verify that the above-mentioned fluorescence changes were directly related to NO itself, analysis using EPR (Fig. 1C) and Griess reagent (Supplementary Fig. S9) were performed. The data presented were also in parallel with those of DAF-FM-associated fluorescence, further indicating that the DAF-FM-associated fluorescence, at least in these experimental conditions, was correlated with the NO production.

Subsequently, it was observed that the NaCl-induced growth inhibition of wild-type, *noa1*, *nia1/2*, and *nia1/2/noa1* plants were significantly rescued by the pretreatment with SNP, suggesting the potential role of NO in *Arabidopsis* salt tolerance (Fig. 1B and Supplementary Fig. S10). Further experiments revealed that three NO-releasing compounds markedly alleviated NaCl-triggered primary root growth inhibition. In contrast, no significant rescuing responses were observed when the degradation products of SNP (Old SNP, NO_2^-/NO_3^-, and $K_3Fe(CN)_6/K_4Fe(CN)_6$) or NONOate (Old NONOate) were applied (Fig. 1B and Supplementary Fig. S11). The treatment with SNP or GSNO alone produced similar positive responses in both the wild-type and *nia1/2/noa1* mutants under non-stressed conditions. A slight but no significant aggravation of NaCl-triggered primary root growth inhibition was observed by the pretreatment with cPTIO. The reversal behaviours of SNP and NONOate in the NaCl-triggered inhibition of seed germination appeared both in the wild-type and in particular the *nia1/2/noa1* mutant (Supplementary Fig. S12). Together, these findings suggested that *NIA1/2/NOA1*-dependent NO production might mediate *Arabidopsis* salt tolerance. Therefore, the *nia1/2/noa1* mutant line was used for further study.

The nia1/2/noa1 *mutant shows the compensatory and decay modes of* HY1*, antioxidant gene expression after short- and long-term salt treatment*

Quantitative real-time PCR was used to monitor the transcript levels of *HY1*, the antioxidant enzyme, and related transcription factors. As a control, expression analysis of *RD29A* and *RAB18*, two well-known stress responsive genes (Lozano-Juste and León, 2010), was also performed.

The results showed that, during a short treatment period (the first 48 h of NaCl treatment; Fig. 2A), *HY1* transcription in triple-mutant plants initially increased and reached a maximum level at 12 h of treatment, and then decreased steadily until 48 h. The upregulation of *RD29A* and *RAB18* gene expression occurred earlier than *HY1*, and their induction was much higher than *HY1* transcription. Further results showed that the accumulation of several antioxidant genes, including *GPX3*, *cAPX1*, *cAPX2*, and *FSD1*, were induced to different degrees, and with different kinetics, upon salt stress exposure in *nia1/2/noa1* mutant plants, with strong upregulation of *cAPX1* and *cAPX2*. In triple-mutant plants was also noticed the biphasic induction of *ZAT12*, which encodes a transcription factor that regulates *cAPX1* expression (Miller *et al.*, 2008), with a primary weak peak at 6 h, followed by a gradual decrease and reaching another stronger peak at 24 h. The transcript levels of *ZAT10*, which encodes a transcription factor responsible for *cAPX2* and *FSD1* expression (Miller *et al.*, 2008), increased 6 h after incubation in NaCl solution, followed by a decreasing tendency until 48 h. However, the induction of transcription in wild-type plants was differentially delayed or blocked.

Contrasting results appeared after long-term salt treatment (7 d of NaCl treatment; Fig. 2B). For instance, with respect to their untreated controls, *HY1* and *cAPX1* expression was elevated by 2 and 39% in the the wild-type subject to salinity stress, respectively, but both correspondingly reduced by 65 and 48% in the *nia1/2/noa1* mutants, indicating that *NIA1/2/NOA1* might be, at least partially, required for proper expression of *HY1* and *cAPX1* after long-term treatment. Interestingly, the weaker induction of *ZAT10* and *ZAT12* was observed in *nia1/2/noa1* mutants. Combined with the significant downregulation of antioxidant genes, these results supported the hypothesis that *ZAT10* and *ZAT12* were required for *cAPX2* and *FSD1*, and *cAPX1* expression, respectively, during salinity-induced oxidative stress.

Salinity hypersensitive responses of nia1/2/noa1 *mutants are reversed by CO*

Previously, pharmacological evidence has supported interactions between NO and CO in salt tolerance (Xie *et al.*, 2008) and the adventitious rooting process (Xuan *et al.*, 2008). Thus, the current study characterized this possibility with respect to salinity response at the genetic and molecular levels. As expected, pretreatment with the CO-releasing compound, CORM-2, as well as SNP (in particular; as a positive control), marginally alleviated the decreases of fresh weight, primary root growth, and chlorophyll contents in *nia1/2/noa1* mutants caused by the following salinity stress (Fig. 3A–D). However, there were no obvious differences in the non-stressed mutants regardless of whether CORM-2 was present or not. Interestingly, pretreatment with SNP alone brought about positive responses. It was also noticed that, with the pretreatment with Old CORM-2, the degradation product of CORM-2, which contained no CO gas, had no influence upon the growth status of *nia1/2/noa1* plants in both control and the salinity stress treatment (Supplementary Fig. S13).

Molecular evidence showed that upon salinity stress, expression of the corresponding genes was altered compared with normal growth conditions (Fig. 3E). Moreover, the pretreatment with CORM-2 or SNP (especially) could differentially weaken or block the downregulation of *cAPX1*, *cAPX2*, *FSD1*, and *GPX3*, and strengthen the upregulation of *ZAT10* gene expression in *nia1/2/noa1* mutants, compared to salinity stress alone. Surprisingly, the expression of *ZAT12* was unaffected by CORM-2 pretreatment, but was clearly induced by SNP. Meanwhile, NaCl treatment led to decreased *HY1*, *HO3*, and *HO4* expression, all of which were weakened or blocked by the application of CO- and in particular NO-releasing compounds. Therefore, these data indicate that salinity hypersensitivity and related gene expression patterns of *nia1/2/noa1* mutants were reversed or blocked differentially by CO (a synergistic mode).

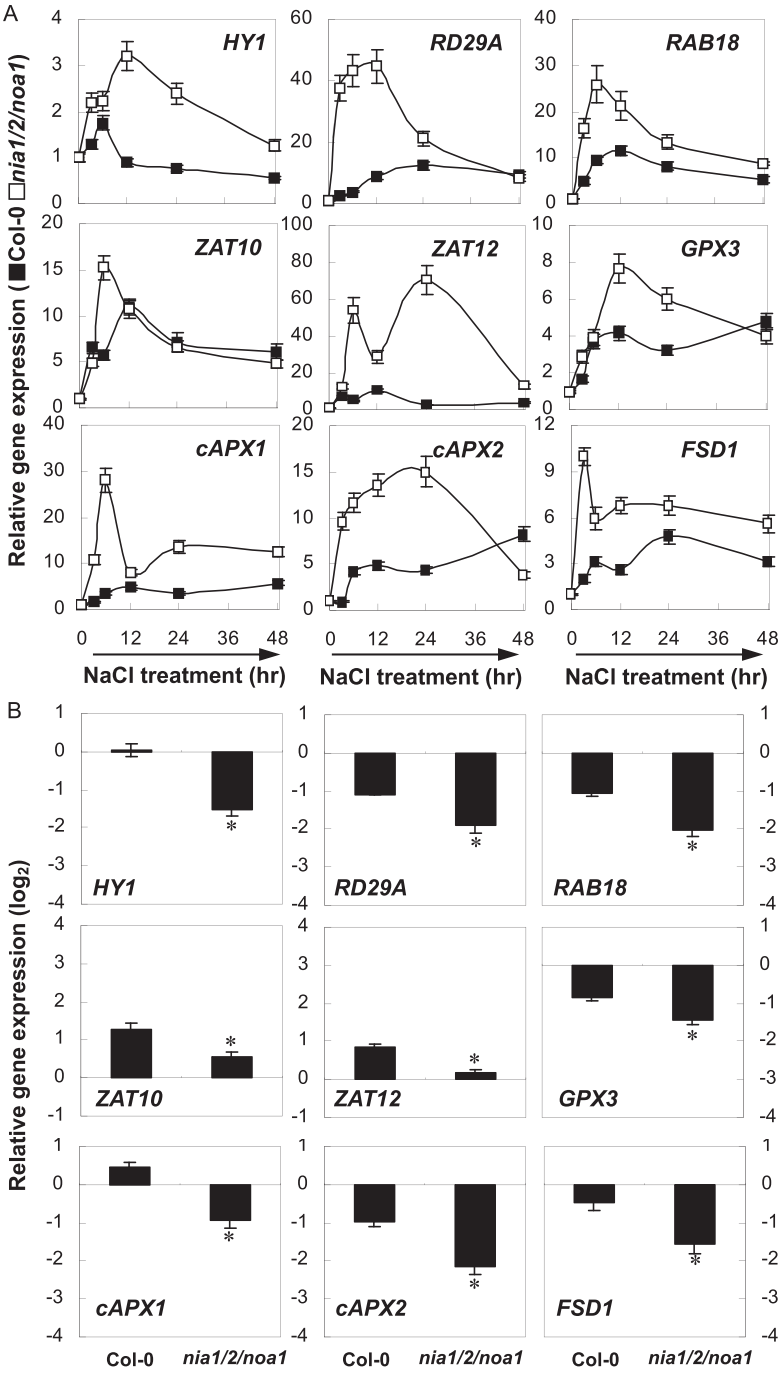

Fig. 2. Short-term compensation (A) and long-term decay (B) of NaCl-stress-related gene expression. The expression levels of representative genes in wild-type and *nia1/2/noa1* mutant seedlings over 48 h (A) or at 7 d (B) of 150 mM NaCl treatment were analysed by real-time RT-PCR. Expression levels were presented as values relative to corresponding untreated control samples at 0 h (A), or 7 d (B) after normalization to *actin2/7* levels. Data are mean ± SE of three independent experiments. Bars with asterisks are significantly different compared with the wild-type at $P < 0.05$ (*t*-test).

Contrasting phenotypes of loss- and gain-of-function mutations of HY1 *are sensitive to NO*

To further investigate the link between HY1 and NO during salt tolerance, *HY1* loss-and-gain mutants were used. Compared with the wild-type plants, a similar but stronger induction of NO appeared after 1 h of salt stress treatment in the HY1 mutant (*hy1-100*), as detected by the DAF-associated fluorescence and the EPR assay (Fig. 4A and Supplementary Fig. S14). Interestingly, the decreased tendency of DAF-FM level during the remainder of the monitored period was somewhat attenuated in the *hy1-100* mutant, compared to the wild-type, although both were below the basal level (dashed line). Comparatively, salt stress brought about stronger but delayed fluorescence accumulation in *35S:HY1-4*, a previously proven gain-of-function in a mutant of HY1 (Xie *et al.*, 2011b).

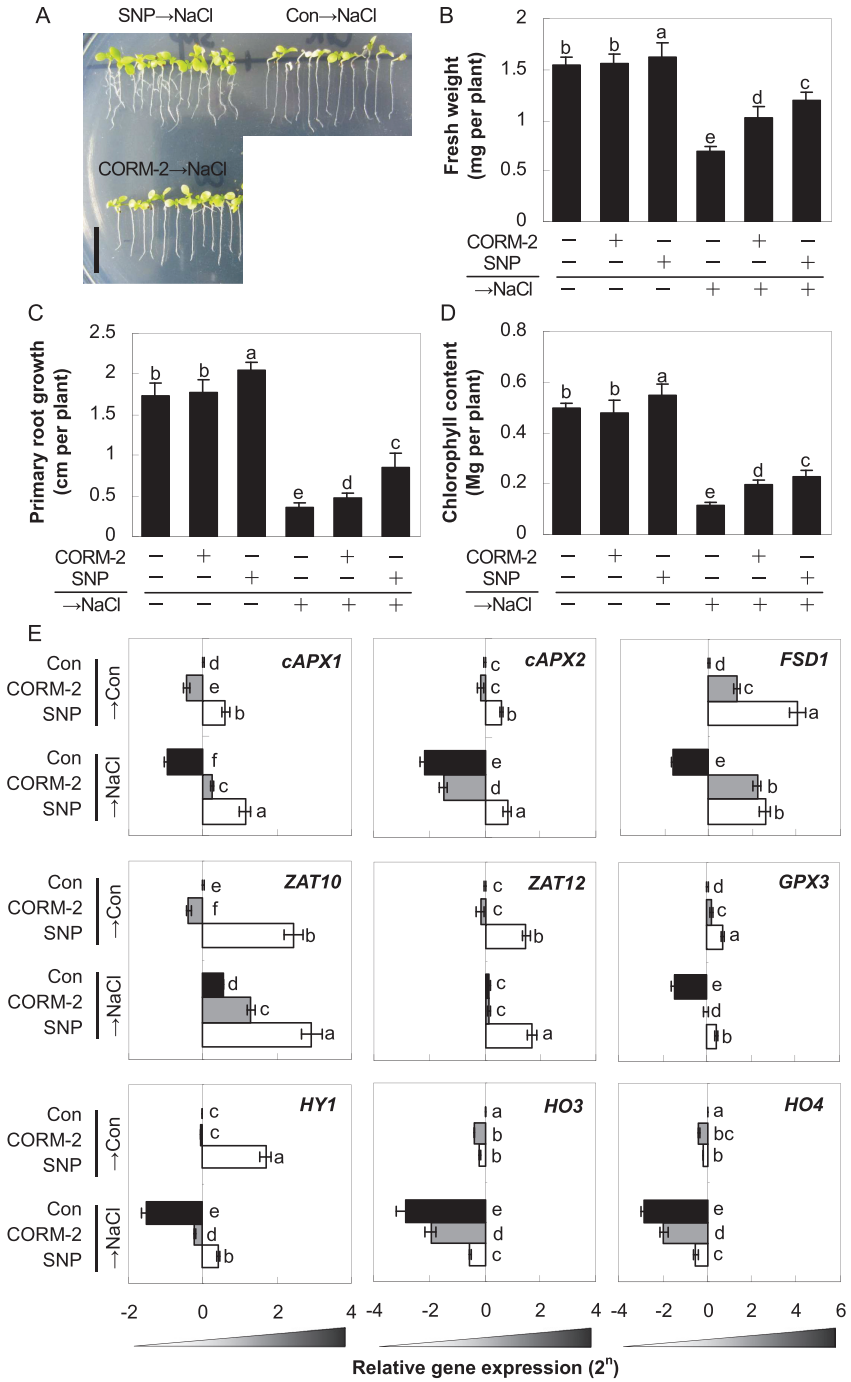

Fig. 3. Effects of CO- and NO-releasing compounds on morphology (A), fresh weight (B), primary root growth (C), chlorophyll content (D), and representative stress-related genes (E) in NO-deficient mutant upon salt stress. *nia1/2/noa1* mutant plants were pretreated for 5 d with or without 0.1 μM CORM-2 or 1.0 μM SNP and then grown with or without 150 mM NaCl for 7 d. Bar = 1 cm (A). The expression levels of representative genes analysed by real-time RT-PCR. Expression levels are presented as values relative to corresponding untreated control samples, after normalization to *actin2/7* levels. Data are mean ± SE of three independent experiments. Bars with different letters denote a significant difference compared with the control according to multiple comparison ($P < 0.05$).

A subsequent experiment showed that compared with wild-type plants, the *hy1-100* mutant exhibited salinity hypersensitivity, as validated by the observed inhibition of seedling growth and changes in primary root length. Contrasting responses were observed in the *HY1* overexpression line. Pretreatment of two *hy1* mutant alleles with two NO-releasing compounds, SNP or NONOate, significantly alleviated salt stress symptoms (Fig. 4B, C and Supplementary Fig. S15). However, no significant changes were observed for the pretreatment with Old SNP or Old NONOate (Fig. 4C). It should be noted that there was no obvious difference in *35S:HY1-4* plants, regardless

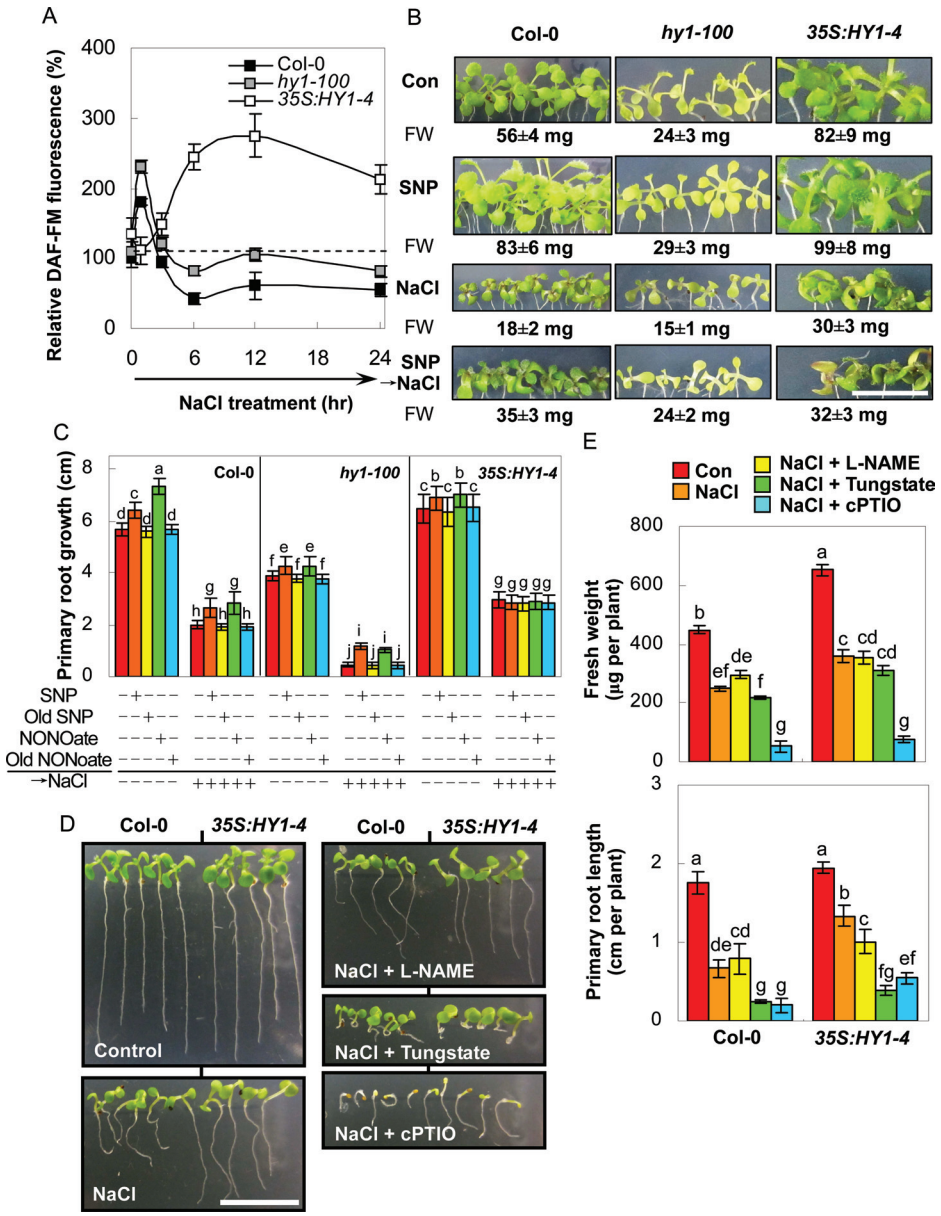

Fig. 4. Contrasting responses of HY1 loss-and-gain mutants were sensitive to NO. (A) Time course of 150 mM NaCl-induced DAF-FM fluorescence in root tips of the wild-type, *hy1-100* mutant, and *HY1* overexpression line *35S:HY1-4* seedlings as detected by LSCM. (B, C) Morphology and total fresh weight (B), and primary root growth (C) of wild-type, *hy1-100*, and *35S:HY1-4* plants pretreated for 5 d with or without 1.0 μM SNP (B), or 1.0 μM SNP, Old SNP, NONOate, or Old NONOate (C) and then grown with or without 150 mM NaCl for 7 d. Bar = 1 cm (B). (D, E) Morphology (D) and fresh weight and primary root length (E) of stratified seeds of wild-type and *35S:HY1-4* plants grown in the presence or absence of 150 mM NaCl with or without 200 μM L-NAME, tungstate, or cPTIO, respectively for 7 d. Bar = 1 cm (D). Data are mean ± SE of three independent experiments. Means were compared by a multiple comparison on the basis of the nested ANOVA, with genotypes nested within different treatments [model: phenotypic indicators = replication + treatment + sample (treatment)]. Bars with different letters denote a significant difference according to multiple comparison ($P < 0.05$).

of whether NaCl was present together with or without NO-releasing compounds.

The alleviating effects of NO-releasing compounds on salt-triggered growth inhibition of the two *hy1* mutant alleles suggested that these phenotypes may result from the reductions of endogenous NO concentrations. To test this hypothesis, the effects of a NO scavenger cPTIO, a mammalian NOS inhibitor (L-NAME), and a plant NR inhibitor (tungstate) on salt tolerance of the *HY1* overexpression line were evaluated. L-NAME had no obvious influence on the fresh weight of the wild-type and *35S:HY1-4* plants upon NaCl stress, whereas the application with tungstate resulted in significant reduced primary root growth (Fig. 4D, E). Meanwhile, there was a slight but non-significant decrease in fresh weight after the addition of tungstate to the stressed wild-type and overexpression line. Combined with the maximum inhibition caused by cPTIO, it was postulated that changes in endogenous NO might be involved in *hy1*-mediated salt hypersensitivity and

that NR-mediated NO production plays a major role in this process (a synergistic mode).

hy1 *mutant also shows the compensatory and decay modes of antioxidant gene expression after short- or long-term salt treatment*

During the first 48 h after NaCl treatment, although there was rapid induction of *ZAT10*, *ZAT12*, and *GPX3* in *hy1-100* mutant, the downregulation of several antioxidant genes, including *cAPX1*, *cAPX2*, and *FSD1*, were modulated to different degrees and with various kinetics (Fig. 5A). The data obtained indicated that *ZAT10*- and *ZAT12*-triggered transcriptional induction of corresponding antioxidant genes during a short treatment period was mostly dependent on HY1- and/or its catalytic products.

After long-term salt treatment, the upregulation of *ZAT10* and *ZAT12* or the downregulation of *GPX3*, *cAPX1*, *cAPX2*, and *FSD1* were aggravated or blocked by SNP and NONOate (both in particular), and CORM-2 in *hy1-100* mutants (Fig. 5B), consistent with the reversing effects of salinity hypersensitivity (Fig. 4B, C; Xie *et al.*, 2011b). Meanwhile, under the control conditions, expression of these genes was induced differentially in mutant plants pretreated with NO- (in particular) or CO-releasing compounds, except for the downregulation of *cAPX1* and *cAPX2* transcripts upon CORM-2 pretreatment.

Salt hypersensitivity phenotypes of NO/HY1-deficient quadruple mutant was blocked by NO and CO

To gain insight into the compensatory and synergistic modes of NR/NOA1-dependent NO and HY1 signal transduction, a quadruple mutant *nia1/2/noa1/hy1-100* was constructed and verified by PCR-based genotyping and Western blot analysis (Supplementary Figs. S2 and S3). The *nia1/2/noa1/hy1-100* mutant plants displayed delayed vegetative growth but promoted reproductive growth throughout its whole life cycle (Supplementary Figs. S4 and S5), for example their true leaves developed significantly later and smaller than those in wild-type plants, but their bolting and flowering times were largely accelerated, both of which were as a result of additive effects from *nia1/2/noa1* and *hy1-100* mutant phenotypes. Comparatively, this mutant showed increased salinity hypersensitivity compared with its parental lines (Supplementary Table S3), which could be differentially reversed by the pretreatment with SNP or NONOate (both in particular), or CORM-2 (Fig. 6A, B). Interestingly, only NO-releasing compounds could obviously induce mutant seedling growth in the absence of NaCl. No significant changes were observed for the pretreatment with their degradation byproducts, respectively (Supplementary Figs. S13 and S16).

Analysis of antioxidant gene expression in NO/HY1-deficient quadruple mutant

Further molecular evidence showed that pretreatment with SNP and NONOate could differentially weaken or block the downregulation of *ZAT12*, *GPX3*, *cAPX1*, *cAPX2*, and *FSD1* or strengthen the upregulation of *ZAT10* gene expression in the quadruple mutant subjected to NaCl treatment for 7 d. Comparatively, no response or only weak responses were found with CORM-2 pretreatment (except *ZAT10*). This finding perfectly matches the changes in corresponding phenotypes (Fig. 6A, B). Upon normal growth conditions, compared to untreated control plants, expression of the some of these genes was differentially upregulated, although *GPX3*, *cAPX1*, and *FSD1* transcripts were inhibited by CORM-2.

Discussion

Arabidopsis hy1-100, nia1/2/noa1, *and* nia1/2/noa1/hy1-100 *mutants exhibit progressive salt hypersensitivity*

In this study, *Arabidopsis hy1*, *nia1/2/noa1*, and *nia1/2/noa1/hy1* mutants exhibited progressive salt hypersensitivity, as evaluated by primary root growth inhibition and fresh weight loss (compared with Figs. 1, 3, 4 and, 6; also shown in Supplementary Table S3).

First, the results (Fig. 4 and Supplementary Fig. S7) confirmed the previous findings that *hy1-100* and *noa1* plants were more salt-sensitive than the wild-type (Zhao *et al.*, 2007; Xie *et al.*, 2011b). It was also observed that the *nia1/2/noa1* mutant was more salinity sensitive than its parental lines, as determined by changes in seed germination and survival rates (Supplementary Figs. S6 and S7), the former of which was confirmed previously by Lozano-Juste and León (2010). It was further observed that salt treatment failed to trigger NO production in *nia1/2/noa1*, as evaluated by the combination of DAF-FM fluorescence, EPR, and Griess reagent assays (Fig. 1A, C and Supplementary Fig. S9). Therefore, it was deduced that both NR and NOA1 were responsible for salt-induced NO production. Except for a rapid burst of stimulation, NO production in the *hy1-100* mutant, however, was always below the basal level during 24 h of salt stress (Fig. 4A).

Subsequent results illustrated that the salinity-induced inhibition of primary root growth was relieved by 33, 156, 141, and 185% in SNP-pretreated wild-type, *hy1-100*, *nia1/2/noa1*, and *nia1/2/noa1/hy1-100* mutants, respectively (Figs. 1B, 3C, 4C, and 6B). There were similar rescuing effects triggered by the pretreatments with other NO-releasing compounds, NONOate and GSNO. Meanwhile, the degradation byproducts of SNP and NONOate produced no such effects (Figs. 1B and 4C and Supplementary Figs. S11 and S16). In contrast, the gain-of-function mutant of HY1 (*35S:HY1-4*) not only led to stronger but delayed NO production, but also displayed tolerance characteristics, which were reversed by the NR inhibitor tungstate and the NO scavenger cPTIO in particular, and slightly by the mammalian NOS inhibitor L-NAME (Fig. 4). These results further demonstrated that *NIA1/2*-dependent NO production mainly mediated *Arabidopsis* salt tolerance. The additive response in salt hypersensitivity of the quadruple mutant *nia1/2/noa1/hy1-100* (Supplementary Table S3) also supported this proposition.

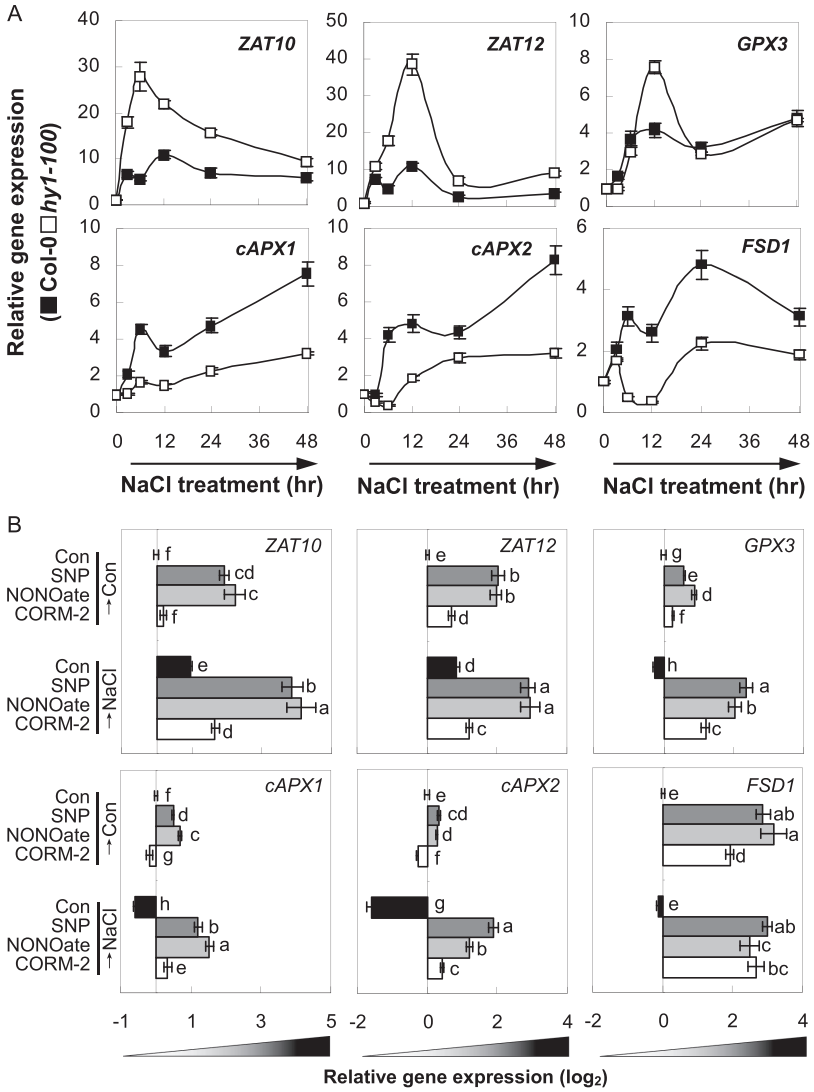

Fig. 5. Short-term and long-term effects of NaCl on antioxidant enzymes and related transcription factors gene expression. (A) Time course analysis of gene expression in wild-type and *hy1-100* mutant seedling roots upon 150 mM NaCl. (B) Effect of pretreatment with SNP, NONOate, or CORM-2 on expression of antioxidant genes and related transcription factors in *hy1-100* mutant seedlings upon NaCl stress. Seedling plants were pretreated for 5 d with or without 1.0 μM SNP or NONOate, or 0.1 μM CORM-2 and then grown with or without 150 mM NaCl for 7 d. Gene expression was analysed by real-time RT-PCR and relative expression levels are presented as values relative to corresponding control samples at the indicated time after normalization to *actin2/7* levels. Data are mean ± SE of three independent experiments. Bars with different letters denote a significant difference compared with the control according to multiple comparison ($P < 0.05$).

The reduced NO accumulation in the *noa1* mutant has been ascribed to its inability to accumulate carbon reserve (sucrose and fumarate, etc); and application of 1% sucrose could rescue its pale green phenotypes (Van Ree *et al.*, 2011). By contrast, a recent study showed that sucrose levels did not contribute to the *noa1*-mediated restoration of *ssi2*-triggered defence phenotypes (Mandal *et al.*, 2012). Here, it was confirmed that, compared with wild-type seedlings, the *noa1* mutant displayed severe inhibition of fresh weight under control growth conditions with 1% sucrose (Supplementary Fig. S7; 38 versus 24% compared with the results of Van Ree *et al.*, 2011). These discrepancies may be explained by the differences in growth conditions, including temperature, light, and nitrogen supplementation. Exogenous application of nitrogen was essential for culturing NR-null mutants (Zhao *et al.*, 2009). In this study, all ecotypes were sown in identical plates containing nitrogen. Notably, the *nia1/2/noa1* mutant was severely impaired in both basal and salinity-induced NO synthesis, and additive hypersensitive phenotypes were observed (Fig. 1 and Supplementary Figs. S6 and S7). Consistently, NIA1/2- and NOA1-mediated pathways of NO biosynthesis were also additive in terms of abscisic acid-mediated inhibition of germination and seedling establishment regardless of the addition or not of sucrose (Lozano-Juste and León, 2010). Strikingly, cold acclimation induced greater freezing tolerance of the *noa1* mutant than wild-type plants (Zhao *et al.*, 2009). Thus, the detailed mechanism of NOA1 in the regulation of NO synthesis and its physiological roles are yet to be fully investigated.

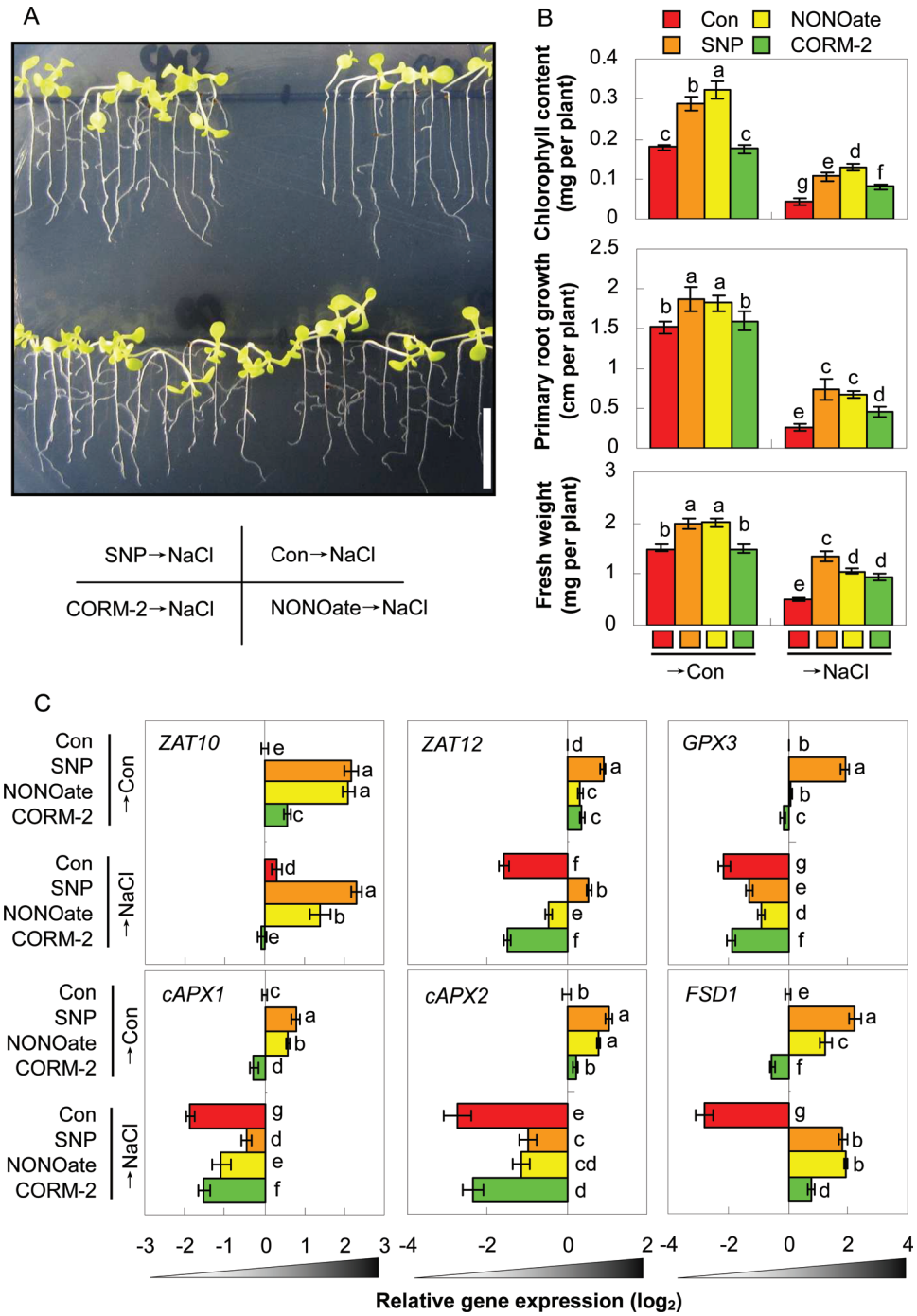

Fig. 6. Rescuing effects of NO/CO-releasing compounds on quadruple mutant *nia1/2/noa1/hy1-100* plants. Plants were pretreated for 5 d with or without 1.0 μM SNP or NONOate, or 0.1 μM CORM-2 and then grown with or without 150 mM NaCl for 7 d. (A) Morphology; bar = 1 cm. (B) Corresponding chlorophyll content, primary root growth, and fresh weight. (C) Expression levels of representative genes analysed by real-time RT-PCR, presented as values relative to corresponding control samples after normalization to *actin2/7* levels. Data are mean ± SE of three independent experiments. Bars with different letters denote a significant difference compared with the control according to multiple comparison ($P < 0.05$).

Compensatory and synergistic modes between NIA/NR/NOA1-dependent NO production and HY1 *expression*

The relationship between NO and HO1/CO, in a number of animal responses, was usually synergistic, complementary, or independent (Otterbein *et al.*, 2003; Bauer *et al.*, 2008; Pae *et al.*, 2010). Since NO and HO1/CO in plants usually have similar physiological roles (Noriega *et al.*, 2007; Xie *et al.*, 2008; Xuan *et al.*, 2008; Santa-Cruz *et al.*, 2010), it is most likely that decreased NO production in plants subjected to environmental stimuli may be complemented by HO1/CO, and vice versa.

In this study's subsequent experiment, although the *nia1/2/noa1* mutant exhibited a more severe hypersensitive response than its parental lines (Supplementary Fig. S3, S4), this triple mutant displayed significantly increased ability to maintain the redox homeostasis – indicated by the upregulation of *HY1*, *GPX3*, *cAPX1/2*, *FSD1*, and *ZAT10/12* transcription after short-term salt treatment (Fig. 2A). Therefore, it was deduced that although the systemic signalling role of NO is associated with plant salt tolerance, the NO-deficient mutants could increase expression of *HY1* and other specific defence genes, at least temporarily, to compensate for the lack of NO. This conclusion was confirmed by the fact that NaCl failed to trigger NO accumulation in *nia1/2/noa1* roots (Fig. 1A, C). A similar compensatory mode was observed for *ZAT10*, *ZAT12*, and *GPX3* expression in the *hy1-100* mutant (Fig. 5A). Bonifacio *et al.* (2011) also reported a compensatory response, showing that rice mutants double-silenced for cytosolic ascorbate peroxidase (APx1/2s) upregulated other peroxidases, which enabled the rice plants to cope with a number of stresses. Similarly, our previous reports demonstrated that cadmium-induced upregulation of *HO1* gene expression was associated with glutathione depletion, leading to transiently enhanced antioxidant capability (Cui *et al.*, 2011).

Normally, plants contain a highly efficient antioxidant defence system that can scavenge/detoxify ROS, thereby increase the salt tolerance (Ashraf, 2009). After long-term salt treatment, the majority of antioxidant gene transcripts were diminished significantly (Figs. 2B and 5B), consistent with corresponding salt hypersensitivity phenotypes (Figs. 1B and 4B, C). These results further illustrated that the temporary compensatory mode of antioxidant defence induction could not make up for the deficiency of NO or HY1 to withstand long-term saline conditions (Fig. 7). Thus, the results confirmed that NO and HY1 were beneficial and essential for plant adaptation to salt stress and that disturbed NO production and HY1 expression reduced plant's long-term adaptation to salt stress. Similarly, previous studies showed that most antioxidants transcripts/activities were increased or decreased upon short- or long-term zinc stress, separately (Xu *et al.*, 2010); the content of a series of amino acids, also increased during short-term NaCl stress, but decreased with long-term treatment periods (Kim *et al.*, 2007).

There was also a synergistic mode, for example pretreatment of *nia1/2/noa1* mutants with the CORM-2 significantly alleviated salt hypersensitivity phenotypes (Fig. 3A–D). Molecular evidence further showed that CORM-2 could differentially

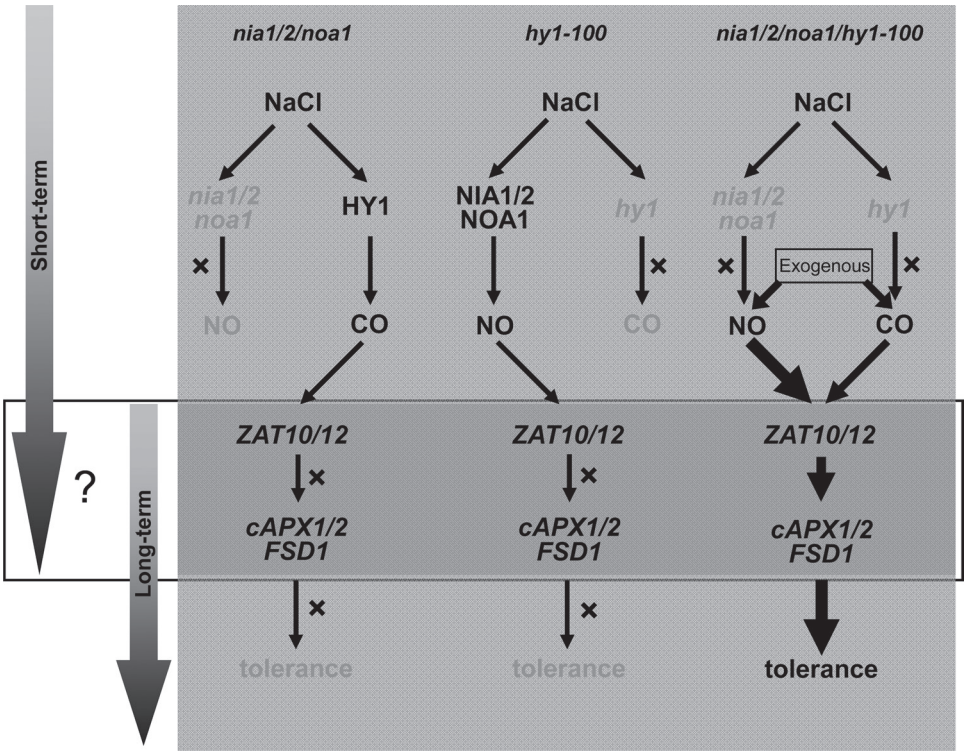

Fig. 7. Schematic representation of the proposed compensatory and synergistic modes of NIA/NR/NOA1-dependent NO production and HY1 expression in salt-tolerance signalling pathway. The relay of short-term signalling and long-term salt adaptation was mediated by unknown mechanisms/factors (question mark). The ZAT10/12-mediated antioxidant defence might be involved in the overlapping. The mutation of NIA/NR/NOA1 or HY1 was accompanied with the compensated upregulation of HY1 expression or NIA/NR/NOA1-associated NO production. However, the short-term compensatory mode followed by the downregulation of antioxidant defence system (*cAPX1/2* and *FSD1*, etc.) was not sufficient for long-term salt tolerance. Hypersensitivity and corresponding parameters could be partially reversed by NO- (in particular) or CO-releasing compounds, a synergistic mode. In the NO/CO-deficient quadruple mutant, the application of NO- (in particular) or CO-releasing compounds could differentially trigger ZAT10/12-mediated long-term antioxidant defence, thus leading to the salt-tolerance phenotype.

rescue the downregulation the expression of HO1 subfamily, antioxidant defence genes, and related transcription factors (Fig. 3E). There were similar rescuing responses in *hy1-100* mutant plants with the addition of NO-releasing compounds (Figs. 4B, C, and 5B). Meanwhile, the salt-tolerant phenotype of *35S:HY1-4* was significantly arrested when NO production was scavenged or inhibited, whereas no additive effects were found when SNP was added (Fig. 4B–E). Finally, the aggravated hypersensitivity symptom and changes in corresponding genes of quadruple mutant *nia1/2/noa1/hy1-100* could be differentially rescued by NO- and CO-releasing compounds, with SNP displaying the maximum responses (Fig. 6). Meanwhile, the degradation byproducts of NO- and CO-releasing compounds produced no such effects (Supplementary Figs. S13 and S16). These results, together with the observations that SNP pretreatment exhibiting the maximal rescuing responses in all mutant ecotypes, suggests that NO might occupy the dominant position in the interaction between NIA/NR/NOA1-dependent NO production and HY1 expression in mediating salt-tolerance signalling (Fig. 7).

How do NIA/NR/NOA1-dependent NO and HY1 work in mediating salt-tolerance signalling?

Transcriptional factor ZAT10/12 was shown to play a key role in modulating plant defence responses (Mittler, 2002; Davletova *et al.*, 2005b; Mittler *et al.*, 2006). The ZAT10/12-dependent increase of *Arabidopsis* tolerance to abiotic stresses could be attributed to the specific activation of ROS-related defence genes, including *cAPX1*, *cAPX2*, and *FSD1* (Miller *et al.*, 2008). Additionally, glutathione peroxidase3 (GPX3) was found to act as a ROS scavenger and specifically relay the H_2O_2 signal as an oxidative signal transducer (Miao *et al.*, 2006). Molecular evidence illustrated that expression levels of the majority of *ZAT10/12* and related ROS-scavenging enzymes in *nia1/2/noa1*, *hy1-100*, and *nia1/2/noa1/hy1-100* mutants were positively regulated by pretreatment with NO/CO-releasing compounds – consistent with the alleviation symptoms (Figs. 1B and 3–6). These findings supported that ZAT10/12-associated ROS metabolism, together with the GPX3, were correlated with the NO/HY1-mediated long-term salt tolerance (Fig. 7).

The opposite induction mode of *ZAT10/12* and *cAPX1/2* in the *hy1-100* mutant during short-term salt stress is noteworthy (Fig. 5A). These results were consistent with this study group's previous reports showing that HY1 is involved in the induction of *cAPX1* expression during salt acclimation (Xie *et al.*, 2011b). In fact, ZAT12 was shown to be required for *cAPX1* expression in response to oxidative stress (Rizhsky *et al.*, 2004; Davletova *et al.*, 2005a). Overexpression of *ZAT12* did not induce *cAPX1* transcript level, further indicating a complex relationship between *ZAT12* and *cAPX1*, and an additional factor(s) may be required for the *cAPX1* expression (Rizhsky *et al.*, 2004). Interestingly, the expression of *ZAT12*, controlling a regulon of 42 different genes involved in abiotic stress, was elevated in a *cAPX1*-knockout mutant during several abiotic stresses (Davletova *et al.*, 2005a). This study further hypothesizes that ZAT12 might be crucial in relaying short-term compensation to long-term tolerance through a complex signalling network, and HY1 as well as other unknown factor(s) might be involved in the ZAT12-regulated *cAPX1* expression (Fig. 7). Further genetic evidence is required to confirm this hypothesis.

In summary, this report provides, as far as is known, the first mechanistic description of the link between *Arabidopsis* HY1 and NIA/NR/NOA1-dependent NO and their functions in salt tolerance and antioxidant defence gene expression. Thus, the ongoing analysis of the role of other HY1/NO target genes should open new window in the understanding of salt signalling.

Supplementary material

Supplementary data are available at *JXB* online.

Supplementary Fig. S1. Morphology, chlorophyll content, and survival rate of stratified seeds of wild-type and *nia1/2* mutant plants grown with or without nitrate and ammonium for 20 d.

Supplementary Fig. S2. Genotyping of the *nia1/2/noa1* and *nia1/2/noa1/hy1-100* mutants.

Supplementary Fig. S3. Detection of HY1 protein level in 5-d-old seedlings of wild-type, *hy1-100*, L*er*, *hy1*, *noa1*, *nia1/2*, *nia1/2/noa1*, and *nia1/2/noa1/hy1-100* by Western blot analysis.

Supplementary Fig. S4. Developmental phenotypes of wild-type, *hy1-100*, *nia1/2/noa1*, and *nia1/2/noa1/hy1-100* plants at the early seedling stage.

Supplementary Fig. S5. Developmental phenotypes of wild-type, *nia1/2/noa1*, *hy1-100*, and *nia1/2/noa1/hy1-100* plants at the vegetative and reproductive growth stages.

Supplementary Fig. S6. Dose-dependent inhibition of seed germination in wild-type, *noa1*, *nia1/2*, and *nia1/2/noa1* induced by increasing NaCl concentrations for 8 d.

Supplementary Fig. S7. Fresh weight and survival rate of stratified seeds of wild-type, *noa1*, *nia1/2*, and *nia1/2/noa1* with or without 150 mM NaCl treatment for 20 d.

Supplementary Fig. S8. DAF-FM-associated fluorescence in seedling roots of wild-type, *noa1*, *nia1/2*, and *nia1/2/noa1*.

Supplementary Fig. S9. Determination of NO content in wild-type and *nia1/2/noa1* seedlings by Griess reagent assay.

Supplementary Fig. S10. Effect of SNP on growth of wild-type, *noa1*, and *nia1/2* plants.

Supplementary Fig. S11. Effect of NO_2^-/NO_3^-, $K_3Fe(CN)_6$/$K_4Fe(CN)_6$ and NaCl on growth of wild-type seedlings.

Supplementary Fig. S12. Germination rate of wild-type and *nia1/2/noa1* mutant seeds with or without 150 mM NaCl and with or without 1 μM SNP or 1 μM NONOate for 3 d.

Supplementary Fig. S13. Effects of CORM-2 and Old CORM-2 on salt hypersensitivity phenotypes of *nia1/2/noa1* and *nia1/2/noa1/hy1-100* mutant plants.

Supplementary Fig. S14. Determination of NO content in wild-type, *hy1-100*, and *35S:HY1-4* seedlings by electron paramagnetic resonance.

Supplementary Fig. S15. Effects of NONOate and SNP on growth and chlorophyll contents of *hy1* mutant plants.

Supplementary Fig. S16. Effects of fresh and old NO-releasing compounds on salt hypersensitivity phenotypes of *nia1/2/noa1/hy1-100* mutant plants.

Supplementary Table S1. Primers for genotyping.

Supplementary Table S2. Primers for real-time RT-PCR.

Supplementary Table S3. Comparative analysis of seedling growth inhibition of wild-type, *hy1-100*, *nia1/2/noa1*, and *nia1/2/noa1/hy1-100* mutants grown with 150 mM NaCl stress for 7 d.

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