Nitric Oxide Controls Constitutive Freezing Tolerance in Arabidopsis by Attenuating the Levels of Osmoprotectants, Stress-Related Hormones and Anthocyanins

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Plant tolerance to freezing temperatures is governed by endogenous constitutive components and environmental inducing factors. Nitric oxide (NO) is one of the endogenous components that participate in freezing tolerance regulation. A combined metabolomic and transcriptomic characterization of NO-deficient nia1,2, noa1–2 mutant plants suggests that NO acts attenuating the production and accumulation of osmoprotective and regulatory metabolites, such as sugars and polyamines, stress-related hormones, such as ABA and jasmonates, and antioxidants, such as anthocyanins and flavonoids. Accordingly, NO-deficient plants are constitutively more freezing tolerant than wild type plants.

Plants ability to tolerate below zero temperatures relies on complex and varied processes that involve the accumulation of endogenous as well as inducible components often regulated by environmental factors. The endogenous components that favor constitutive freezing tolerance have been extensively studied and mainly refer to metabolites with osmoprotective activities to limit freeze-induced dehydration and to avoid ice nucleation inside the cells, with hormonal activities or with antioxidant functions. Moreover, other regulatory molecules such as polyamines, lipids, reactive oxygen species and nitric oxide (NO) have also been described to be involved in freezing tolerance.

NO is endogenously produced in diverse living organisms and regulates a wide array of biological processes including many responses of plants to environmental abiotic and biotic stresses. In plants, NO is generated through both oxidative and reductive biosynthetic pathways, which are enhanced under stress. NO is a free radical that react with metals and reactive oxygen species (ROS), thus contributing to redox homeostasis and alleviating oxidative stress. It has been also reported that NO improves the antioxidant capacity of plants. However, NO can potentiate or attenuate oxidative stress in plants when acts either in a chronic or acute mode. Besides redox-related functions, NO has also the potential of triggering post-translational modifications (PTMs) of many target proteins, which then display often altered function, activity, stability and/or subcellular localization. NO induces S-nitrosylation of Cysteine and nitrination of Tyrosine, but also ubiquitylation of Lysine and phosphorylation of Serine, Threonine and Tyrosine. In addition, many of those PTMs also alter the stability of the target proteins through the regulation of proteolytic degradation.

Genetic approaches using the Arabidopsis nia1,2 mutant plants, impaired in nitrate reductase-mediated synthesis of NO, as well as pharmacological treatments with NO donors, inhibitors and scavengers allowed proposing a role for NO in constitutive freezing tolerance in Arabidopsis. However, the contribution of nitrate-independently produced NO to low temperature responses has been barely addressed. In this work, we

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have studied the constitutive freezing tolerance and the capacity to cold acclimate of triple nia1,2noa1–2 mutant plants that are impaired in nitrate-dependent and nitrate-independent NOA1-associated pathways and thus contain very low levels of NO\textsuperscript{21}. Dissection of the regulatory roles exerted by NO on constitutive freezing tolerance has been aided by an integrative approach combining metabolomic and transcriptomic analyses.

**Results**

The transcriptome of NO-deficient nia1,2noa1–2 mutant plants is enriched in cold-related transcripts. We previously reported that nia1,2noa1–2 mutant plants, carrying mutations in both NIA1 and NIA2 nitrate reductases, as well as in the NO-Associated 1 (NOA1) protein, accumulated very low levels of endogenous NO under control and stressed conditions\textsuperscript{21}. The strong NO deficiency of the mutant plants correlated with hypersensitivity to ABA in seed germination, stomata closure and tolerance to dehydration\textsuperscript{21}. Intriguingly, our transcriptome analysis of nia1,2noa1–2 mutants grown at 20 °C (GEO identification number GSE41958)\textsuperscript{22} revealed that around 20% (88/465) of the genes that were up-regulated in the mutant compared to wild-type plants (>2-fold; FDR < 0.05) had been related to cold responses\textsuperscript{23,24}. Among those genes (Table S1), some coded for Late Embryogenesis Abundant (LEA) proteins and for transcription factors belonging to the ERF/DREB, Zinc finger and WRKY families. Cold-induced BCH2 and NCED3 genes, encoding β-carotene hydroxylase 2 and 9-cis-epoxy-carotenoid dioxygenase 3 enzymes involved in ABA biosynthesis, as well as LOX4 and OPRI coding for jasmonate biosynthesis enzymes were also up-regulated in NO-deficient plants (Table S1). ABA and jasmonates have been reported to positively regulate freezing tolerance in Arabidopsis\textsuperscript{25–28}. Furthermore, a Gene Ontology analysis performed with the Arabidopsis thaliana dataset of the Gene Ontology Consortium (http://www.geneontology.org/) showed that 7 out of 19 and 15 out of 67 genes (20- and 12-fold enrichment with p-values of 2.51E-04 and 1.71E-08) involved in the anthocyanin and flavonoid metabolism functional categories, respectively, were up-regulated in the NO-deficient mutant plants. Accordingly, we found the anthocyanin and flavonoid biosynthesis and metabolism genes CHS, F3’H/TT6, DFR/TT3, PAP1/MYB75 and UF3GT among the cold-induced genes that were up-regulated in nia1,2noa1–2 plants (Table S1). In addition, genes coding for SUS3, SSP2, and ADC2 enzymes involved in the biosynthesis of sugars and polyamines, respectively, were among the cold-inducible genes up-regulated in NO-deficient plants (Table S1). Sugars and polyamines had been reported to enhance plant-freezing tolerance\textsuperscript{27–29}. To assess the robustness of the over-representation of cold-inducible genes detected in our transcriptomic analysis, the expression levels of 11 cold-induced genes, including ADH1, ZAT10, NCED3, HAI1, SnRK2.9, LEA7, LEA4–5, LTI65, PAP1/MYB75, DFR and CYP707A3, were determined by RT-qPCR in independent RNA samples from the triple nia1,2noa1–2 mutant and Col-0 plants grown at 21 °C. In all cases, the transcript levels were significantly higher in mutant than in wild-type plants (Fig. 1a), thus validating the microarray data. These observations indicated that, under control conditions, NO functions as a negative regulator of cold-induced gene expression in Arabidopsis.

**Enhanced biosynthesis of ABA, JA and osmoprotective metabolites in NO-deficient plants.**

Data from microarray analyses strongly suggested that NO-deficient mutants should have increased levels of ABA, JA, anthocyanins, flavonoids, sugars and polyamines. Ultra Performance Liquid Chromatography-Mass Spectrometry analysis confirmed that, in fact, the levels of ABA and JA were around 2-fold higher in nia1,2noa1–2 than in wild-type plants (Fig. 2a). On the other hand, a combination of GC-MS and LC-MS techniques allowed quantifying 180 biochemicals including amino acids, carbohydrates, lipids, cofactors and prosthetic groups, nucleotides and secondary metabolites in wild-type and NO-deficient mutant plants (Table S2). As expected from the microarray data, the endogenous levels of flavonoids, anthocyanins, polyamines and sugars were significantly higher in mutant than in wild-type plants (Fig. 2b–d). The content of the flavonoids/anthocyanins dihydrokaempferol and naringenin in mutant plants were around 6- and 2-fold higher than in wild-type plants, respectively (Fig. 2b; Table S2). Similarly, the polyamines agmatine and putrescine were around 30- and 14-fold higher, respectively, in mutant than in wild-type plants (Fig. 2c; Table S2). Finally, the levels of glucose,
glucose-1-phosphate, glucose-6-phosphate, sucrose, fructose and maltose were increased from 2- to 18-fold in nia1,2noa1–2 when compared to wild-type plants (Fig. 2d; Table S2). As shown in Fig. 3a, the increased levels of polyamines correlated with reduced content of arginine and ornithine and increased levels of citrulline. On the other hand, the increased levels of sugars in nia1,2noa1–2 plants reflected a general accumulation of glycolis metabolites and phosphoglycerate-derived amino acids of the serine family (Fig. 3b). In turn, metabolites of the tricarboxylic acids (TCA) cycle were significantly less abundant in nia1,2noa1–2 than in wild-type plants (Fig. 3b). Accordingly, the levels of α-ketoglutarate-derived amino acids of the glutamate synthetase-glutamine oxoglutarate aminotransferase (GS-GOGAT) cycle were also lowered in nia1,2noa1–2 plants (Fig. 3b), likely as a reflection of the impaired nitrate assimilation of the mutant plants. In summary, NO seems to exert a metabolic brake in the production of ABA, JA, anthocyanins, flavonoids, sugars and polyamines under standard conditions.

Increased levels of antioxidant metabolites in nia1,2noa1–2 plants. As shown in Table S2, the ascorbate and oxidized glutathione (GSSG) were both elevated in nia1,2noa1–2 plants. Moreover, other metabolites with antioxidant activity such as the flavonoids dihydrokaempferol and naringenin (Fig. 2b) as well as sinapate (Table S2) accumulated also in NO-deficient plants. We also found around 3-fold accumulation of the oxylipins 9-hydroxyoctadecadienoic acid (9-HODE) and 13-hydroxyoctadecadienoic acid (13-HODE).
Glycolysis and TCA cycle metabolite ratios between nia1,2noa1–2 and Col-0 plants. Metabolites in red and green were significantly more or less abundant in nia1,2noa1–2 than in Col-0 plants, respectively. Metabolites in blue were not significantly changed. Values indicated for each metabolite are the mean of six independent replicates performed in the complete metabolomic analyses described in Table S2.

Figure 3. Glycolysis and TCA cycle metabolite ratios between nia1,2noa1–2 and Col-0 plants. Metabolites in red and green were significantly more or less abundant in nia1,2noa1–2 than in Col-0 plants, respectively. Metabolites in blue were not significantly changed. Values indicated for each metabolite are the mean of six independent replicates performed in the complete metabolomic analyses described in Table S2.

NO negatively regulates constitutive freezing tolerance of Arabidopsis. The results described above strongly suggested that NO should have a negative role in the constitutive freezing tolerance of Arabidopsis. To test this possibility, we analyzed the constitutive freezing tolerance of 2-week-old wild-type and nia1,2noa1–2 plants. Freezing tolerance was determined as the percentage of surviving plants after exposure to different freezing temperatures for 6 h. Figure 5a shows that triple nia1nia2noa1–2 mutant plants displayed significantly greater freezing tolerance than did wild-type plants, the LT50 (temperature that causes 50% lethality) value being −5.6 °C and −4.5 °C, respectively. However, the double nia1nia2 mutant plants (LT50 −4.6 °C) were not significantly different than wild-type plants and the single noa1–2 mutant plants were slightly more tolerant (LT50 −4.8 °C) than wild-type plants. Despite nia1,2noa1–2 plants being slightly delayed in their development compared to Col-0 plants, the increased freezing tolerance manifested by the mutant with respect to the wild-type plants was very apparent (Fig. 5b). The endogenous NO levels of wild-type and mutant plants were measured by staining with the NO-specific fluorophore staining FAF-FM DA and we found that, as expected, nia1,2noa1–2 plants contained
significantly less NO than Col-0 plants (Fig. 5c). These results demonstrated that NO negatively regulates constitutive freezing tolerance in Arabidopsis, in all likelihood, by controlling the levels of osmoprotectant, hormones and redox metabolites.

**Discussion**

Genetic approaches using the Arabidopsis nia1,2 mutant plants suggested that NO is required for the adequate constitutive freezing tolerance and also for the full development of the cold acclimation process. Nevertheless, our data reported here using nia1,2noa1–2 triple mutant plants, which are impaired not only in nitrate reductase-mediated but also in NOA1-associated production of NO, showed that nia1,2noa1–2 plants were constitutively more tolerant to freezing than wild-type plants. Remarkably, neither the double nia1,2 nor the single noa1–2 mutant plants were significantly more tolerant than wild type plants under our freezing conditions. The discrepancy between our data and those obtained by using nia1,2 plants could be due to several reasons. First, the experimental conditions employed to grow plants, which have been described to be critical for correct hormone

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**Figure 4.** Endogenous content of ascorbate-glutathione cycle metabolites in wild type and NO-deficient plants. A diagram of the ascorbate-glutathione cycle is shown at top of the figure. Metabolites in red and green were significantly more or less abundant in nia1,2noa1-2 than in Col-0 plants, respectively. The box plots corresponding to the metabolites significantly different in both genotypes are shown in the bottom part of the figure. Values indicated for each metabolite are the mean of six independent replicates performed in the complete metabolomic analyses described in Table S2.
Figure 5. Constitutive freezing tolerance of Col-0 and nia1,2noa1-2 plants. (a) Freezing tolerance of 2-week-old plants exposed for 6 h to the indicated freezing temperatures was estimated as the percentage of plants surviving each specific temperature after 7 d of recovery under control conditions at 21 °C. Data are expressed as means of three independent experiments with around 50 plants each indicated genotype ± standard deviation. Asterisks indicate significant differences between nia1,2noa1-2 and wild-type plants (p-value < 0.05). (b) Upper panels show individual plants of wild type and mutant genotypes before freezing to show the difference in size. The bottom panel shows a representative image of plants from both genotypes after freezing at −5 °C and recovery at standard growing temperature for additional 7 days. (c) NO levels in Col-0 and nia1,2noa1-2 plants. Plants were maintained at standard growing conditions for 14 days. The fluorescence of DAF-FM DA-treated plants was detected by confocal microscopy. Shown images are representative of four to six different analyzed plants per genotype and condition, and the quantification values are the mean ± standard error. *Indicates significant differences between nia1,2noa1-2 and wild-type plants (p-value < 0.05).
signaling in cold acclimation, were very different. Indeed, previous work with nia1,2 plants was performed using plants grown in Petri dishes on sucrose supplemented MS media, which implies that they were exposed to a high relative humidity. Our freezing tolerance experiments, however, were carried out with plants grown on soil. As an indicator of the differences in the performance of both experimental systems, the increase in NO content was significant during the first hour after 24 h of exposure to 4 °C in wild-type plants. Yet, under our experimental conditions, wild-type plants registered a very slight likely non-significant increase in the endogenous NO content by 1 day at 4 °C but large increases were detected only after 7 days under cold conditions, when acclimation has been reported to be completed. As expected, the cold-induced increase in NO was largely abolished in nia1,2,2 plants. On the other hand, our previous analyses comparing the transcriptomes of nia1,2,2 and nia1,2 to that of wild-type plants pointed to a potentiated enhancement of the differentially expressed genes in the triple mutant (GE identification number GSE41958), which correlated with the stronger reduction in NO content. Together, these data suggest that the effects of NO-deficiency were additive in the triple mutant. Finally, another indication of nia1,2 and nia1,2,2 mutants being different in terms of cold response comes from their different accumulation of proline, whose content has been shown to positively correlate with freezing tolerance in Arabidopsis. Regarding this, whereas Zhao et al. observed an increase in proline content in wild-type but not in nia1,2 plants exposed to 4 °C, we did not find significant differences in proline content between nia1,2,2 and wild-type plants. Nevertheless, although increases in proline content have been reported during cold acclimation in different Arabidopsis accessions, there was no correlation with enhanced freezing tolerance. Actually, our results revealed lower levels of proline metabolites, such as N-acetylproline, trans-4-hydroxyproline and 5-oxoprolne, in nia1,2,2 than in wild-type plants.

The increased constitutive freezing tolerance of NO-deficient plants described in this work is fully consistent with the changes observed when the transcriptomes and metabolomes of nia1,2,2 and wild-type plants, grown under control conditions, were compared. The up-regulated transcriptome of nia1,2,2 plants contained a large number of transcripts that have been previously reported to be cold-induced. In addition, mutant plants also accumulated high amounts of osmoprotective metabolites such as and sugars, polyamines, and antioxidant metabolites, including anthocyanins and flavonoids, which, in all likelihood, limit the impact of the freezing imposed damage. Many of the cold-inducible transcripts that were up-regulated in nia1,2,2 plants were also significantly more expressed in nia1,2 plants (GE identification number GSE41958), thus suggesting that the metabolic changes could be determinant for the enhanced freezing tolerance displayed by the triple mutant. For instance, nia1,2,2 plants contained 14 times the content of the polypeptide putrescine in wild-type but not in nia1,2 plants exposed to 4 °C, we did not find significant differences in proline content between nia1,2,2 and wild-type plants. Nevertheless, although increases in proline content have been reported during cold acclimation in different Arabidopsis accessions, there was no correlation with enhanced freezing tolerance. Actually, our results revealed lower levels of proline metabolites, such as N-acetylproline, trans-4-hydroxyproline and 5-oxoprolne, in nia1,2,2 than in wild-type plants.

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several of the enzymes involved in that process undergo NO-related PTMs. This mechanism based on NO trig-
gerated PTMs would represent a potential control point of oxidative stress, which participates in plant responses to
a wide array of environmental stresses.

The data presented in this work suggest that NO would scavenge reactive oxygen species and also would
attenuate metabolic changes leading to increases in the levels of polyamines, sugars, anthocyanins, flavonoids,
ABA and JA that are essential for the adequate constitutive freezing tolerance (Fig. 6), thus explaining why
NO-deficient plants display an increased constitutively freezing tolerance.

**Methods**

**Plant materials and growth conditions.** The *Arabidopsis thaliana* Col-0 ecotype was the wild-type
genetic background used in this work. The triple *nia1,2noa1–2* mutant seeds were obtained by crossing *nia1nia2*
(N2356) and *noa1–2* (SAIL_507_E11), obtained from NASC seed bank, as previously reported. Genotyping by
PCR and Cleaved Amplified Polymorphic Sequences (CAPS) with specific primers (Table S3) were used to select
triple homozygous mutant plants. Seeds were grown in soil mixture or MS media as previously described and
experiments were performed with 2-week old plants.

**Freezing tolerance assays.** Seeds from the different genotypes were sown in soil-containing pots and
allowed to develop for 7 days. Then, several plants for each pot were removed in order to leave a similar num-
ber (25–30) of plants, homogenously distributed in all pots. Before being subjected to freezing temperatures,
plants were exposed for 1 h to 4 °C in the freezing chamber. Then, temperature was progressively decreased
(−1 °C/30 min) until reaching the indicated freezing temperatures. After exposing plants to the appropriate freez-
ing temperature for 6 h, temperature was gradually increased to 4 °C (+1 °C/30 min). One hour later, plants were
transferred to 20 °C under long-day light regime for recovering and subsequent survival evaluation 7 days later.

**RNA isolation and quantitative transcript analysis.** Total RNA was isolated from 10 to 12 days old
seedlings, separated, and analyzed by RT-qPCR techniques as previously described with specific primers
(Table S3). The identity and full name annotation of every gene analyzed in this work is as follows: ALCOHOL
DEHYDROGENASE 1 (ADH1, AT1G77120); SALT TOLERANCE ZINC FINGER (STZ/ZAT10, AT1G27730);
NINE-CIS-EPOXYCAROTENOID DIOXYGENASE 3 (NCED3, AT3G14440); HIGHLY ABA-INDUCED
PP2C GENE 1 (HAI1/SAG113, AT5G59220); SNF1-RELATED PROTEIN KINASE 2.9 (SnRK2.9, AT2G23030);
LATE EMBRYOGENESIS ABUNDANT 7 (LEA7, AT1G52690); LATE EMBRYOGENESIS ABUNDANT 4–5
(LEA4–5, AT5G06760); LOW-TEMPERATURE-INDUCED 65/RESPONSIVE TO DESICCATION
29B (LTI65/RD29B, AT5G52300); PRODUCTION OF ANTHOCYANIN PIGMENT 1/MYB DOMAIN
PROTEIN 75/ANTHOCYANIN ACCUMULATION 1 (PAP1/MYB75/SIAA1, AT1G56650); DIHYDROFLAVONOL 4-REDUCTASE
(DFR/TT3, AT5G42800); CYTOCHROME P450, FAMILY 707, SUBFAMILY A, POLYPEPTIDE 3
(CYP707A3, AT5G45340); NITRATE REDUCTASE 1 (NR1/NIA1, AT1G77760); NITRATWE REDUCTASE 2
(NR2/NIA2, AT1G37130); NITRIC OXIDE ASSOCIATED
PROTEIN 1 (NOA1/RIFI/SVR10, AT3G47450).

**NO detection by fluorescence and confocal microscopy.** The endogenous levels of NO in shoots
were determined by staining with 10 μM DAF-FM DA fluorescein as described with some modifications.
Fluorescence was detected by confocal microscopy with a CLSM LEICA TCS SP5, using unchanged parame-
ters for every measurement. The DAF-FM DA fluorescence intensities were analyzed using Adobe Photoshopto quantifying green pixels in 3 to 6 replicate images of every genotype and condition from three independent
experiments. The mean value ± standard error is shown.

**Metabolomic analyses.** The sample preparation process was carried out using the automated MicroLab
STAR® system from Hamilton Company. Recovery standards were added prior to the first step in the extraction
process for quality control (QC) purposes. Sample preparation was conducted by series of organic and aqueous
extractions to remove the protein fraction while allowing maximum recovery of small molecules. The resulting
extract was divided into two fractions; one for analysis by Liquid Chromatography (LC) and one for analysis by
Gas Chromatography (GC). Samples were placed briefly on a TurboVap® (Zymark) to remove the organic solvent.
Each sample was then frozen, dried under vacuum and prepared for either LC/MS or GC/MS. More details on the metabolomics methodology are described in the Methods data sheet in Table S2.

**Quantification of anthocyanins.** Anthocyanins were spectrophotometrically determined in methanolic extracts by reading their absorbance at 530 nm as described56.

**Statistical analyses.** For metabolomic analyses, following log transformation and imputation with minimum observed values for each compound, Welch's two-sample t-test was used to identify biochemicals that differed significantly between experimental groups. An estimate of the false discovery rate (q-value) was calculated to take into account the multiple comparisons. Statistical analyses are performed with the program “R” http://cran.r-project.org/. Statistically significant differences in hormone quantification and transcript analyses were computed based on Student's t-tests.

**Phytohormone quantification.** Four independent biological replicate samples of around 150–200 mg fresh weight of either non-acclimated or cold-acclimated Col-0 and nia1,2noa1–2 seedlings were suspended in 80% methanol-1% acetic acid containing internal standards and mixed by shaking during one hour at 4°C. The extract was kept a –20°C overnight, centrifuged, the supernatant dried in a vacuum evaporator, and the dry residue was dissolved in 1% acetic acid and passed through an Oasis HLB (reverse phase) column as described51. The dried eluate was dissolved in 5% acetonitrile-1% acetic acid, and the hormones were separated using an autosampler and reverse phase UHPLC chromatography (2.6 µm Accucore RP-MS column, 50 mm length x 2.1 mm i.d.; ThermoFisher Scientific) with a 5 to 50% acetonitrile gradient containing 0.05% acetic acid, at 400 µL/min over 14 min.

The phytohormones were analyzed with a Q-Exactive mass spectrometer (Orbitrap detector; ThermoFisher Scientific) by targeted Selected Ion Monitoring (SIM). The concentrations of hormones in the extracts were determined using embedded calibration curves and the Xcalibur 2.2 SP1 build 48 and TraceFinder programs. The internal standard for quantification of ABA was the deuterium-labelled hormone. For JA, dihydrojasmonate (dhJA) was used as internal standard.

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Author Contributions
J.L., A.C.-B., C.P.-R. and J.S. conceived and designed the experiments; A.C.-B., C.P.-R., M.C.C., M.F.R. and J.L. performed the experiments; J.L. analyzed the transcriptome and metabolome data; J.L. wrote the article with the contribution of J.S.

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