Nitric Reductase-Dependent Nitric Oxide Production Is Involved in Cold Acclimation and Freezing Tolerance in Arabidopsis

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Nitric oxide (NO) is an important signaling molecule involved in many physiological processes in plants. We evaluated the role of NO in cold acclimation and freezing tolerance using Arabidopsis (Arabidopsis thaliana) wild type and mutants nia1nia2 (for nitrate reductase [NR]-defective double mutant) and Atnoa1/rif1 (for nitric oxide associated/resistant to inhibition by fosmidomycin1) that exhibit defects in NR and reduced NO production, respectively. Cold acclimation induced an increase in endogenous NO production in wild-type and Atnoa1/rif1 leaves, while endogenous NO level in nia1nia2 leaves was lower than in wild-type ones and was little changed during cold acclimation. Cold acclimation stimulated NR activity and induced up-regulation of NIA1 gene expression. In contrast, cold acclimation reduced the quantity of NOA1/RIF1 protein and inhibited NO synthase (NOS) activity. These results indicate that up-regulation of NR-dependent NO synthesis underpins cold acclimation-induced NO production. Seedlings of nia1nia2 were less tolerant to freezing than wild-type plants. Pharmacological studies using NR inhibitor, NO scavenger, and NO donor showed that NR-dependent NO level was positively correlated with freezing tolerance. Furthermore, cold acclimation up- and down-regulated expression of P5CS1 and ProDH genes, respectively, resulting in enhanced accumulation of proline (Pro) in wild-type plants. The stimulation of Pro accumulation by cold acclimation was reduced by NR inhibitor and NO scavenger, while Pro accumulation by cold acclimation was not affected by the NOS inhibitor. In contrast to wild-type plants, cold acclimation up-regulated ProDH gene expression in nia1nia2 plants, leading to less accumulation in nia1nia2 plants than in wild-type plants. These findings demonstrate that NR-dependent NO production plays an important role in cold acclimation-induced increase in freezing tolerance by modulating Pro accumulation in Arabidopsis.

Freezing temperature is a major factor limiting geographical distribution of plants and crop yield. Many plants from temperate and cold climates have evolved a mechanism to enhance their freezing tolerance during exposure to low, nonfreezing temperatures in a process known as cold acclimation (Guy, 1990). Identification and characterization of genes involved in cold acclimation have advanced our knowledge of the molecular mechanisms underlying cold acclimation and freezing tolerance in plants (Chinnusamy et al., 2006). The dehydration and cold response gene family genes have been identified as key transcriptional activators to activate the associated downstream cold-regulated genes, conferring tolerance of plants to freezing (Thomashow, 1999; Iba, 2002). There are numerous changes in biochemical and physiological processes during cold acclimation, ranging from accumulation of osmolytes and cryoprotectants (Xin and Browse, 1998) to disruption of reactive oxygen species homeostasis (Suzuki and Mittler, 2006). Several messenger molecules such as abscisic acid (ABA; Xiong et al., 2001), hydrogen peroxide (H₂O₂; Prasad et al., 1994), and cytosolic Ca²⁺ activity (Tahtiharju et al., 1997) are involved in perception and transduction of low temperature signal to mediate cold acclimation-dependent changes in physiological processes. Nitric oxide (NO) is emerging as an important signaling molecule with multiple biological functions in plants (Neill et al., 2003; Besson-Bard et al., 2007; Wilson et al., 2008). NO has been demonstrated to be associated with abiotic stresses, including drought (Maia and Lamattina, 2001), salt stress (Zhao et al., 2007), heat (Song et al., 2006), UV-B radiation stress (Shi et al., 2005), and metal toxicity (Rodrı´guez-Serrano et al., 2006; Tian et al., 2007). In addition, the NO-dependent signaling network is often closely related to the plant hormones ABA (Desikan et al., 2004) and ethylene (Ederli et al., 2006) and other signaling molecules such as H₂O₂ (Neill, 2007), cytosolic Ca²⁺ (Lamotte et al., 2006) and other signaling molecules such as H₂O₂ (Neill, 2007), cytosolic Ca²⁺ (Lamotte et al.,...
2004), and phosphatidic acid (PA; Distefano et al., 2008). Interestingly, those molecules are also involved in cold acclimation and freezing tolerance in plants: ABA (Gusta et al., 2005), ethylene (Yu et al., 2001), \( \text{H}_2\text{O}_2 \) (Prasad et al., 1994), cytosolic \( \text{Ca}^{2+} \) (Knight et al., 1996), and PA (Li et al., 2004). Thus, it is not unexpected that NO is involved in cold acclimation and freezing tolerance in plants. However, there has been no detailed study to evaluate the role of NO in cold acclimation and freezing tolerance in plants.

In mammals, NO production is mainly mediated by NO synthase (NOS), which catalyzes the conversion of l-Arg to l-citrulline and NO (Furchgott, 1995). A NOS-like activity has been suggested by pharmacological studies in plants (Durner and Klessig, 1999; Corpas et al., 2004, 2006, 2008; Jasid et al., 2006; Tian et al., 2007). However, the molecular identity of plant NOS is unknown. An Arabidopsis (Arabidopsis thaliana) AtNOS1 gene that encoded a protein with sequence similarity to a protein that is involved in NO synthesis in the snail was isolated by Guo et al. (2003). However, further studies have discounted the possibility that AtNOS1 per se is an Arg-dependent NOS enzyme (Crawford et al., 2006; Zemojtel et al., 2006). Associated1 (Crawford et al., 2006). Very recently, Flores-Pérez et al. (2008) demonstrated that the accumulation of plastid-targeted enzymes of the methylerythritol pathway conferring resistance to fosmidomycin in an isolated noa1 mutant may be accounted for by pleiotropic effects of defective plastids (Moreau et al., 2008). Despite the uncertainties regarding the function of plant NOA1/RIF1, the Atnoa1/rif1 mutant is insensitive to NO donor, thus suggesting that the loss of NOA1/RIF1 function affects physiological processes unrelated to NO synthesis. Several studies have shown that NOA1/RIF1 is a plastid GTPase that is not directly related to NO production; rather, it may be required for proper protein synthesis in plastids (Flores-Pérez et al., 2008; Moreau et al., 2008). The reduced endogenous NO levels in the Atnoa1/rif1 mutant may be accounted for by pleiotropic effects of defective plastids (Moreau et al., 2008). Despite the uncertainties regarding the function of plant NOA1/RIF1, the Atnoa1/rif1 mutant with reduced endogenous NO level has been shown to be a valuable tool to study the physiological function of NO in plants (Guo et al., 2003; Zhao et al., 2007; Ahlfors et al., 2009).

In addition to NOS-mediated NO production, several additional NO biosynthesis pathways may operate in plant cells (Neill et al., 2003; Wilson et al., 2008). Among them, nitrate reductase (NR), which is usually associated with nitrogen assimilation, can also mediate NO production from nitrite in an NAD(P)H-dependent manner (Dean and Harper, 1988; Yamasaki et al., 1999; Rockel et al., 2002). Furthermore, NO can also be generated in the apoplast of plant cells in a nonenzymatic way (Bethke et al., 2004). The involvement of NR-mediated NO production in physiological processes in plants has been demonstrated using Arabidopsis mutants defective in NR activity (Desikan et al., 2002; Bright et al., 2006; Modolo et al., 2006). In Arabidopsis, NR was encoded by two genes (NITRATE REDUCTASE1 [NIA1] and NIA2; Wilkinson and Crawford, 1993). NIA2 accounts for 90% of the total NR activity, while NIA1 is responsible for the remaining 10% of NR activity (Wilkinson and Crawford, 1993). The observations that expression of NIA1, but not NIA2, is sensitive to hormonal and developmental cues (Yu et al., 1998) and that NIA1 is responsible for NO production in guard cells (Bright et al., 2006) are in line with the proposition that NIA1 may be a key component underlying NR-mediated NO production.

It is well known that higher plants accumulate free Pro in response to a number of abiotic stresses such as drought, salinity, and freezing (Hare et al., 1999; Ashraf and Foolad, 2007). The accumulation of Pro under stressed environments can result from enhanced biosynthesis and/or reduced degradation of Pro. In plants, biosynthesis of Pro is catalyzed by P5C synthase (P5CS) and P5C reductase (Kishor et al., 1995; Hare and Cress, 1997). P5CS is coded by two closely related P5CS genes in Arabidopsis. The two genes have different functions: P5CS1 is responsible for Pro accumulation under osmotic stress, while P5CS2 acts as a housekeeping gene associated with seed development (Szekely et al., 2008). Degradation of Pro is mediated by Pro dehydrogenase (ProDH) and P5C dehydrogenase (Kiyosue et al., 1996; Deuschle et al., 2004). In Arabidopsis, freezing tolerance is positively correlated with enhanced accumulation of Pro due to down-regulation of Pro degradation and/or up-regulation of Pro synthesis (Xin and Browse, 1998; Nanjo et al., 1999). The elevated Pro may act either as a compatible solute to protect plants from dehydration or contribute to stabilizing subcellular structures and scavenging free radicals, thus conferring tolerance of plants to abiotic stress (Ashraf and Foolad, 2007). The capacity of Pro to scavenge free radicals resembles the function of NO, because NO can also serve as an antioxidant under conditions of various stresses (Beligni et al., 2002). In this context, a recent study revealed that NO is involved in metal toxicity-induced Pro production in Chlamydomonas reinhardtii (Zhang et al., 2008). However, whether cross talk between NO and Pro exists in cold acclimation and freezing tolerance remains unknown.

To evaluate the role of NO in cold acclimation and freezing tolerance of plants, we investigated the responses of endogenous NO level in leaves of Arabidopsis wild type and mutants that exhibit defects in NR (nia1nia2) and reduced NO levels (Atnoa1/rif1) to cold acclimation. We further studied the effect of NO on freezing tolerance by manipulating endogenous NO levels using NO donor, scavengers and antagonists that inhibit NOS and NR activity. Finally, we examined the role of NO in Pro accumulation during cold acclimation. Our results demonstrate that NR-mediated NO production plays an important role in cold acclimation and freezing tolerance in Arabidopsis.
RESULTS

Cold Acclimation Induced an Increase in Endogenous NO Level

To investigate whether NO is involved in cold acclimation and freezing tolerance, the response of endogenous NO level in leaves of Arabidopsis was monitored by labeling NO using the cell-permeable, NO-specific fluorescent probe 4-amino-5-methylamino-2’,7’-difluorofluorescein diacetate (DAF-FM DA) and imaging by confocal microscopy. The DAF-FM-dependent fluorescence intensity in detached Arabidopsis leaves was abolished by the NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethyl-imidazoline-1-1-oxyl-3-oxide (cPTIO), and the fluorescence was markedly increased by treatment with the NO donor sodium nitroprusside (SNP; Supplemental Fig. S1). These results confirm that the DAF-FM-dependent fluorescence is related to endogenous NO level in Arabidopsis leaves; thus, the fluorescence was used to report endogenous NO level throughout this study. The endogenous NO level in Arabidopsis leaves displayed a time-dependent increase during cold acclimation at 4°C, and it reached steady level after 7 d at 4°C (Fig. 1, A and B), while the NO level in leaves grown in non-cold-acclimated temperature during the same period was relatively constant (Fig. 1B), indicating that the cold acclimation-induced increase in NO level is not due to development-dependent changes in NO level. Because NR and NOS are two key enzymes responsible for plant NO biosynthesis in plants, we examined the effects of the NR inhibitor okadaic acid (OA; Kaiser et al., 2002; Rockel et al., 2002) and the NOS inhibitors Nω-nitro-L-arginine (L-NNA) and L-NAME on NO production during cold acclimation. Treatment with OA, L-NNA, and L-NAME markedly reduced cold acclimation-induced increase in endogenous NO level in Arabidopsis leaves (Fig. 1, A and C), suggesting that the cold acclimation-induced increase in NO may result from enhanced activities of both NR and NOS.

To determine the contribution of NR and NOS to the cold acclimation-induced increase in NO level, we also adopted a genetic approach using Arabidopsis mutants exhibiting null NR activity (nia1nia2; Wilkinson and Crawford, 1993), reduced NR activity by approximately 90% (nia2; Wilkinson and Crawford, 1991), and reduced endogenous NO level (Atnoa1/rif1; Guo et al., 2003). As shown in Figure 2, NO level in nia2 mutant leaves was comparable to that in wild-type leaves, while NO level was lower in leaves of nia1nia2 and Atnoa1/rif1 plants than in wild-type leaves under non-cold-acclimated conditions. Similar to wild-type plants, NO level in leaves of nia2 plants also increased when cold acclimated at 4°C (Fig. 2). In contrast to the wild-type, nia2, and Atnoa1/rif1 plants, NO level in leaves of nia1nia2 plants showed little change under the identical conditions of cold acclimation (Fig. 2). These findings imply that an increase in NIA1-dependent NR activity is likely to underlie the increased NO level in Arabidopsis leaves during cold acclimation.

Cold Acclimation Increased Activity of NR and Reduced Activity of NOS

To further verify the involvement of NR in cold acclimation-induced increase in NO level in Arabidopsis, we also measured the effects of cold acclimation on activities of NR and NOS. As shown in Figure 3A,
NOS activity in wild-type plants was marginally increased after 3 d of cold acclimation, but it was markedly reduced after 5 d of cold acclimation at 4°C. Consistent with the reduction in NOS activity during cold acclimation, cold acclimation also reduced the quantity of NOA1/RIF1 proteins as revealed by western-blot analysis (Fig. 3B). In contrast to NOS activity, cold acclimation induced increases in NR activities in both wild-type and Atnoa1/rif1 plants, and the enhanced NR activities in both wild-type and Atnoa1/rif1 plants increased with the duration of cold acclimation, reaching maximum level after cold acclimation at 4°C for 7 d (Fig. 3C). No changes in NR activities in both wild-type and Atnoa1/rif1 plants were observed when grown at non-cold-acclimated temperature (Fig. 3C). NR activity was not detected in nia1nia2 plants (data not shown). In addition, we also studied responses of NIA1, NIA2, and NOA1/RIF1 gene expression to cold acclimation by quantitative real-time PCR. NIA2 and NOA1/RIF1 were the highest and lowest expressed genes in nonacclimated, control conditions, respectively, and the expression of NIA1 and NIA2 genes was increased during cold acclimation (Fig. 3, D–F). For instance, after cold acclimation for 72 h, transcripts of NIA1 and NIA2 were increased by 10- and 2-fold, respectively (Fig. 3, D and E). NOA1/RIF1 had low expression level in control conditions, and it increased slightly by cold acclimation (Fig. 3F). In contrast to cold-acclimated plants, expression of NIA1, NIA2, and NOA1/RIF1 in non-cold-acclimated plants remains relatively constant (Fig. 3, D–F). These results indicate that an increase in NR activity due to increased expression of NIA1 may account for the cold acclimation-induced increase in endogenous NO level in Arabidopsis leaves.

NR Null Mutant nia1nia2 Was Less Tolerant to Freezing

To determine whether the cold acclimation-induced increase in NO level plays a role in freezing tolerance, we assayed whole plant freezing survival rate of Arabidopsis wild-type and nia1nia2 mutant seedlings in responses to freezing treatment. Wild-type and nia1nia2 plants grown on half-strength Murashige and Skoog medium were directly frozen at −7°C for 4 h without cold acclimation (nonacclimation) or frozen at −7°C for 8 h after cold acclimation at 4°C for 3 and 7 d. The survival rate for those plants directly
frozen at –7°C for 4 h was 73% and 45% for non-acclimated wild-type and \textit{nia1nia2} plants, respectively (Fig. 4, A and C), suggesting that \textit{nia1nia2} plants are inherently less tolerant to freezing than wild-type plants. The survival rate for wild-type and \textit{nia1nia2} seedlings that were cold acclimated for 3 d and then frozen at –7°C for 8 h was 25% and 6%, respectively (Fig. 4, B and D). The survival rate for wild-type and \textit{nia1nia2} seedlings frozen at –7°C for 8 h was increased to 58% and 11%, respectively, after 7 d of cold acclimation (Fig. 4, B and D). In contrast, for the non-acclimated wild-type and \textit{nia1nia2} seedlings, none of them survived after exposure to –7°C for 8 h (Fig. 4, B and D). On the other hand, the survival rate for \textit{nia2} plants that were cold acclimated and frozen at –7°C for 8 h was comparable to that for wild-type plants (Supplemental Fig. S2), while the survival rate for \textit{Atnoa1/rif1} seedlings was greater than that of wild-type and \textit{nia2} plants after cold acclimation for 3 and 7 d (Supplemental Fig. S2).

In addition to survival rate, sensitivity of wild-type and \textit{nia1nia2} plants to freezing was also evaluated by measuring electrolyte leakage from their leaves. Electrolyte leakage, a commonly used parameter for freezing injury (Gilmour et al., 2000), increased as temperatures decreased from 0°C to –12°C for both wild-type and \textit{nia1nia2} plants irrespective of cold acclimation. Cold acclimation led to reductions in electrolyte leakage for wild-type and \textit{nia1nia2} seedlings such that cold-acclimated wild-type and non-acclimated \textit{nia1nia2} plants exhibited the lowest and highest electrolyte leakage, respectively (Fig. 4E). Accordingly, the LT$_{50}$ values, which are freezing temperatures that cause release of 50% of tissue electrolytes, were –6.7°C ± 0.3°C and –5.7°C ± 0.2°C for wild-type and \textit{nia1nia2} plants, respectively (Fig. 4E). The LT$_{50}$ values for wild-type and \textit{nia1nia2} plants were reduced to –10.2°C ± 0.1°C and –6.2°C ± 0.5°C, respectively, after 7 d of acclimation at 4°C (Fig. 4E). These findings reveal that wild-type plants are more tolerant to freezing than \textit{nia1nia2} plants and that cold acclimation enhances freezing tolerance for wild-type plants but not for \textit{nia1nia2} plants.

The greater survival rate and lower LT$_{50}$ values for wild-type than \textit{nia1nia2} plants and differences in endogenous NO level between wild-type and \textit{nia1nia2}
plants during cold acclimation (Fig. 2) prompted us to hypothesize that the endogenous NO may play a role in conferring freezing tolerance in Arabidopsis. To test this hypothesis, we explored the relationship between freezing survival rate and endogenous NO level in leaves of Arabidopsis wild-type plants by treating plants with NO scavenger (cPTIO), NR inhibitor (OA), and NOS inhibitor (L-NNA and L-NAME). As shown in Figure 5A, freezing survival rate was reduced by cPTIO, OA, L-NNA, and L-NAME, with cPTIO and OA being more effective in reducing the survival rate than L-NNA and L-NAME. For instance, the survival rate exposed to a freezing regime of 4 h at $-7^\circ$C was decreased from 73% to 21%, 0%, and 0%, respectively, after treatment with L-NNA, OA, and cPTIO (Fig. 5A). On the other hand, survival rate for wild-type plants exposed to $-7^\circ$C for 4 h was increased from 74% to 91% by treatment with NO donor SNP, while the same treatment led to an increase in the survival rate from 44% to 90% for nia1nia2 plants (Fig. 5A). These results indicate that the NR-dependent endogenous NO levels in Arabidopsis are positively correlated with freezing tolerance of Arabidopsis and that the enhancement of freezing tolerance through cold acclimation is likely to be achieved by up-regulation of NIA1-mediated endogenous NO level.

Figure 4. Freezing tolerance of wild-type (WT) and nia1nia2 plants. A, Survival rates of nonacclimated wild-type and nia1nia2 plants grown at 23°C for 20 d exposed to $-7^\circ$C for 4 h. B, Survival rates for wild-type and nia1nia2 plants exposed to $-7^\circ$C for 8 h after cold acclimation at 4°C for 3 or 7 d. NS indicates none survived. C, Representative nonacclimated plants exposed to $-7^\circ$C for 4 h. D, Representative images of plants that were cold acclimated (3 or 7 d at 4°C) and exposed to $-7^\circ$C for 8 h. Freezing survival rates were estimated as the percentage of plants surviving after 7 d of recovery at room temperature of 23°C. Data were expressed as means of three independent experiments with 60 plants each. Photographs were taken after 7 d of recovery. E, LT50 values for leaves from plants cold acclimated for different durations presented as means ± SE of three independent experiments.

NO Is Involved in Cold Acclimation-Induced Pro Accumulation

Accumulation of free Pro during cold acclimation has been observed as a mechanism to enhance freezing tolerance (Xin and Browse, 2000). To test whether cold acclimation-induced NO production plays a role in Pro accumulation, we first studied the effect of cold acclimation on Pro content in leaves of wild-type, nia1nia2, and Atnoa1/rif1 plants. Under standard growth conditions, Pro content in rosette leaves of nia1nia2 plants was much lower than that of wild-type and Atnoa1/rif1 plants (Fig. 6A). Pro content increased from 147 to 443 mg g$^{-1}$ fresh weight (fw) in wild-type plants after 3 d of cold acclimation at 4°C. Atnoa1/rif1 plants exhibited comparable Pro content to that of wild-type plants under both non-cold-acclimated and cold-acclimated conditions (Fig. 6A). The same cold acclimation protocol led to an increase in Pro content in nia1nia2 plants from 18 to 59 mg g$^{-1}$ fw (Fig. 6A). This difference in cold acclimation-induced Pro accumulation between wild-type and nia1nia2 plants may suggest that NIA1 and NIA2 proteins play a role in modulation of Pro accumulation during cold acclimation. Given that NO level was lower in nia1nia2 mutant than in wild-type plants under both...
non-cold-acclimated (control) and cold-acclimated conditions (Fig. 2), it is envisaged that accumulation of Pro may be regulated by endogenous NO in Arabidopsis leaves. To test this possibility, we further investigated the relationship between endogenous NO level and Pro content in leaves of wild-type plants during cold acclimation by manipulating endogenous NO level using NO scavenger (cPTIO) and NR inhibitor (OA). Figure 6B shows that the NO donor SNP stimulated Pro accumulation in Arabidopsis leaves under nonacclimated conditions. On the other hand, cold acclimatition-induced increase in Pro content was markedly inhibited by treatment with cPTIO and OA (Fig. 6C). These results indicate that an elevated NO level may function as a trigger to evoke Pro accumulation during cold acclimation. Accumulation of Pro during cold acclimation can result from enhanced biosynthesis and/or reduced degradation. We thus evaluated the effect of cold acclimation and NO on the expression of genes encoding P5CS1 and ProDH, two key enzymes responsible for Pro synthesis and degradation. Figure 7A shows that expression of P5CS1 was lower in nia1nia2 than in wild-type plants in non-cold-acclimated conditions and that P5CS1 expression in both wild-type and nia1nia2 plants increased during cold acclimation. Expression of P5CS1 in wild-type plants was approximately three times higher than that in nia1nia2 plants under conditions of both nonacclimation and cold acclimation (Fig. 7A). Expression of ProDH was also higher in wild-type than nia1nia2 plants in non-cold-acclimated conditions, and cold acclimation induced a decrease in ProDH expression in wild-type plants (Fig. 7B). By contrast, ProDH expression in nia1nia2 plants was enhanced during the cold acclimation (Fig. 7B). These results imply that greater up-regulation of P5CS1 and down-regulation of ProDH expression may account for the greater Pro accumulation in wild-type than nia1nia2 plants during cold acclimation. Consistent with this hypothesis was that P5CS1 expression was enhanced by SNP, and this effect was alleviated by the NO scavenger cPTIO (Fig. 7C). In contrast to P5CS1 expression, ProDH expression was relatively insensitive to SNP (Fig. 7C). Furthermore, expression of P5CS1 and ProDH in Atnoa1/rfl1 plants exhibited similar up- and down-regulation to those in wild-type plants before and after cold acclimation (Fig. 7, D and E), and the cold acclimation-dependent expression of P5CS1 and ProDH in wild-type and Atnoa1/rfl1 plants was insensitive to the NOS inhibitor l-NNA (Fig. 7, D and E).

**DISCUSSION**

There has been ample experimental evidence in support of the involvement of NO in abiotic stresses in plants (for recent reviews, see Neill et al., 2003; Besson-Bard et al., 2007). However, there has been no detailed study to evaluate the role of NO in cold acclimation.
and freezing tolerance in plants. In this study, we demonstrated that cold acclimation elicited a marked increase in endogenous NO production in Arabidopsis leaves (Fig. 1). More specifically, we identified that cold acclimation-induced NO production resulted from enhanced NR activity due to up-regulation of NIA1 gene expression (Fig. 3). The involvement of NR in cold acclimation-induced NO production was further corroborated by the findings that NR antagonist inhibited NO generation (Fig. 1, A and C) and cold acclimation failed to elicit NO production in NR double mutant nia1nia2 plants (Fig. 2). Furthermore, pharmacological studies using NR inhibitor, NO scavenger, and NO donor revealed that NR-dependent NO level was positively correlated with freezing tolerance (Fig. 5). The findings that the NR-defective mutant nia1nia2 exhibited lower endogenous NO level and less tolerance to freezing than wild-type plants are also in agreement with this proposition. In addition, we found that cold acclimation-induced NO may be associated with the accumulation of Pro by transcriptionally promoting Pro synthesis and suppressing Pro degradation (Fig. 6), thus contributing to enhanced freezing tolerance in Arabidopsis. Taken together, these findings provide unambiguous evidence in support of the idea that NR-dependent NO plays an important role in cold acclimation and freezing tolerance in Arabidopsis.

Rapid NO generation in response to hormonal and environmental stimuli has been reported in the literature. This includes enhanced NO production in response to cytokinin (Tun et al., 2008), auxin (Pagnussat et al., 2002; Kolbert et al., 2008), ABA (Bright et al., 2006; Ribeiro et al., 2009), salicylic acid (Zottini et al., 2007), hypoxia (Rockel et al., 2002), and mechanical stress (Garces et al., 2001). Similarly to our findings in this paper, both pharmacological and genetic studies using Arabidopsis mutants defective in NR (nia1nia2) and reduced NO levels (Atnoa1/rif1) have demonstrated that elevated NO production in response to treatment with ABA (Desikan et al., 2002) and auxin (Kolbert et al., 2008) results from up-regulation of NR-catalyzed NO generation. By contrast, salicylic acid- and mechanical stress-induced NO production can be attributed to a NOS-mediated pathway (Garces et al., 2001; Zottini et al., 2007). In the case of cytokinin-dependent NO production, neither NR nor NOS is involved (Tun et al., 2008). The NR-defective mutant nia1nia2 has been widely used to study NO production and physiological NO function (for review, see Wilson et al., 2008). In this study, we found that both nia1nia2 and Atnoa1/rif1 plants exhibited lower DAF-FM-dependent fluorescence in their leaves than their wild-type counterparts under control conditions (Fig. 2), suggesting that these plants have reduced endogenous NO level. A similar lower endogenous NO in nia1nia2 leaves than in wild-type plants has been observed in other studies (Modolo et al., 2006; Tun et al., 2008). The lower endogenous NO in nia1nia2 leaves may result from reduced intracellular Arg content due to aberrant nitrogen metabolism, leading to a decrease in NOS-dependent NO production (Crawford, 2006; Modolo et al., 2006). However, several lines of evidence seem to discount this possibility. NIA2 is by far the more abundant protein (Wilkinson and Crawford, 1993), and expression of the NIA2 gene.
in leaves was much higher than that of the NIA1 gene (Fig. 3D; Yu et al., 1998). Furthermore, the nia2 mutant did not differ from wild-type plants in terms of cold acclimation-induced NO generation (Fig. 2) and freezing tolerance (Supplemental Fig. S2). Stomatal closure was elicited by elevated NO production during the transition from light to dark in both wild-type and nia2 mutant plants, but nia1 mutant stomata failed to close under the same conditions (Wilson et al., 2008). Therefore, the low NO level and failure to generate NO in nia1nia2 mutant plants in response to cold acclimation are unlikely to result from impaired nitrogen metabolism. A similar argument discounting the disruption of nitrogen metabolism in nia1nia2 plants has been used to account for the failure of induction of NO in guard cells by ABA (Ribeiro et al., 2009). These results, together with those of Yu et al. (1998) and Bright et al. (2006), highlight the novel function of NIA1 in the mediation of NO production, whereas NIA2 may mainly involve nitrogen assimilation. A low temperature-induced increase in NR activity due to de novo transcription of NR and an increased amount of NR protein has been reported in tomato (Solanum lycopersicum; Tucker and Ort, 2002). A recent study by Corpas et al. (2008) reported low temperature-induced NO in pea (Pisum sativum) leaves. However, in contrast to our findings, these authors found that the increase in NO induced by low temperature is ascribed to NOS-dependent NO production. The discrepancy in sources of low temperature-induced NO production between this study and those reported by Corpas et al. (2008) may be accounted for by differences in plant species (Arabidopsis versus pea), low-temperature treatments (4°C versus 8°C), and light intensity (60 versus 210 μmol m⁻² s⁻¹). Regardless of the discrepancy, both studies revealed that low temperature induced an increase in NO level in leaves. Our study demonstrated the physiological significance of the increased NR activity in stimulation of NO production, which in turn plays a role in cold acclimation-dependent increase in freezing tolerance in plants.

Previous studies have revealed that the NO level in Atnoa1/rif1 roots was lower than in wild-type roots (Guo et al., 2003; Zhao et al., 2007). In this study, we found that endogenous NO level in Atnoa1/rif1 leaves was much lower than in wild-type plants (Fig. 2) and that Atnoa1/rif1 exhibited much more reduced endogenous NO level in leaves than in roots (Zhao et al., 2007; Fig. 2). This may be accounted for by localization of NOA1/RIF1 in chloroplasts (Flores-Pérez et al., 2008). The lower NO level in nia1nia2 leaves than in wild-type leaves under nonstressed, control condition...
also indicates the essential role of NR in controlling basal NO level in leaves. Despite uncertainty and controversy on the function of NOA1/RIF1 (Zemojtel et al., 2006; Moreau et al., 2008), our finding that the Atnoa1/rif1 mutant displayed a much reduced endogenous NO level, particularly in leaves (Fig. 2), proves Atnoa1/rif1 to be a valuable material to study the physiological function of NO in plants, as it provides an alternative way to control endogenous NO level. The quantity of NOA1/RIF1 protein was reduced during cold acclimation (Fig. 3B), which may account for the observed reduction in NOS activity (Fig. 3A). Note that there was a marginal increase in NOS activity after 3 d of cold acclimation at 4°C (Fig. 3A).

The cold acclimation-induced NO production was effectively inhibited by the NOS inhibitors L-NNA and l-NAME (Fig. 1). These findings suggest that NOS may also play a role in the cold acclimation-dependent increase in NO production. However, the observations that the Atnoa1/rif1 mutant exhibited reduced NOS activity (Zhao et al., 2007) and a similar increase in NO production to wild-type plants during cold acclimation (Fig. 2) do not agree with this argument. Another interesting observation is that cold acclimation induced a greater tolerance of the Atnoa1/rif1 mutant to freezing than wild-type plants (Supplemental Fig. S2). We do not have an explanation for this finding, but it may suggest that greater tolerance of Atnoa1/rif1 is not directly related to NOS-dependent NO production.

The involvement of NO in salt stress in several plant species has been demonstrated using pharmacological agents to manipulate endogenous NO level (Zhao et al., 2004; Zhang et al., 2006) and using Atnoa1/rif1 mutant plants (Zhao et al., 2007). Similar to freezing tolerance, tolerance to salt stress is also positively correlated with endogenous NO level, such that treatments with NO donor and NO scavenger alleviate and exaggerate salt stress symptoms, respectively (Zhao et al., 2004, 2007; Zhang et al., 2006). However, in contrast to freezing stress, salt stress inhibits NOS activity, thus reducing endogenous NO level in roots (Zhao et al., 2007). The reduction of endogenous NO level under salt stress may facilitate accumulating more Na\(^+\) and fewer K\(^+\) ions and enhancing associated oxidative stress, thus rendering plants more vulnerable to salt stress (Zhao et al., 2007). The mitigating effect of exogenous NO on plants under salt stress may lie in up-regulation of H\(^+\)-ATPases in the plasma membranes and tonoplasts, minimizing Na\(^+\) accumulation and/or compartmentation into vacuoles (Zhao et al., 2004; Zhang et al., 2006). It remains unknown whether the NO-dependent increase in freezing tolerance is associated with plant H\(^+\)-ATPases.

Another important observation in this study is that nia1nia2 plants had much lower Pro contents than wild-type plants before and after cold acclimation (Fig. 6A). The lower Pro contents in nia1nia2 plants than in wild-type plants are likely to result from the disruption of Pro biosynthesis due to less expression of P5CS1 in control conditions (Fig. 7). Moreover, the cold acclimation-dependent increase in Pro contents in wild-type plants can be markedly reduced in the presence of NR inhibitor and NO scavenger (Fig. 6C). Therefore, these findings are indicative that induction of Pro accumulation may be a downstream component of cold acclimation-induced NO production. In plants, Pro contents are maintained by transcriptional regulation of both biosynthesis and degradation (Hare et al., 1999). The rapid up-regulation of expression of the P5CS1 gene and down-regulation of the ProDH gene by cold acclimation indicate that both enhanced synthesis and reduced degradation are responsible for the enhanced Pro accumulation. Like wild-type plants, expression of P5CS1 was also up-regulated in nia1nia2 plants by cold acclimation (Fig. 7A). In contrast, expression of ProDH was up-regulated in nia1nia2 plants during cold acclimation (Fig. 7B). These findings suggest that the lower accumulation of Pro in nia1nia2 plants than in wild-type plants is due to less enhanced synthesis and greater degradation of Pro during cold acclimation. There have been several reports showing that NO can stimulate P5CS1 activity and up-regulate the expression of P5CS1 genes in plants (Uchida et al., 2002; Ruan et al., 2004; Zhang et al., 2008). Similar to our findings, Ruan et al. (2004) also found that SNP stimulates the activity of P5CS1 in wheat (Triticum aestivum). The observations that the NO donor SNP can mimic cold acclimation to up-regulate expression of the P5CS1 gene, and that this effect can be abolished by the NO scavenger cPTIO (Fig. 7C), highlight the critical role of NO in cold acclimation-induced Pro accumulation. Although cold acclimation-induced Pro accumulation has been widely observed in plants, varying responses of expression of P5CS1 and ProDH genes to cold acclimation have been reported in the literature (Hare et al., 1999). Nevertheless, the up-regulation of P5CS1 and down-regulation of ProDH expression by cold acclimation reported in this study are comparable to the findings of Xin and Browse (1998).

The elevated Pro content in wild-type plants can act as a compatible osmolyte to protect plants from dehydration, thus enhancing freezing tolerance (Xin and Browse, 2000). However, we did not observe a significant increase in osmolality of both wild-type and nia1nia2 plants during cold acclimation (data not shown), suggesting that the ameliorative effect of NO on freezing tolerance cannot be explained by osmoregulation of accumulated Pro content. It has also been shown that Pro can function as a molecular chaperone to stabilize the structures of proteins and play a role in regulation of the antioxidant system and cellular redox potential (Hare et al., 1999; Székely et al., 2008). Further studies to elucidate the mechanism underlying NO-dependent Pro accumulation in freezing tolerance are warranted. In addition, a recent study revealed that NO can induce PA accumulation by activation of phospholipase C and phospholipase D (PLD; Distefano et al., 2008). In this context, Li et al. (2004) demonstrated that increased accumulation of
PA by overexpressing plasma membrane-bound PLD leads to enhanced freezing tolerance in Arabidopsis. Therefore, it is plausible that the cold acclimation-dependent NO production may contribute to freezing tolerance by up-regulation of PLD-dependent production of PA.

In conclusion, we demonstrate that cold acclimation induced a marked increase in endogenous NO level in Arabidopsis leaves resulting from up-regulation of NIA1 expression. The elevated NO may function as a signal to evoke Pro accumulation via enhanced synthesis and reduced degradation, conferring tolerance of Arabidopsis to freezing stress. Therefore, these findings highlight the involvement of NR-dependent NO production in cold acclimation-induced freezing tolerance in plants.

MATERIALS AND METHODS

Plant Material, Treatment, and Freezing Survival Analyses

Seeds of wild-type (Columbia), nia2, nia2nia3, and Attnao1/riffffff Arabidopsis (Arabidopsis thaliana) plants were sterilized and cultured in petri dishes (5.5 cm in diameter) on half-strength Murashige and Skoog basal medium (pH 5.8). The nitrogen in the medium includes 1 mM NH₄Cl and 1.94 mM NO₃⁻. Seedlings were grown in a greenhouse under conditions of 23°C, 12 h of light, and 120 μmol m⁻² s⁻¹. Twenty-day-old seedlings were used in this study. Cold acclimation was conducted by exposing Arabidopsis seedlings to low temperature (4°C, 12 h of light, with light intensity of 60 μmol m⁻² s⁻¹) for varying periods. The following chemicals were used to treat the seedlings during cold acclimation or nonacclimation by adding 2 mL of the solutions containing individual chemicals into each petri dish in which 15 seedlings were grown. The chemicals used for treatments were 400 μM OPD, 1 μM OA, 300 μM l-NA, 300 μM l-NAME, 200 μM sodium ferrocyanide, and 0 to 1,000 μM SNP. The concentrations used in this study were determined from preliminary experiments in which the chemicals did not have any substantial effect on normal growth of Arabidopsis (data not shown). All chemicals were purchased from Sigma-Aldrich. Control plants were treated with equal amounts of deionized water only. For assay of freezing tolerance, seedlings were subjected to an 8-h freezing regime at −7°C for cold-acclimated and non-cold-acclimated plants. After gradually increasing the temperature to 4°C and incubating for 12 h at 4°C in the dark, the plants were returned to the greenhouse conditions, and surviving plants were scored after transferring for 7 d. Survival rates were calculated from the results of three independent experiments.

Electrolyte Leakage Assay

Electrolyte leakage was assayed according to the method of Gilmour et al. (2000) with some modifications. Briefly, tubes containing six to eight leaves detached from 20-d-old plants acclimated for varying periods at 4°C were placed in a low-temperature-bath (Grant) set at 0°C. Ice chips were added to each tube after 1 h of incubation at 0°C. The bath temperature was lowered to 0°C over 2°C h⁻¹. Tubes were removed at defined temperatures and thawed overnight at 4°C in the dark, then incubated with 6 mL of deionized water in a shaker with shaking speed of 200 rpm and at 25°C for 2 h. Electrical conductivity in the bathing solution was first determined (C₀), and thereafter the samples were heated at 100°C for 30 min and conductivity was determined again in the bathing solution (Cₜ). Relative ion leakage was expressed as a percentage of the total conductivity after heating at 100°C (i.e., relative ion leakage: % = (C₀/Cₜ) × 100).

Determination of Endogenous NO

Endogenous NO level was detected by imaging the NO-specific fluorescent probe DAF-FM DA (Molecular Probes) by confocal microscopy. After washing the excised leaves with buffer solution (20 mM HEPES-NaOH, pH 7.4), the leaves were incubated in the buffer solution containing 25 μM DAF-FM DA for 1 h at room temperature. The incubated leaves were visualized using a laser confocal scanning microscope (LSM510; Zeiss) after washed thoroughly with buffer solutions to remove excess fluorophore. Excitation was at 488 nm, and emission was at 515 nm. The fluorescence intensity of the individual leaf was determined by delineating the whole leaf using the Zeiss LSM510 software, and the fluorescence was expressed in pixel numbers on a scale ranging from 0 to 255.

Determination of NR Activity

The NR activity was assayed following the method of Scheible et al. (1997) with some modifications. Briefly, about 1 g of leaves was ground with liquid N₂ and then resuspended in extraction buffer containing 100 mM HEPES-KOH (pH 7.5), 1 mM EDTA, 0.5% (v/v) glycerol, 5 mM diithiothreitol, 0.1% Triton X-100, 0.5 mM phenylmethylsulfonyl fluoride, 20 μM FAD, 1 μM leupeptin, 5 μM Na₂MoO₄, and 1% polyvinylpyrrolidone. After centrifuging at 10,000g for 20 min at 4°C, the supernatant was used for NR determination. The NR activity was measured by mixing 1 volume of extract with 5 volumes of prewarmed (25°C) assay buffer (100 mM HEPES-KOH, pH 7.5, 300 mM NaCl, 0.25 mM KNO₃, and 0.02 mM NADH). The reaction was started by the addition of assay buffer, incubated at 25°C for 30 min, and then stopped by adding 0.1 ml zinc acetate. After centrifugation, the tubes were centrifuged at 13,000g for 5 min. The nitrite produced was measured colorimetrically at 520 nm by adding 1 mL of 1% (w/v) sulfitolamid in 3% HCl plus 1 mL of 0.02% (v/v) N-(1-naphthyl)-ethylenediamine in distilled water.

Determination of NOS Activity

NOS activity was determined as described by Zhao et al. (2007). Briefly, approximately 3 g of leaves, together with 50 mg of polyvinylpyrrolidone, were ground with liquid N₂ and then resuspended in extraction buffer (50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 320 mM Suc, 1 mM dithiothreitol, 1 μM leupeptin, 1 μM pepstatin, and 1 mM phenylmethylsulfonyl fluoride). After centrifuging at 10,000g for 30 min at 4°C, the supernatant was used to determine NOS activity by the citrulline assay using the NOS assay kit (Cayman Chemical). Reaction mixture (50 μL) contained 25 mM Tris-HCl, pH 7.4, 3 μM l-arginine, 25 μM sodium ferrocyanide, and 0.1 mM l-NAME. After centrifuging for 30 min at 37°C, the reaction was stopped by adding 400 μL of stop buffer (50 mM HEPES, pH 5.5, and 5 mM EDTA). A 100-μL resin slurry was added to the reaction mixture, and the resin was removed by centrifugation. Flow-through (400 μL) was added to 5 mL of scintillation liquid and radioactivity was counted (LS 6000; Beckman). The protein contents in the supernatants were determined according to the method of Bradford (1976) with bovine serum albumin as a standard.

NOA1/RIIF1 Antibody and Western-Blot Analysis

Anti-NOA1/RIIF1 rabbit polyclonal antibody was made against the N-terminal peptide containing 100 amino acids of NOA1/RIIF1. Protein extraction was shown as NOS activity determination. The supernatant was examined by SDS-PAGE, and protein on the gel was blotted electrically to nitrocellulose membranes. Membranes were blocked with TTBS (25 mM Tris-HCl, pH 7.5, 137 mM NaCl, and 0.2% Tween 20) containing 5% nonfat dry milk for at least 1 h and incubated with affinity-purified polyclonal NOS1 antibody (1:1,000) for 2 h in TTBS containing 1% nonfat dried milk. After multiple washes with TTBS, bound antibodies were detected with peroxidase-conjugated secondary antibodies and a chemiluminescence kit (Amersham Pharmacia).

Pro Determination

Pro accumulation in Arabidopsis leaves was determined by the method described previously (Bates et al., 1973) using i-Pro as standard. Briefly, leaves were harvested, weighed, and extracted in 3% sulfosalicylic acid. An aliquot of each extract (2 mL) was incubated with 2 mL of ninhydrin reagent (2.5% [w/v] ninhydrin, 60% [v/v] glacial acetic acid, and 40% 6 M phosphoric acid) and 2 mL of glacial acetic acid at 100°C for 40 min, and the reaction was terminated in an ice bath. Toluene (5 mL) was added, followed by vortexing and incubation at 23°C for 24 h. The absorbance was measured at 520 nm.
Analysis of Gene Transcription

Leaves from 20-d-old plants were collected in liquid nitrogen at the indicated times following cold (4°C) or 300 μM i-NNA, 200 μM SNP, 200 μM BP, and 400 μM cPTIO treatment. The gene expression pattern was studied using real-time quantitative reverse transcription (RT)-PCR. Total RNA was extracted from Arabidopsis leaves with Trizol reagent (Invitrogen) and treated with RNase-free DNase I (Promega). The total RNAs were reverse transcribed into first-strand cDNA with SuperScript II reverse transcriptase (Invitrogen), and the cDNAs obtained were used as templates for PCR amplification with specific primers. Gene-specific primers used for real-time quantitative RT-PCR were 5'-AGTACGGTAAATCTGCTGCTGTTG-3' and 5'-CCCTCTCTCTTATTGACGTTCTGTTGACGTTCTGTTGACTC-3' for ANI1A (At1g37130), 5'-GGATTGTGATGGTATCTTATTGACGTTCTGTTGACGTTCTGTTGACTC-3' and 5'-TATACCAACTTTAATACATCAAGACG-3' for NOA1/RIFT (At3g47450), 5'-CTTGGTACGATGATGCAAGACG-3' and 5'-CTTGGTACGATGATGCAAGACG-3'. The same amplification reaction was conducted with an Arabidopsis Actin11 gene and used as template RNA loading control.

Sequence data from this article has been deposited in the GenBank/EMBL data library under accession numbers NM_104425 (ANIIA1), NM_103364 (AtIIA2), NM_180335 (ANIIA1RIFT), NM_201912 (P5CS1), NM_113981 (ProDH), and NM_112046 (Actin11).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Effect of cPTIO and SNP on endogenous NO levels in wild-type leaves.

Supplemental Figure S2. Freezing tolerance of wild-type, nia2, and Atnoia1/2 plants.

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LITERATURE CITED

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Nitric Oxide and Cold Acclimation in Arabidopsis

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