Putrescine Is Involved in Arabidopsis Freezing Tolerance and Cold Acclimation by Regulating Abscisic Acid Levels in Response to Low Temperature[1]

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The levels of endogenous polyamines have been shown to increase in plant cells challenged with low temperature; however, the functions of polyamines in the regulation of cold stress responses are unknown. Here, we show that the accumulation of putrescine under cold stress is essential for proper cold acclimation and survival at freezing temperatures because Arabidopsis (*Arabidopsis thaliana*) mutants defective in putrescine biosynthesis (*adc1*, *adc2*) display reduced freezing tolerance compared to wild-type plants. Genes *ADC1* and *ADC2* show different transcriptional profiles upon cold treatment; however, they show similar and redundant contributions to cold responses in terms of putrescine accumulation kinetics and freezing sensitivity. Our data also demonstrate that detrimental consequences of putrescine depletion during cold stress are due, at least in part, to alterations in the levels of abscisic acid (ABA). Reduced expression of *NCED3*, a key gene involved in ABA biosynthesis, and down-regulation of ABA-regulated genes are detected in both *adc1* and *adc2* mutant plants under cold stress. Complementation analysis of *adc* mutants with ABA and reciprocal complementation tests of the *aba2-3* mutant with putrescine support the conclusion that putrescine controls the levels of ABA in response to low temperature by modulating ABA biosynthesis and gene expression.

The polyamines putrescine, spermidine, and spermine are ubiquitous polycationic aliphatic compounds that are present in all eukaryotic cells (Pegg and McCann, 1982). There is general agreement that polyamines in animals are essential for cell proliferation. Polyamines may sustain growth through interaction with macromolecules (e.g. DNA, RNA, proteins) by virtue of their polycationic structure (Feuerstein et al., 1986; Yuki et al., 1996). Polyamines play a role in maintenance of conformation and protection of DNA (D'Agostino et al., 2005) and RNA stabilization (Marton and Feuerstein, 1986), and act as key factors in protein synthesis (Park et al., 1996) and cell cycle progression (Thomas and Thomas, 1994). In plants, the mechanism of polyamine action has been less investigated, but numerous studies indicate that polyamines are involved in a wide range of physiological processes, such as control of development, senescence, and stress responses (for review, see Evans and Malmberg, 1989).

Putrescine in plants can be formed either directly from Orn in a single reaction catalyzed by Orn decarboxylase (ODC) or by decarboxylation of Arg via the Arg decarboxylase (ADC) pathway (for review, see Tiburcio et al., 1997). The ADC pathway consists of three enzymatic steps catalyzed by sequential actions of ADC, agmatine iminohydrolase (AIH), and *N*-carbamoylputrescine amidohydrolase (CPA). Spermidine and spermine are formed by sequential addition of aminopropyl groups to putrescine and spermidine, respectively, by spermidine synthase (SPDS) and spermine synthase (SPMS). The aminopropyl groups are generated from *S*-adenosylmethionine (SAM) by SAM decarboxylase (SAMDC; Tiburcio et al., 1997).

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With the exception of ODC, genes encoding all of these enzymes have been characterized in Arabidopsis (*Arabidopsis thaliana*), which has no detectable ODC activity (Hanfrey et al., 2001). In Arabidopsis, where putrescine is produced exclusively through the ADC pathway, two genes encode ADC (*ADC1* and *ADC2*), whereas single genes code for AIH and CPA (Janowitz et al., 2003; Piotrowski et al., 2003). Duplicated genes code for SPDS (*SPDS1* and *SPDS2*) and for SPMS (*SPMS* and *ACL5*; Hanzawa et al., 2000; Panicot et al., 2002), whereas SAMDC is encoded by at least four genes (*SAMDC1*, *SAMDC2*, *SAMDC3*, and *SAMDC4*; Urano et al., 2004).

Numerous studies demonstrated that major changes in polyamine metabolism occur in response to various abiotic stresses (for review, see Bouchereau et al., 1999). Putrescine accumulation under abiotic stresses has been traditionally correlated with changes in ADC activity in several plant systems (Flores and Galston, 1982; Chattopadhyay et al., 1997; Shen et al., 2000). In Arabidopsis, recent studies have shown that *ADC2* expression is strongly induced by dehydration, high salinity, and K^+ deficiency (Perez-Amador et al., 2002; Urano et al., 2003; Armengaud et al., 2004; Alcazar et al., 2006a), whereas *ADC1* seems to be mainly induced by cold (Hummel et al., 2004). Because no significant increase in *CPA* and *AIH* expression is observed under any stress condition tested (Alcazar et al., 2006b), it seems that ADC is the key enzyme responsible for stress-induced putrescine accumulation in plants.

Recently, alterations in the metabolite profiles of Arabidopsis plants challenged with cold stress have been investigated and the diamine putrescine was found to increase in plants subjected to low temperature (Cook et al., 2004; Kaplan et al., 2004). Analogous changes in metabolic profiles of polyamines and other osmoprotectants during cold stress suggests a role for the polyamines as cellular compatible solutes (Kaplan et al., 2004). Despite these reports, the biological functions of polyamines in low-temperature response are still unknown. The availability of insertion mutations in genes of the Arabidopsis polyamine biosynthetic pathway, together with very sensitive HPLC-based methodologies for precise metabolomic quantification, prompted us to study the function of these compounds in freezing tolerance and cold acclimation. Our results reveal an important role of putrescine in modulating the levels of abscisic acid (ABA) in response to low temperature.

RESULTS

Polyamine Content and Expression of Genes Involved in Polyamine Biosynthesis in Response to Low Temperature

To investigate the roles of polyamines in plant responses to low temperature, we first measured the levels of free polyamines in soil-grown Arabidopsis plants exposed to 4°C for different time periods. Figure 1A shows that a significant increase in free putrescine occurred 24 h after the plants were transferred to low temperature and the increased putrescine levels remained constant even 72 h after the onset

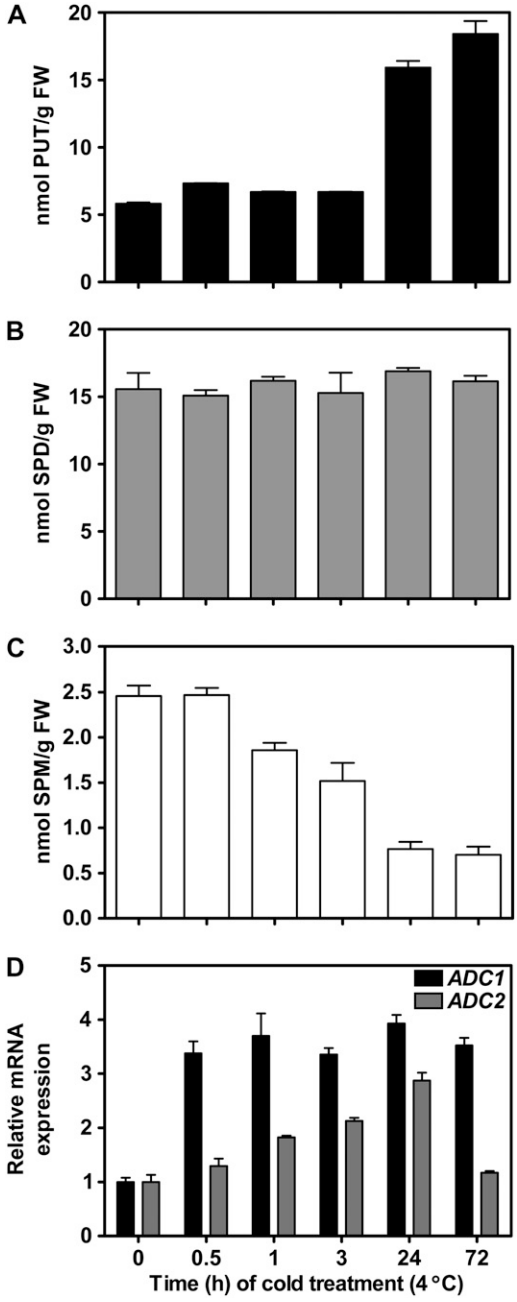

Figure 1. Polyamine profile and expression of *ADC* genes under cold treatment. Free putrescine (A), spermidine (B), and spermine (C) levels of soil-grown wild-type Arabidopsis plants challenged with low-temperature treatment. Values are means of three biological replicates. Experiments were repeated at least twice with similar results. D, Relative mRNA levels of key putrescine biosynthesis genes in the same experiment. Values are the means of four independent determinations expressed as relative to *EF1* values ± SD and normalized respect to the sample without treatment at time 0 h.

of cold treatment. The profile of free spermidine shown in Figure 1B was not affected by the low-temperature treatment. However, the other main high-M_r polyamine, spermine, displayed a slow but continuous decline in response to low temperature, reaching its lowest level during the first 24 h of cold treatment (Fig. 1C).

Because putrescine levels increase over 2-fold in response to cold stress, we used quantitative reverse transcription (qRT)-PCR to monitor the expression of *ADC1* and *ADC2* genes (encoding the key ADC enzymes controlling putrescine biosynthesis), in plants exposed to low temperature. Shoot tissue samples collected at different time points showed a very similar induction of *ADC1* and *ADC2* transcription, with a rapid response starting as early as 30 min after imposing the cold stress (Fig. 1D). However, the amplitude of the response at the level of steady-state mRNAs was higher for *ADC1* than *ADC2* at every point of the time kinetics. In samples collected at 72 h, *ADC2* expression was restored to unstressed levels, whereas *ADC1* expression was maintained at the induced level.

Mutants Defective in Putrescine Biosynthesis Are Affected in Freezing Tolerance and Cold Acclimation

To confirm the distinguished role of the diamine putrescine in low-temperature response, we screened two different T-DNA insertional mutant collections and identified knockout mutations in both *ADC1* and *ADC2* genes. Figure 2A shows a schematic representation of positions of T-DNA insertions in the mutant alleles named according to previously reported mutant alleles in the literature. Northern RNA hybridization analysis demonstrated lack of wild-type *ADC1* and *ADC2* transcripts in the homozygous *adc1* and *adc2* mutants (data not shown). To confirm this result with more sensitive methodologies, we used qRT-PCR under inductive and noninductive conditions and demonstrated that all four *adc* mutations can indeed be considered as null alleles (Fig. 2B).

The *adc1* and *adc2* mutant plants did not display any apparent developmental alteration (data not shown). Therefore, next we compared the accumulation of putrescine in wild-type and mutant plants under low temperature. Figure 3 shows that the characteristic

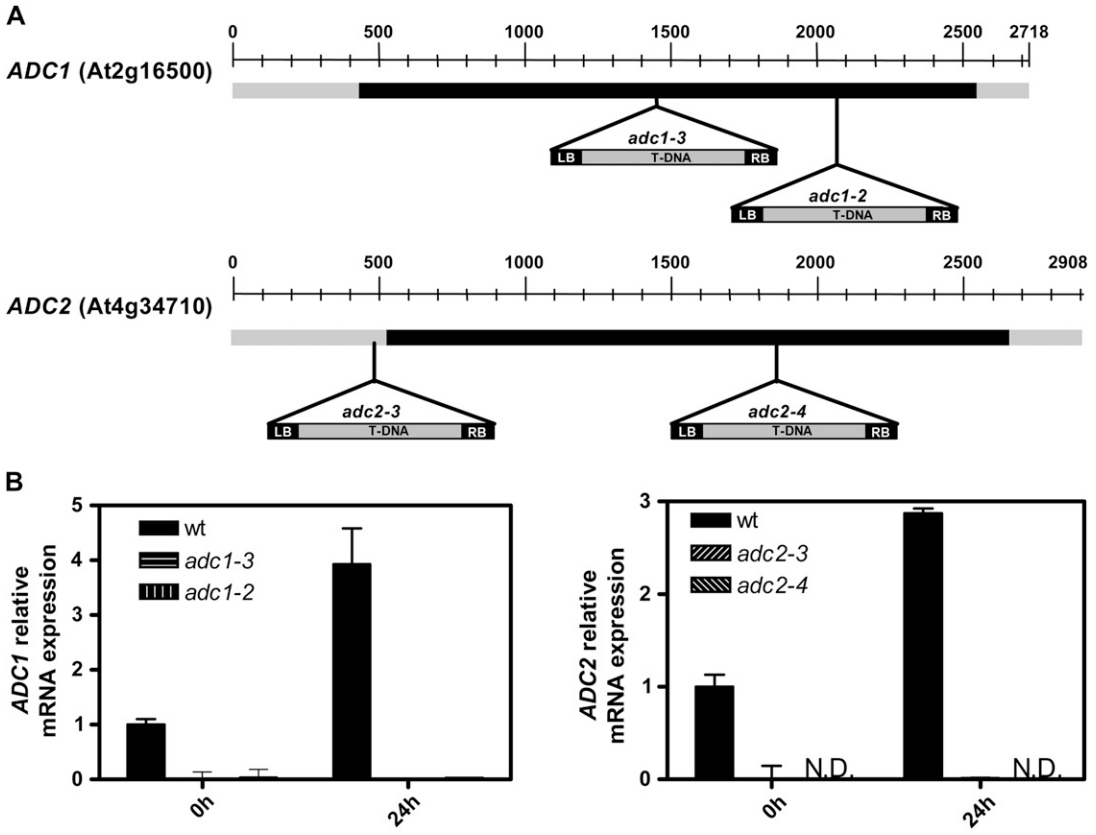

Figure 2. Isolation and characterization of *adc* mutant plants. A, Schematic representation of the positions of T-DNA insertions in the *adc1* and *adc2* mutant alleles. The *adc1-3* and *adc2-3* mutants are from the Cologne collection, whereas *adc1-2* and *adc2-4* mutants are from the NASC. B, qRT-PCR analysis for *ADC1* and *ADC2* in wild type and homozygous mutants in nonstressed plants (time 0 h) and under cold-inductive conditions (24 h of cold treatment). Values are the means of four determinations expressed as relative to *EF1* value ± SD and normalized respect to the wild-type sample at time 0 h of treatment. N.D., Not determined.

profile of putrescine accumulation in wild-type plants in response to low temperature was significantly altered in every mutant tested and demonstrated that the *adc1* and *adc2* mutants accumulated less free putrescine than the wild-type control.

To evaluate the physiological role of putrescine in freezing tolerance and cold acclimation, we further tested the freezing tolerance of nonacclimated and cold-acclimated (7 d, 4°C) *adc* mutant and wild-type plants (Fig. 4, A–H). Loss of function of either *ADC1* or *ADC2* led to similar consequences of reduced freezing tolerance in both nonacclimated and cold-acclimated plants. However, the differences in freezing tolerance were more striking in cold-acclimated plants than in nonacclimated plants.

To assay for complementation of freezing tolerance phenotypes of *adc* mutants, we performed putrescine feeding of seedlings grown in petri dishes. As shown in Figure 4, I and J, the freezing hypersensitive phenotype of *adc* mutants was restored to wild type when putrescine was provided exogenously in the growing medium. These data demonstrated that the freezing sensitive phenotypes were indeed caused by the *adc* mutations in the putrescine biosynthesis genes. It is remarkable that putrescine also improved the freezing tolerance of both nonacclimated and cold-acclimated wild-type plants, suggesting that exogenous putrescine application could effectively alleviate the damage caused by freezing conditions.

Defects in Putrescine Biosynthesis Lead to Alterations in ABA Metabolism in Response to Low Temperature

We next asked whether the reduced freezing tolerance of the *adc* mutants defective in putrescine biosynthesis could be linked to alterations in any of the well-studied signaling pathways that control low-temperature responses. Figure 5 shows no severe alteration in the cold-induction profiles of the genes (CBF regulon) regulated by the *CBF/DREB1* genes that code for transcriptional activators with important roles in cold acclimation (Van Buskirk and Thomashow, 2006). Nevertheless, for several genes, such as *RD29A*, *COR47*, and *KIN1*, lower transcript levels were detected in *adc* mutants compared to wild type. Therefore, we next tested the cold-regulated expression of the *CBF* genes in the *adc* mutants by qRT-PCR (Fig. 6). We did not observe significant changes in the expression of both *CBF1* and *CBF2*, and only reduced expression levels for *CBF3* in *adc* mutants compared to wild type after 8 h of cold treatment. Because some of the affected CBF regulon genes (*RD29A*, *COR47*, and *KIN1*) as well as *CBF3* are regulated by ABA (Knight et al., 2004; Sanchez et al., 2004), and the expression of some cold-responsive genes is mediated by ABA (Viswanathan and Zhu, 2002), we analyzed the expression of several representative genes of two ABA-inducible pathways. The gene *RD22* belongs to an ABA-dependent pathway that uses MYC and MYB transcription factors that bind to MYCR/MYBR elements (Abe et al., 1997), whereas the ABA-dependent cold gene induction of *RD29B* has been shown to depend exclusively on a bZIP family of transcription factors (ABA-responsive element [ABRE]-binding factors [AREBs]) that bind to the ABREs (Uno et al., 2000; Hannah et al., 2005). The results obtained for the *adc1-3* and *adc2-3* mutant alleles, which are representative of all alleles tested, indicated a minor decrease in the late *RD22* cold induction that occurs after 48 h of treatment (Fig. 7A). However, as shown in Figure 7B, ABRE-dependent gene expression of *RD29B* showed a more dramatic effect of reduced cold induction after 24 h of treatment, coincident with the endogenous ABA increase (Lang et al., 1994). To confirm the involvement of polyamines in the control of ABA-dependent cold-induced gene expression, we analyzed the expression of *NCED3*, a key gene in ABA biosynthesis that responds to cold (Iuchi et al., 2001), in the *adc* mutants exposed to 4°C for different time periods. As shown in Figure 7C, we have observed a notable dependence of a proper cold-induction profile of *NCED3* on ADC1 and ADC2 functions, suggesting that the *adc* mutations could affect transient increase of ABA levels during the first 24 h of cold treatment. Therefore, we examined the accumulation of ABA in the *adc* mutants grown in soil after 24 h of low-temperature treatment. A significant reduction of ABA level in the *adc1-3* and *adc2-3* mutants compared to wild-type was detected (Fig. 7D), suggesting that putrescine levels modulate ABA synthesis in response to low temperature. To demonstrate the specificity of the observed response, we designed complementation studies in petri dishes (Fig. 8). We first noticed that cold-induced increase in ABA content was delayed in plate-grown wild-type plants compared to soil-grown plants (compare Figs. 7D and 8A). This is not surprising because previous observations have reported different cold stress responses depending on the growth conditions (Vogel

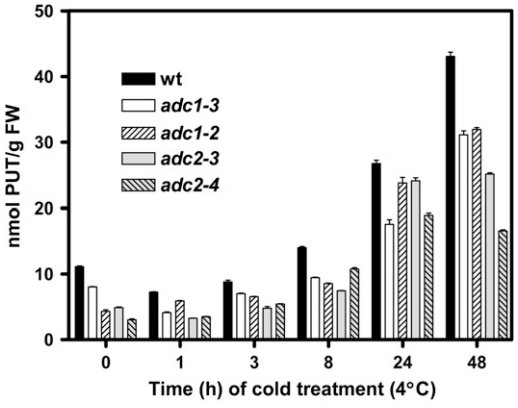

Figure 3. Putrescine accumulation under cold treatment. Free-putrescine content was measured in wild-type plants (black), *adc1-3* mutant (white), *adc1-2* mutant (dashed-white), *adc2-3* mutant (gray), and *adc2-4* (dashed-gray) plants grown for 3 weeks in soil at 4°C. Values are the means of three biological replicates. Experiments were repeated twice with similar results.

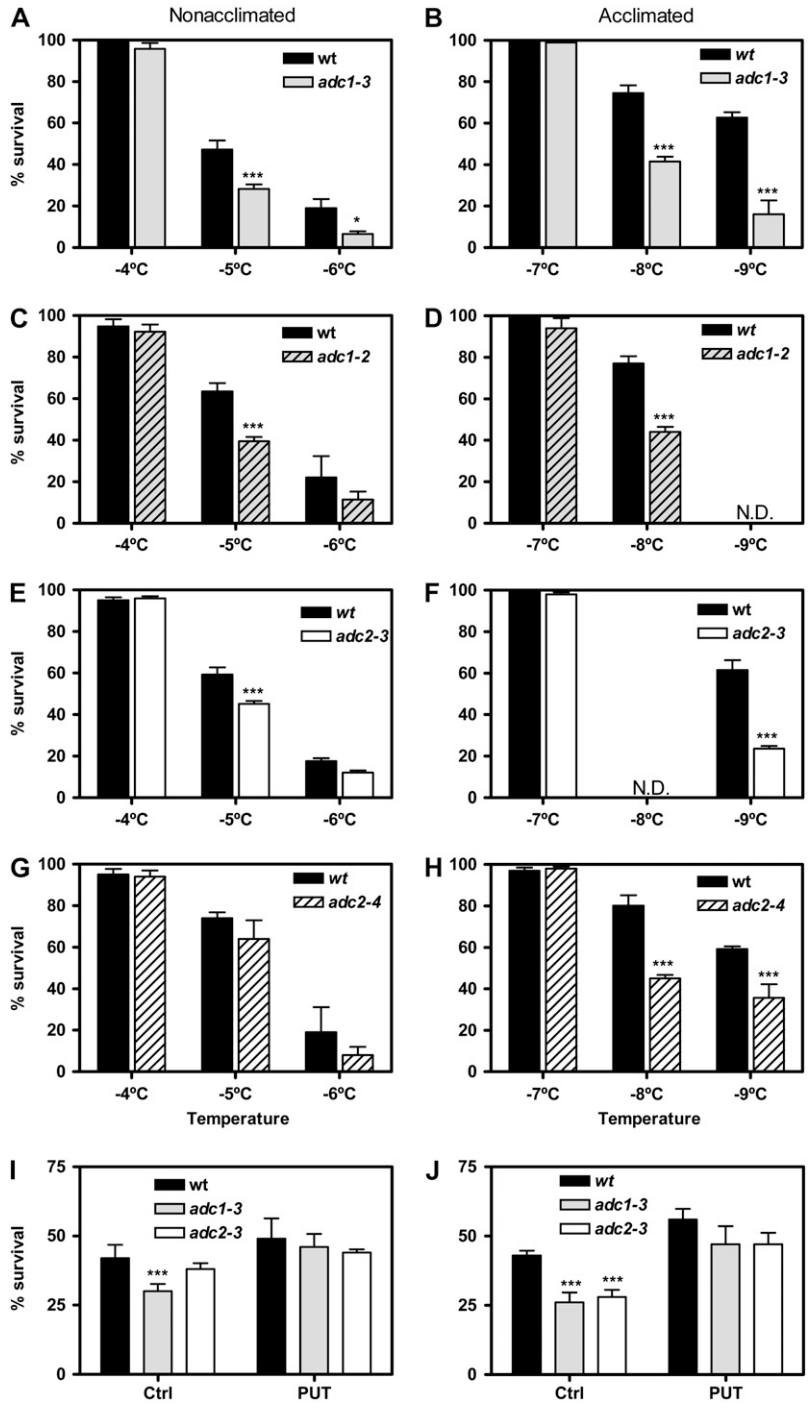

Figure 4. Freezing tolerance of *adc* mutants. A to H, Two-week-old wild-type (dark), *adc1-3* (gray), *adc1-2* (dashed-gray), *adc2-3* (white), and *adc2-4* (dashed-white) plants grown in soil under long-day photoperiods at 20°C were exposed to different freezing temperatures for 6 h. Data are expressed as means of three independent experiments with 50 plants each. Bars indicate SD. N.D., Not determined. I and J, Two-week-old wild-type (dark), *adc1-3* (gray), and *adc2-3* (white) plants grown on Murashige and Skoog plates under long-day (16 h light/8 h dark) photoperiod at 20°C and exposed to −6°C (nonacclimated) and −12°C (cold-acclimated) freezing temperatures for 1 h in the absence (Ctrl) or presence of putrescine. Data are expressed as means of three to seven replicates with 50 plants each. Bars indicate SD. Two-way ANOVA and Bonferroni posttest were assayed; significant difference with wild type is stated with one or three stars (for $P < 0.05$ and $P < 0.001$, respectively).

et al., 2005). Moreover, the reduction of ABA accumulation in *adc* mutants was restored to normal levels when putrescine was added to the medium (Fig. 8A). The restoration of ABA levels upon providing putrescine exogenously correlated with an increase of *NCED3* mRNA level as shown in Figure 8B. The data depicted in Figures 7 and 8 strongly suggested that putrescine accumulation participates in the increase of ABA levels, which occurs in response to low temperature through the control of *NCED3* expression.

Putrescine Positively Modulates ABA Accumulation in Response to Low Temperature

To confirm that a rapid increase in putrescine levels is required for ABA accumulation in response to low temperature, we performed reciprocal complementation studies of freezing tolerance in plate-grown seedlings. With this purpose, ABA was added to *adc1-3* and *adc2-3* mutants and putrescine to the ABA-deficient mutant (*aba2-3*). Figure 9 shows that the freezing

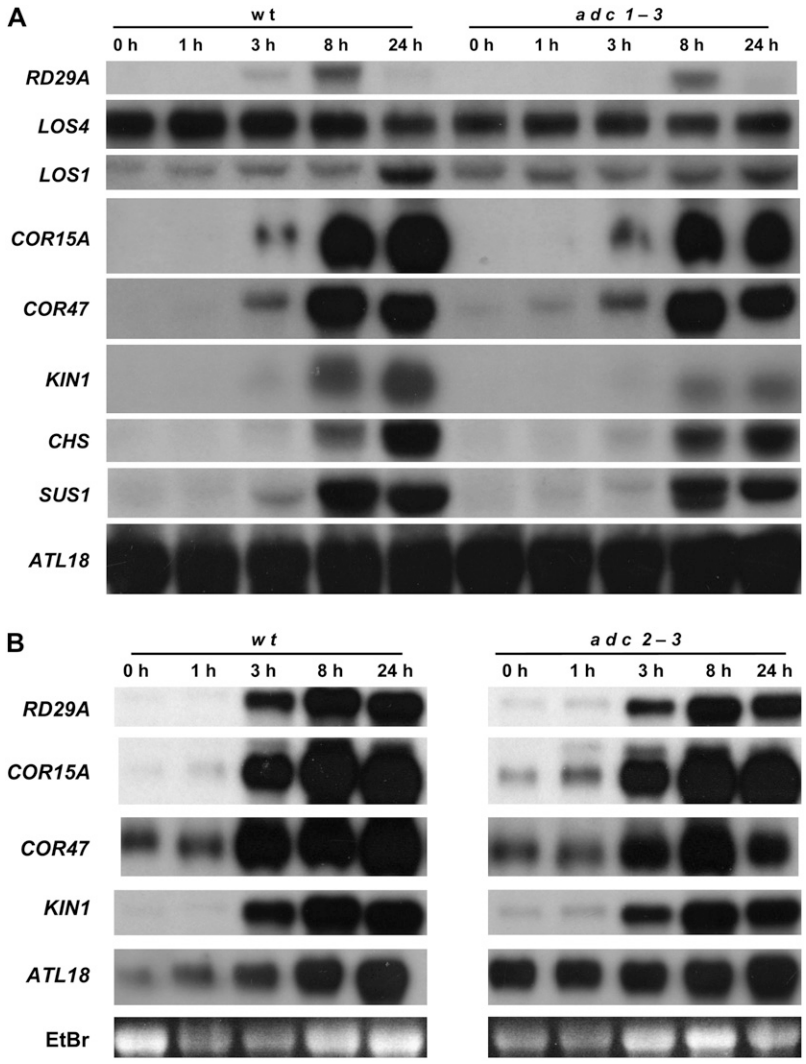

Figure 5. Gene expression of CBF regulon in *adc* mutants under low temperature. Northern analysis was performed with 20 μg of total RNA from wild type and either *adc1-3* (A) or *adc2-3* mutants (B) grown in soil and subjected to different periods of cold treatment as indicated.

tolerance phenotypes of *adc* mutants were completely restored when ABA was added to the growth medium using either nonacclimated or cold-acclimated plants (Fig. 9, A and B). On the contrary, freezing tolerance of the *aba2-3* mutant could not be restored to wild-type levels by putrescine supplementation, but was complemented by ABA addition to the medium (Fig. 9, C and D). Thus, although *NCED3* expression was stimulated by putrescine addition, the *aba2-3* mutation that affects the next enzymatic step in ABA biosynthesis (Schwartz et al., 1997) abolished the capacity of Arabidopsis seedlings to tolerate freezing constitutively and to cold acclimate. These data indicated that cold-induced increase in putrescine synthesis acts positively on ABA accumulation by modulating, either directly or indirectly the expression of *NCED3* involved in ABA biosynthesis.

DISCUSSION

In this article, we have studied changes in polyamine synthesis in response to short- and intermediate-term low-temperature treatments as defined by Kaplan et al. (2004). We found that the levels of free putrescine sharply increase during the intermediate phase (24–72 h) in response to cold (Fig. 1A). Despite stimulation of putrescine accumulation, the levels of free spermidine and spermine (for which putrescine is a precursor) remained either unchanged or slowly decreased, especially during the intermediate phase of cold response (Fig. 1, B and C). Because conjugated spermidine and spermine levels did not significantly change under low-temperature conditions (data not shown), the observed decrease in free soluble spermine levels likely reflects an increase in its catabolism (Cona et al., 2006). Putrescine accumulation accompanied by reduced or unchanged levels of spermidine and spermine levels has already been observed in oat (*Avena sativa*) plants subjected to osmotic stress (Tiburcio et al., 1993) and in transgenic Arabidopsis plants overexpressing *ADC2* (Alcazar et al., 2005). In this regard, it has been proposed that cellular levels of spermidine and spermine are homeostatically regulated and more tightly than those of putrescine (Bhatnagar et al., 2001). It is possible

that the supramolecular organization of aminopropyl transferases (*SPDS1*, *SPDS2*, and *SPMS*) in the form of a metabolon contributes to this regulation (Panicot et al., 2002).

Because putrescine is the only polyamine whose levels increase in response to low temperature, we studied cold regulation of *ADC1* and *ADC2* gene expression. These genes encode two isoforms of Arabidopsis ADC, which is considered a key enzyme of putrescine synthesis under stress conditions (Alcazar et al., 2006b). Accumulation of *ADC1* and *ADC2* mRNAs under cold stress (Fig. 1D) is correlated with the increase of putrescine during the intermediate phase of cold response (Fig. 1A). Cold induction of *ADC1* occurs earlier and it is more pronounced than

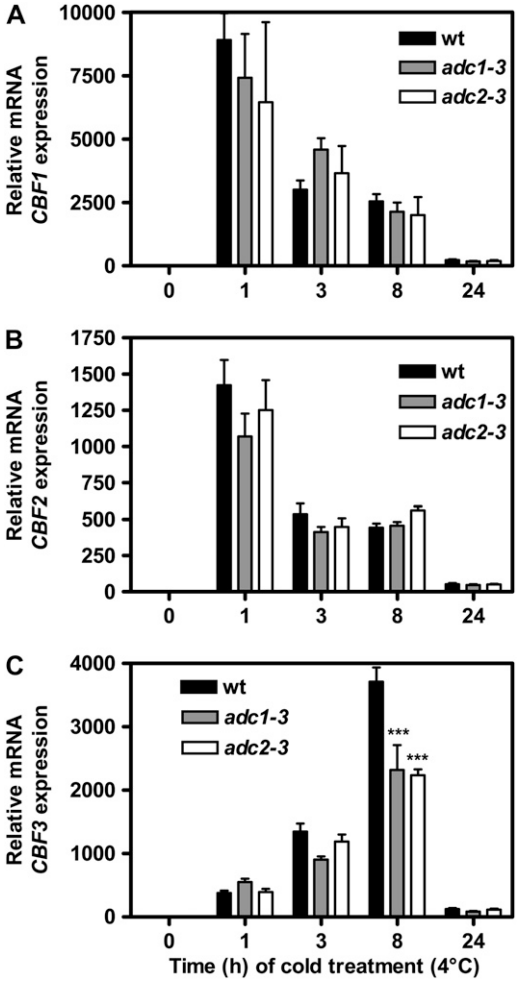

Figure 6. Expression of CBF genes in *adc* mutants under low temperature. qRT-PCR determination was performed in 3-week-old wild-type (black), *adc1-3* (gray), and *adc2-3* (white) plants subjected to different periods of cold stress. Values are the means of four determinations expressed as fold change normalized to *EF1*. Bars indicate SD. Values are means of three to four biological replicates. Three stars indicate significant difference with wild-type background (at $P < 0.001$). Experiments were repeated at least twice with similar trends observed.

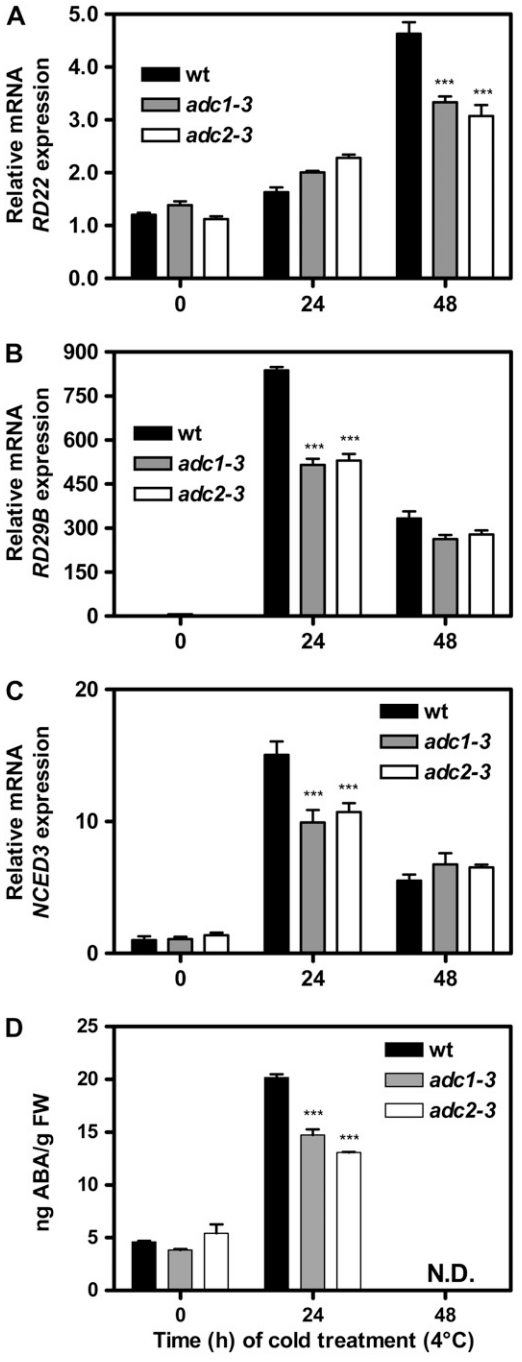

Figure 7. Expression of ABA-related genes in the *adc* mutants and ABA measurements. A to C, qRT-PCR was performed for the indicated genes in 3-week-old wild-type (black), *adc1-3* (gray), and *adc2-3* (white) plants subjected to different periods of cold stress. Values are the means of four determinations expressed as fold change normalized to *EF1* and relative to wild type at time 0 h of treatment. D, ABA levels were quantified by HPLC-MS in wild-type (black), *adc1-3* (gray), and *adc2-3* (white) 3-week-old soil-grown plants under cold stress (4°C). N.D., Not determined. Bars indicate SD. Values are means of three to four biological replicates. Three stars indicate significant difference with wild-type background (at $P < 0.001$). Experiments were repeated at least twice with similar trends observed.

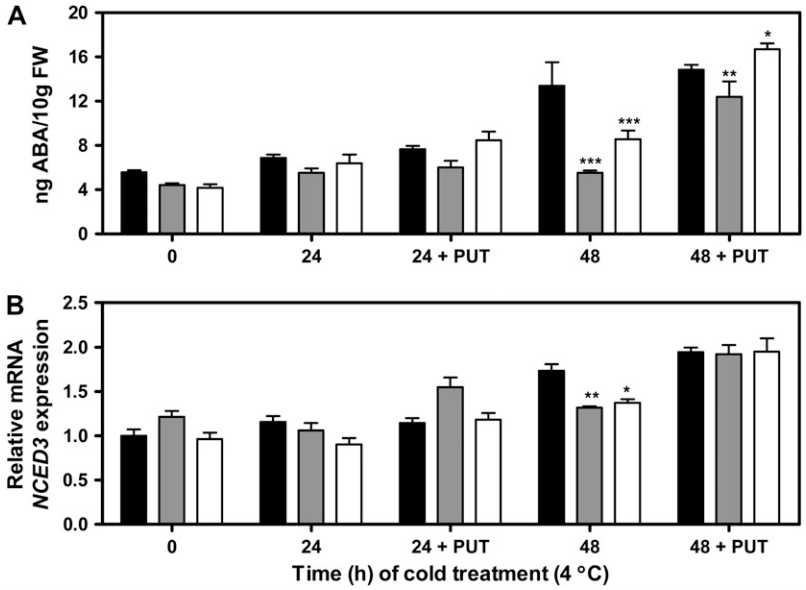

Figure 8. ABA levels and *NCED3* expression in *adc* mutant plants under cold treatment. A, ABA levels were scored by HPLC-MS for 14-d-old plants grown in petri dishes under cold stress (4°C) with or without addition of putrescine in wild-type (black), *adc1-3* (gray), and *adc2-3* (white) plants. Values are the means of four independent determinations expressed as fold change normalized to *EF1* and relative to wild type at time 0 h of treatment. Bars indicate SD. B, The same experiment as for A was scored by qRT-PCR to determine *NCED3* expression. Values are means of three to four biological replicates. One, two, or three stars indicate significant difference with wild-type background (at $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively). Experiments were repeated at least twice with similar trends observed.

that of *ADC2*, and this is probably related to a different promoter context (Hummel et al., 2004). In contrast to *ADC1*, *ADC2* promoter contains several ABRE cis-elements (Alcazar et al., 2006b) and it is inducible by drought stress in an ABA-dependent manner, whereas *ADC1* does not respond to dehydration (Alcazar et al., 2006a). Interestingly, expression of the *SAMDC2* gene also increases by cold (Urano et al., 2003), but it does not lead to increases in spermidine and spermine (Fig. 1, B and C). Particular mechanisms of posttranscriptional control that have been reported for *SAMDC* genes (Hu et al., 2005) may explain the observed responses to low temperature.

Our data provide genetic evidence for a requirement of putrescine accumulation to achieve full development of freezing tolerance in Arabidopsis before and after cold acclimation (Figs. 2–4). Two independent mutant alleles for both *ADC1* and *ADC2* genes were used in our studies because double mutants completely devoid of ADC activity are not viable in Arabidopsis (Urano et al., 2005). We observed that putrescine accumulation in response to cold is impaired in plants carrying the recessive *adc* mutations (Figs. 2 and 3). The *adc1* and *adc2* mutants are defective in freezing tolerance before and after cold acclimation (Fig. 4, A–H), and these phenotypes are reversed by addition of putrescine (Fig. 4, I–J). The meaningful increase in putrescine levels for both *adc1* and *adc2* mutants, together with the similar phenotypic response to freezing stress for both mutations, suggest that both *ADC1* and *ADC2* are partially redundant in the response to low temperatures. Similar observa-

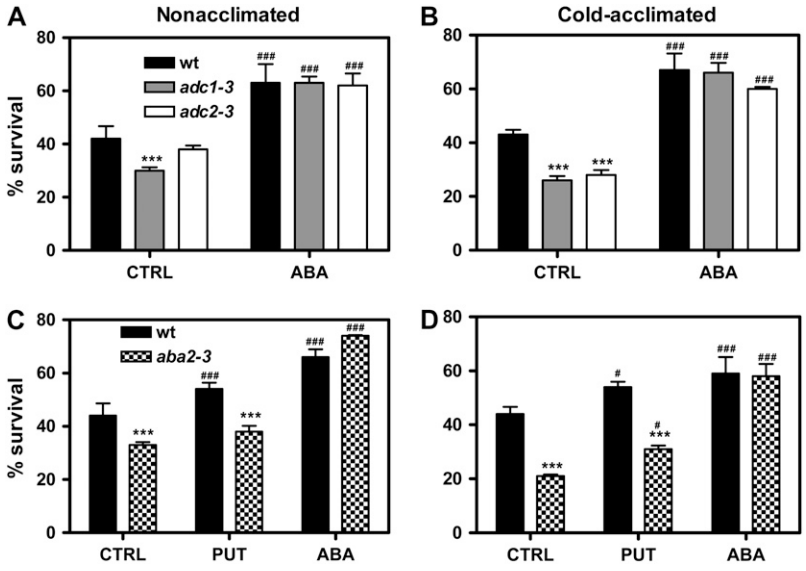

Figure 9. Chemical complementation of freezing tolerance in *adc* and *aba* mutants. Two-week-old wild-type (black), *adc1-3* (gray), *adc2-3* (white), and *aba2-3* (dotted) plants grown in Murashige and Skoog plates under long-day photoperiod at 20°C were supplemented with putrescine and exposed to −6°C (nonacclimated) and −12°C (72 h at 4°C, acclimated) freezing temperatures for 1 h. Freezing tolerance was estimated as the percentage of plants surviving each specific temperature after 3 d of recovery under unstressed conditions. Data are expressed as means of three to five replicates with 50 plants each. Bars indicate SD. Two-way ANOVA and Bonferroni posttest were assayed; significant difference versus wild type is stated with three stars ($P < 0.001$), whereas significant differences for each genotype in control versus treatment is indicated with one or three hash symbols (for $P < 0.05$ and $P < 0.001$, respectively). The experiments were repeated at least twice with similar trends observed.

tions of functional redundancy for *ADC1* and *ADC2* have been reported during seed development (Urano et al., 2005). In addition, putrescine also seems to increase freezing tolerance of wild-type plants under both nonacclimated and cold-acclimated conditions (Fig. 4, I–J), thus reinforcing the protecting role of putrescine against freezing stress.

To gain more insight into the role played by putrescine in low-temperature response, we analyzed the expression of different cold-induced genes in mutants with reduced ability to synthesize putrescine. No relevant alteration in the CBF-dependent pathway was found in any of the mutants, with the exception of minor changes for ABA-regulated genes (Fig. 5). Moreover, quantitative studies of CBF gene expression showed that only *CBF3* is altered in *adc* mutants (Fig. 6). Because *CBF3* is also responsive to ABA, we speculated that reduction in putrescine content for *adc* mutants would cause alterations in cold-induced ABA-dependent gene expression. In agreement with this, we could show that genes whose expression is controlled by ABA displayed reduced induction upon cold treatment in the *adc* mutants when compared to wild type (Fig. 7). Of particular interest was the reduced induction of *NCED3*, a key gene of ABA biosynthesis (Nambara and Marion-Poll, 2005). Close relationship between ABA and low-temperature responses is well documented in a great variety of plant species (Daie and Campbell, 1981; Chen et al., 1983; Anderson et al., 1994). An initial study of possible connection between ABA and polyamines has been carried out recently in cold-stressed tomato (*Solanum lycopersicum*; Kim et al., 2002), and a functional role for both ABA and polyamines in low-temperature response has been suggested. However, Kim et al. proposed that ABA and polyamines act independently to control cold responses, which is in disagreement with our observations in Arabidopsis. Two main arguments could explain this apparent discrepancy. First, Kim et al. did not use genetic tools to dissect the functional role of polyamines in cold-stressed tomato. Rather, they used pharmacological inhibitors that target only the ODC pathway for putrescine biosynthesis in tomato and this may not be as precise as the use of knockout mutations affecting the unique ADC pathway present in Arabidopsis. Second, they used the *flacca* tomato mutant, which retains a detectable amount of ABA (Sagi et al., 1999). Our studies indicate that genetic alterations in the putrescine biosynthesis pathway reduce the accumulation of ABA in plants subjected to cold (Fig. 7D), and this can be restored upon addition of putrescine to the growth medium (Fig. 8). Moreover, reciprocal chemical complementation studies using putrescine and ABA supplementation to both *adc* and *aba2-3* mutants showed that, although ABA addition can complement putrescine deficiency to survive freezing, the opposite is not the case (Fig. 9). This failure in the putrescine complementation of the *aba2-3* freezing sensitivity strongly suggests that the diamine requirement to full development of freezing tolerance in Arabidopsis is, at least in part, mediated by ABA, and places putrescine upstream of ABA in the hypothetical signaling pathway.

The results presented above point to a functional role for the diamine putrescine in freezing tolerance and cold acclimation that goes beyond a mere physicochemical protecting function. In this regard, it has been suggested that polyamines in mammals may participate in loops involving interaction with signal transduction pathways and activation/repression of proteins that may control either cell death or cell growth (Pignatti et al., 2004). In plants, a similar suggestion has been made regarding the role of spermine acting as a signaling molecule to transduce defense responses, including hypersensitive response cell death in tobacco (*Nicotiana tabacum*; Yamakawa et al., 1998). Our observations strongly suggest that putrescine acts as a signaling molecule interacting with ABA-dependent signaling pathways involved in cold stress. However, we note that the freezing sensitivity of the *adc* mutants described in this article may not be explained solely by the alterations in ABA synthesis, and, at this point, we cannot exclude other protective functions for putrescine not elucidated here. A relevant remaining question raised by our observations is how the increments in putrescine may account for a change in *NCED3* gene expression. Although there are several steps related to the control of gene expression at which polyamines can execute their physiological actions (Childs et al., 2003), the elucidation of how the specificity, intensity, and timing of such control is achieved will certainly require additional scientific effort.

MATERIALS AND METHODS

Plant Materials, Growth Conditions, and Treatments

Twelve- to 14-d-old sterile seedlings and 2- and 3-week-old soil-grown plants of Arabidopsis (*Arabidopsis thaliana*) ecotype Columbia (Col-0) were used in this work. To obtain sterile seedlings, Cl_2 vapor-sterilized seeds were germinated on polyester mesh (33-μm opening, 40-μm fiber diameter; Bückmann GmbH) in petri dishes containing one-half-strength Murashige and Skoog medium (Murashige and Skoog, 1962), supplemented with 250 mg/L MES solidified with 1% (w/v) plant agar (Duchefa Biochemie). Seeds were sown in pots containing a mixture of soil and vermiculite (3:1 [v/v]), and irrigated with water and Hoagland-based mineral solution. All seedlings and plants were grown at 21°C ± 1°C under long-day photoperiods (16 h of cool-white fluorescent light, photon flux of 70–90 μmol m^{-2} s^{-1}). Low-temperature treatments were performed by transferring plants to a growth chamber set to 4°C ± 1°C for different periods of time under the same light and photoperiodic conditions. All cold experiments were started after 1 h of light, and plant material was collected at the indicated times.

Freezing assays were carried out in a temperature-programmable freezer. Nonacclimated or cold-acclimated (7 d, 4°C) 2-week-old plants were exposed to 4°C for 30 min in darkness and subsequently the temperature was lowered at a rate of 2°C/h. The final desired freezing temperature was maintained for 6 h and then the temperature was increased again to 4°C at the same rate. After thawing at 4°C for 4 h in the dark, plants were returned to their original growth conditions (see above). Tolerance to freezing was determined as the capacity of plants to resume growth after 14 d of recovery under control conditions. Also, nonacclimated or cold-acclimated (72 h, 4°C) 12- to 14-d-old seedlings were transferred to the programmable freezer set at −1°C. After 1 to 2 h, the plates were sprinkled with ice chips and maintained at −1°C for at

least 16 h. Temperature was lowered with a rate of 1°C/h and plates were removed (−4°C to −8°C for nonacclimated and −9°C to −13°C for acclimated plants). After removal, the plates were maintained in the dark at 4°C for 12 to 20 h for thawing (Verslues et al., 2006). The polyester mesh with plants was transferred to new medium and returned to the original growth conditions. Tolerance to freezing was determined as the capacity of plants to resume growth after 3 d of recovery under control conditions.

ABA treatments were performed by transferring the mesh with seedlings to medium supplemented with 1 μM ABA. An ABA stock solution (20 mM) was prepared in 2 M KOH; no change in pH due to the addition of KOH was monitored previously. For putrescine treatment, the polyester mesh with seedlings was transferred to new petri dishes containing agar medium supplemented with 400 μM putrescine (from a 200 mM stock solution in 1 mM MES, pH 5.8) for different periods of time. After treatments, plants were immediately frozen in liquid N_2 and stored at −80°C until their use.

Identification of ADC Mutants

The *adc1-3* and *adc2-3* mutants were identified by screening 90,000 T-DNA-tagged lines by PCR, using gene- and T-DNA-specific primers (Rios et al., 2002). For the *ADC1* gene 5′-AGAAGAAGAAGATGCCTGCTCTAGCTT-TTG-3′ and 5′-GAACTAGGATCGTAATCAATCGTA-3′ and for the *ADC2* gene 5′-TAGATTCATCTTCTGTCTTCTCCTGAGG-3′ and 5′-TCACGCAGA-GATGTAATCGTAGACGTCTTC-3′ gene-specific primers were used in combination with the T-DNA-specific primers FISH1 5′-CTGGGAATGGCGA-AATCAAGGCATC-3′ and FISH2 5′-CAGTCATAGCCGAATAGCCTC-TCCA-3′. DNA sequencing showed that the T-DNA insertions were 1,100 bp downstream of the *ADC1* starting codon and 1,301 bp downstream of the *ADC2* starting codon. The *adc1-2* and *adc2-4* mutants were ordered from the Nottingham Arabidopsis Stock Centre (NASC; N585350 and N647171, respectively; Alonso et al., 2003). T-DNA-specific primers (SALK-LB 5′-TTT-GGGTGATGGTTCACGTAGTGGG-3′ and FISH2) and the same gene-specific primers as above were used for determining the position of SALK T-DNA tags. DNA sequencing indicated that the T-DNA insertions were 1,496 bp downstream of the *ADC1* starting codon and 98 bp downstream of the *ADC2* starting codon. Homozygous plants were identified by PCR. Northern blot showed no detectable transcripts in any of the four homozygous mutants. Southern blot, antibiotic resistance (when feasible), and PCR segregation analysis revealed that the *adc1-3*, *adc1-2*, and *adc2-4* mutant lines carry single T-DNA insertions.

Gene Expression Analysis

For northern blots, total RNA was isolated from 3-week-old wild-type and mutant plants, according to Logemann et al. (1987). RNA-blot hybridizations were performed using standard protocols (Sambrook et al., 1989). The *ADC1* and *ADC2* probes were PCR fragments of 261 and 265 bp obtained from the 5′-noncoding region of these genes, respectively. Probes were obtained by PCR from genomic DNA templates using the primers *ADC1*-5′-FW 5′-TCT-CTTCCCTGAAACTTTCCCGAGAAA-3′ and *ADC1*-5′-RV 5′-ACCCCCTCA-GAAGGAAGCAGGA-3′, and *ADC2*-5′-FW 5′-ATTTTCCCCAGAATTCCTT-CACA-3′ and *ADC2*-5′-RV 5′-CCCCCTCAGGAGAAGACAGAA-3′. Specific probes for the *RD29A*, *COR15A*, *COR47*, *KIN1*, *CHS*, *SUS1*, and *ATL18* genes have been described previously (Novillo et al., 2004, and refs. therein). *LOS1* and *LOS4* probes were synthesized as described by Guo et al. (2002) and Gong et al. (2005), respectively.

To facilitate qRT-PCR measurement of transcripts of all investigated genes under standard reaction conditions, primers were designed using Primer-Express 2.0 software (Applied Biosystems) and the following criteria: melting temperature of 60°C to 65°C and PCR amplicon lengths of 60 to 200 bp, length of primer sequences ranging from 19 to 25 nucleotides, and guanine-cytosine content of 40% to 60%. The specificity of primer pair sequences was checked against the Arabidopsis transcript database using The Arabidopsis Information Resource BLAST (http://www.arabidopsis.org/Blast). Specificity of the primer amplicons was checked by melting-curve analysis performed by the PCR machine after 45 amplification cycles and by gel-electrophoretic analysis. The PCR products were resolved on 3.5% (w/v) agarose gels (NuSIEVE agarose; CAMBREX). *ADC1*, *ADC2*, *SPDS1*, *SPDS2*, *SPMS*, *SAMDC1*, *SAMDC2*, and *RD29A* primers were described previously (Alcazar et al., 2006a). *EF1*, *CBF1*, and *CBF2* primers were described by Rohde et al. (2004). Other primers used were *CBF3*-FW 5′-GCCGATCAGCCTGTCTCAATT-3′ and *CBF3*-RV 5′-CGAAGCCATGATCCGTCGT-3′; *RD29B*-FW 5′-GGAGC-GGTCACTTCTTGGCT-3′ and *RD29B*-RV 5′-GGTGGTGCCAAGTGATTGTG-3′; and *NCED3*-FW 5′-CAGCTTGTAGCTTTTGGGCTGTA-3′ and *NCED3*-RV 5′-TAACAGAAACCAGCTGAGCTCGA-3′. The amplification efficiency of every primer pair was determined empirically by 5- to 10-fold serial dilutions of genomic DNA or cDNA, and only primer pairs with efficiency over 0.85 were used.

Total RNA was isolated from rosette leaves of 3-week-old Arabidopsis plants using the TRIzol reagent (Invitrogen). One hundred micrograms of total RNA was purified with RNeasy plant mini kit columns (Qiagen GmbH) following the manufacturer's instructions. DNase digestion, genomic DNA contamination check, cDNA synthesis, and quality check of cDNA were performed as described (Czechowski et al., 2005). All samples in the same experiment were within 2 threshold cycle (Ct) units. Quantitative real-time PCR with the SYBR Green PCR Mastermix (Applied Biosystems) was performed using a 10-fold cDNA dilution as template in a 384-well platform system (model 7900; Applied Biosystems). For quantification, no-template-control, calibration curves, and unknown reactions were run in quadruplicate. The *EF1* gene was used as a housekeeping control (Rohde et al., 2004). Experiments were repeated at least twice. Data were analyzed using the SDS 2.0 software (Applied Biosystems). To generate a baseline-subtracted plot of the logarithmic increase in fluorescence signal (ΔR_n) versus cycle number, baseline data were collected between cycles 3 and 12. All amplification plots were analyzed with an R_n threshold of 0.2 to obtain Ct values. Expression values of each gene of interest were calculated using the equation described by Pfaffl (2001).

Polyamine Analysis

Free polyamines were analyzed by HPLC separation of dansyl chloride derivatives. The extraction and determination methods have been described previously (Marce et al., 1995).

ABA Analysis

ABA was measured by liquid chromatography-mass spectrometry as described previously by Lopez-Carbonell and Jauregui (2005). Briefly, 300 to 500 mg of 3-week-old rosette leaves and 5 to 10 g of plants grown in Murashige and Skoog medium were frozen in liquid N_2, powdered with mortar and pestle, and extracted with acetone:water:acetic acid (80:19:1 [v/v]) at −20°C. Then, samples were vortexed and centrifuged at 10,000*g*, 4°C for 10 min. Supernatants were collected and pellets reextracted with the extraction solvent. The second extraction was centrifuged and then supernatants were combined and dried under a nitrogen stream. The dried samples were kept at −20°C until analysis. The extracts were reconstituted in 200 μL of water:acetonitrile:acetic acid (90:10:0.05 [v/v]), stirred, vortexed, centrifuged (10,000*g*, 5 min), filtered through a 0.45-μm polytetrafluoroethylene filter (Waters), and 5 μL was injected into the liquid chromatography-tandem mass spectrometry system. Quantification was done by the standard addition method by spiking control plant samples with ABA solutions (ranging from 5–100 ng mL^{-1}).

Statistical Analysis

ANOVA with Bonferroni's posttest analysis and *t* test were performed using GraphPad Prism, version 4.00, for Windows (GraphPad Software).

Arabidopsis seed stocks deposited at the NASC include *adc1-2* (N9658), *adc1-3* (N9657), *adc2-3* (N9659), and *adc2-4* (N9660).

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