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eskimo1 mutants of *Arabidopsis* are constitutively freezing-tolerant

(cold acclimation/frost tolerance)

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ABSTRACT **Temperate plants develop a greater ability to withstand freezing in response to a period of low but nonfreezing temperatures through a complex, adaptive process of cold acclimation. Very little is known about the signaling processes by which plants perceive the low temperature stimulus and transduce it into the nucleus to activate genes needed for increased freezing tolerance. To help understand the signaling processes, we have isolated mutants of *Arabidopsis* that are constitutively freezing-tolerant in the absence of cold acclimation. Freezing tolerance of wild-type *Arabidopsis* was increased from −5.5°C to −12.6°C by cold acclimation whereas the freezing tolerance of 26 mutant lines ranged from −6.8°C to −10.6°C in the absence of acclimation. Plants with mutations at the *eskimo1* (*esk1*) locus accumulated high levels of proline, a compatible osmolyte, but did not exhibit constitutively increased expression of several cold-regulated genes involved in freezing tolerance. RNA gel blot analysis suggested that proline accumulation in *esk1* plants was mediated by regulation of transcript levels of genes involved in proline synthesis and degradation. The characterization of *esk1* mutants and results from other mutants suggest that distinct signaling pathways activate different aspects of cold acclimation and that activation of one pathway can result in considerable freezing tolerance without activation of other pathways.**

Freezing temperatures represent a major environmental constraint for all living organisms. For example, frost damage limits the geographical distribution and growing season of many plant species and results in significant crop losses (1, 2). Many tropical and subtropical plants are incapable of surviving freezing. By contrast, most temperate species respond to a period of low but nonfreezing temperatures by developing greater ability to withstand subsequent freezing through a cell-autonomous process of cold acclimation (1). Cold acclimation also occurs in other organisms such as nematodes and insects (3, 4). The extent of increased freezing tolerance achieved varies among species. For example, winter rye improves freezing tolerance from an LT_{50} (the temperature that kills 50% of plants) of −6°C in nonacclimated plants to −21°C (5), whereas spinach improves from −6 to −10°C (6). Nonacclimated *Arabidopsis* were killed at −3°C, whereas a 2-day exposure to 4°C increases the freezing tolerance to −10°C (7–9).

The process of cold acclimation involves numerous physiological and biochemical changes. The most notable changes include reduction or cessation of growth, reduction of tissue water content (1), a transient increase in abscisic acid (ABA) (10), changes in membrane lipid composition (5, 11), and the accumulation of compatible osmolytes such as proline, betaine, and soluble sugars (12, 13). Cold acclimation is associated with complex changes in gene expression (6). Many genes have been cloned by differential screening of cDNA libraries constructed from cold-acclimated plant species (9). Several lines of evidence indicate that some of these genes have roles in freezing tolerance (14, 15). In general, however, there is no clear understanding of the relative importance of each of these activated genes or indeed which are more general responses to low, nonfreezing temperatures but not specifically involved in freezing tolerance. For example, in *Arabidopsis* transcripts for alcohol dehydrogenase, phenylalanine ammonia-lyase and chalcone synthase are all strongly induced by low temperature, but mutants deficient in these activities are able to cold acclimate as fully as wild type (8, 16). These studies demonstrate that some cold-induced genes are not required for development of freezing tolerance. To understand cold acclimation, it is essential to determine the relative importance of these cold-induced genes in providing increased freezing tolerance.

Determining how cold acclimation is initiated and coordinated is also a challenging task. In broad terms, it can be assumed that a temperature transducer responds to low temperature by activating a signaling pathway, which, in turn, institutes the biochemical and gene expression events needed for increased freezing tolerance. In the last few years, evidence has accumulated to indicate that classical signaling processes, including protein phosphorylation (17, 18), calcium fluxes (19, 20), and hormone action (21, 22), are all involved in activating cold acclimation. However, very little is known about the organization, degree of complexity, or the sequence within the signaling pathway. To address these issues, and to clone genes controlling cold acclimation, we have isolated a series of *Arabidopsis* mutants that are constitutively freezing tolerant in the absence of any low-temperature treatment. Isolation of these mutants and characterization of one of them, *eskimo1*, suggest that at least four separate signaling cascades operate in cold acclimation. The mutants offer new tools to investigate the signaling process mediating cold acclimation and will allow cloning of terminal genes critical for freezing tolerance.

METHODS

Petri Dish Freezing Tolerance Assay. Sterilized *Arabidopsis* seeds were sown on Petri dishes containing Gamborg basal salts (23) solidified with 0.9% agar. The Petri dishes with seeds were placed at 4°C for 2 days to promote germination and grown at 22°C under 90 μmol quanta $m^{-2} \cdot s^{-1}$ continuous light. The freezing test was conducted in the dark. Ten days after germination, Petri dishes of plants were transferred to a chamber set to −1 ± 0.1°C. To achieve uniform freezing, it was necessary to incubate the Petri dishes of plants with ice chips at −1°C for at least 16 hr before further lowering the temperature. In control experiments to establish that this incubation did not induce cold acclimation, the freezing tolerance of wild-type seedlings held in

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Abbreviations: ABA, abscisic acid; LT_{50}, lethal temperature (50); P5CS, Δ^1-pyrroline-5-carboxylate synthase.

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the dark at −1°C was monitored over 2 days. No increase in freezing tolerance was observed at any temperature during the 2 days of treatment. Thus, for all experiments the Petri dishes were maintained at −1°C for 16 hr after the addition of ice crystals before the chamber was programmed to cool at 1°C per hr. The temperature of the culture medium was monitored by a thermocouple. Dishes of plants were removed at desired temperatures, thawed at 4°C for 12 hr in the dark, and then returned to the original growth conditions. Two days later, survival of plants was scored visually.

Mutant Screening. M_2 seeds of ethyl methanesulfonate mutagenized populations of *Arabidopsis thaliana* ecotype Columbia were either purchased from Lehle Seeds (Round Rock, TX) or generated according to ref. 24. Ten-day-old M_2 seedlings grown on Petri dishes were frozen to −8°C as described above, and the Petri dishes were maintained at −8°C for an additional 3 hr before thawing at 4°C. Plants that survived such treatment were transplanted to soil to produce M_3 seeds. Constitutive freezing tolerance was retested by using 32 M_3 plants.

Electrolyte Leakage Assay. Wild-type and *esk1* seeds were grown on commercial potting mix at 22°C under a diurnal light regime that included 10 hr of illumination at 150 μmol quanta $m^{-2}{\cdot}s^{-1}$. The fourth and fifth leaves from 30-day-old plants were assayed for ion leakage after freezing at various temperatures as described (7, 25).

Genetic Analysis. To determine the dominance of *esk1* mutation, *esk1* plants were reciprocally crossed to wild type. Freezing tolerance assays were conducted with 32 F_1 plants. To study the segregation of freezing tolerance, one of the resulting F_1 hybrids was allowed to self-fertilize, the resulting F_2 plants were grown to maturity and the seeds were used to generate F_3 families. The genotype of each F_2 plant at the *esk1* locus was determined by testing the freezing tolerance of 32 F_3 seedlings grown on Petri dishes. For genetic mapping of the *esk1* mutation, an *esk1* plant was crossed to a wild-type plant of the *Arabidopsis thaliana* Landsberg erecta ecotype and an F_1 hybrid was allowed to self-fertilize. A total of 672 F_2 plants were used to map *esk1* mutation. A single leaf from each F_2 plant was sampled and stored at −70°C. After sampling, the plants were self-fertilized to produce F_3 seeds. The genotype of each F_2 plant at the *esk1* locus was determined by testing the freezing tolerance of 32 F_3 seedlings derived from the corresponding F_2 plant. Genomic DNA from homozygous *esk1/esk1* or *ESK1/ESK1* plants was extracted according to ref. 26. The chromosome location of *esk1* mutation was determined by cosegregation of freezing tolerance with two types of PCR-based polymorphic DNA markers—codominant cleaved amplified polymorphic sequence (CAPS) markers (27) and simple sequence-length polymorphic (SSLP) markers (28).

Proline and Sugar Measurement. Lyophilized leaf tissue was extracted in 75% ethanol with constant stirring at 4°C for 24 hr. After centrifugation at 20,000 × *g* for 5 min, an aliquot of the supernatant was dried under vacuum. Amino acids were analyzed with an Amino Acid Analyzer (Bioanalytical Center, Washington State University). The above extract also was used to determine the soluble sugars according to ref. 29.

Osmotic Pressure Determination. The fourth and fifth leaves from 30-day-old plants were assayed for osmotic pressure of leaf cell sap. The leaves were sampled at the midday and frozen immediately in liquid nitrogen. Cell sap was collected by centrifugation at 20,000 × *g* for 5 min at 4°C. The osmotic pressure was measured with a Wescor Vapor Pressure Osmometer (Wescor, Logan, UT).

RNA Isolation and Hybridization. Total RNA was isolated according to ref. 30. Hybridization and washing conditions were the same as described in ref. 31.

Relative Growth Rate. Plants were grown on commercial potting mix in 4-inch pots under 150 μmol quanta $m^{-2}{\cdot}s^{-1}$ continuous light and 22°C. At 3-day intervals from 4 to 25 days after germination, samples of five plants were harvested and the dry weight of above-ground parts were measured. The relative growth rate was calculated as the slope of the natural logarithm of dry weight versus time in days. To measure the relative growth rate at 4°C, 7-day-old plants were transferred to 4°C. At 7-day intervals between 1 and 63 days after transfer, samples of five plants were harvested and the dry weight of above-ground parts were used to calculate relative growth rate.

RESULTS

Isolation of Mutants. We established a freezing-survival protocol that can clearly distinguish cold-acclimated from nonacclimated plants by using seedlings of *Arabidopsis* grown on Petri dishes. In this assay, nonacclimated wild-type seedlings had a LT_{50} of −5.5°C and were completely killed by freezing at −7°C (Fig. 1*B*). After a 2-day acclimation at 4°C,

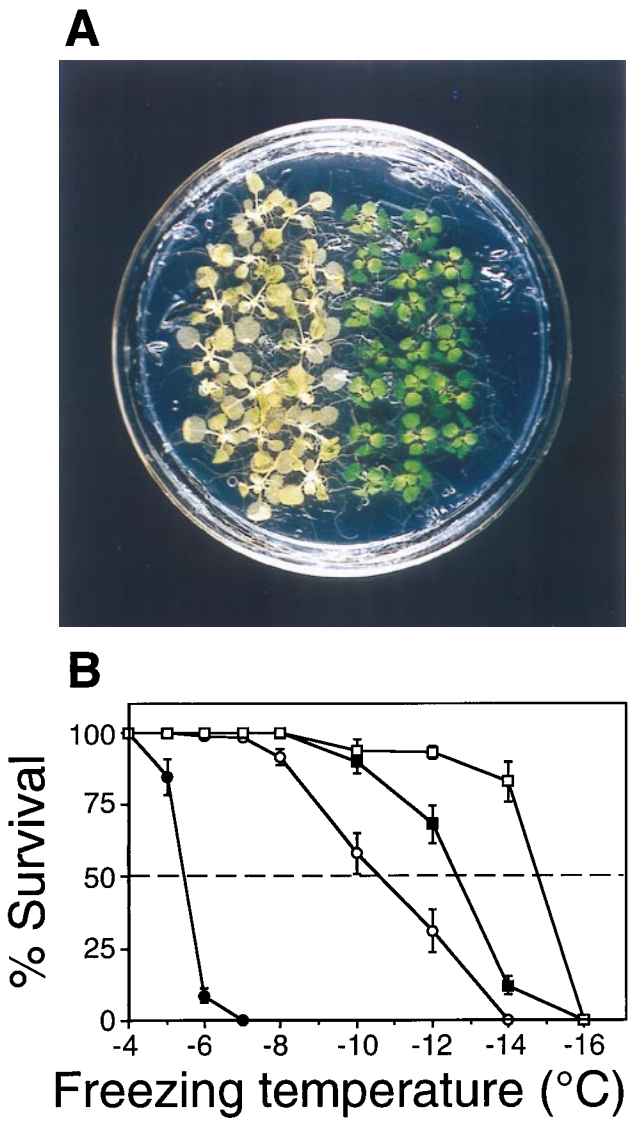

FIG. 1. Freezing survival of wild-type and *esk1* plants. (*A*) Nonacclimated wild-type (*Left*) and *esk1* plants were frozen at −8°C under the protocol described under *Methods*. (*B*) Percent survival of nonacclimated and acclimated plants after freezing to different temperatures. Nonacclimated wild-type (●) and *esk1* (○) were frozen in a temperature-controlled chamber as described under *Methods*. At the temperatures shown, samples of plants were removed from the chamber, allowed to recover, and scored for survival. Alternatively, wild-type (■) and *esk1* (□) plants were cold acclimated at 4°C for 2 days before being subjected to the same freezing test. The data are means ± SE for three separate experiments.

seedlings did not show any visible injury at temperatures above −8°C, 90% of plants survived freezing at −10°C, and 68% of the plants survived freezing at −12°C (Fig. 1*B*). This intrinsic ability of *Arabidopsis* to acclimate and then withstand lower freezing temperatures suggested that it might be possible to discover mutants that are constitutively freezing tolerant in the absence of cold acclimation.

From ethyl methanesulfonate-mutagenized populations of *Arabidopsis*, 800,000 plants were screened for their ability to survive freezing at −8°C, which reliably killed nonacclimated wild-type plants. The plants that survived this freezing treatment were transferred to soil to produce M_3 seeds. The constitutive freezing tolerance of each line was measured again with 32 M_3 plants. Mutants confirmed in this way were backcrossed to wild-type plants and reselected from the resulting F_2 progeny. After several rounds of screening and selection, we obtained 26 mutant lines that demonstrated heritable increases in constitutive freezing tolerance. Allelism tests among the first nine lines demonstrated that they represent mutations at six separate loci. One of these mutants, designated *eskimo1* (*esk1*), was chosen for detailed analysis. A second allele at this locus, *esk1–2*, was subsequently isolated from an independently mutagenized population. All the characteristics of the *esk1–1* mutants described here also have been demonstrated in *esk1–2* plants.

Genetic Analysis of *esk1*. To determine the genetic basis of the *esk1* mutation, *esk1* plants were reciprocally crossed to wild type. Resulting F_1 seedlings from either cross were freezing-sensitive like the wild type, indicating that the *esk1* mutation is recessive. One of the resulting F_1 hybrids was allowed to self-fertilize to produce F_2 seeds, which were planted individually to generate 167 F_3 families. When 32 seedlings from each F_3 family were tested for constitutive freezing tolerance, it was found that 41 families showed 100% survival, 80 families showed 18–30% survival, and 46 families showed no surviving individuals. This ratio is a good fit to the Mendelian expectation of 1:2:1 ($\chi^2 = 0.59, P = 0.75$) for a single recessive mutation. The *esk1* mutation was mapped to 71.4 ± 0.3 cM on chromosome 3 between CAPS markers TT5 and BGL1 on the Recombinant Inbred Map of Lister and Dean updated on October 2, 1997 (http://genome-www.stanford.edu/Arabidopsis/ww/Vol4ii).

Freezing Tolerance of Wild-Type and *esk1* Plants. When wild-type and *esk1–1* plants were grown side by side and then frozen to −8°C without acclimation, all the wild type were killed whereas none of the *esk1* plants showed significant damage (Fig. 1*A*). Mutant plants were capable of continued growth after freezing and completed their life cycle. In the same experiment, 2 days of cold acclimation at 4°C allowed 100% survival of both wild-type and *esk1* plants after freezing to −8°C (not shown). To determine the extent of freezing tolerance that is constitutively activated in *esk1* mutants, samples of plants grown on agar were frozen to temperatures ranging from −4°C to −16°C (Fig. 1*B*). These experiments indicated that nonacclimated wild-type showed 50% survival at a temperature of −5.5°C. Full acclimation of wild-type plants increased survival to −12.6°C whereas nonacclimated *esk1* plants showed 50% survival at −10.6°C. These results indicate that *esk1* mutation has instituted approximately 70% of the freezing tolerance generated by full acclimation of wild-type *Arabidopsis*. Interestingly, cold acclimation of *esk1* plants increased their tolerance beyond that of acclimated wild type to −14.8°C, indicating that other signaling pathways, in addition to the one constitutively activated in *esk1* plants, are also likely to be involved in cold acclimation.

When the freezing tolerance of plants grown on soil was measured using an ion leakage assay, the temperature causing 50% ion leakage was −7.9°C for *esk1* compared with −2.8°C for nonacclimated wild type. The extent of improved freezing tolerance, 5.1°C, was the same as that observed in plants grown on agar in Petri dishes, although the absolute temperatures are higher than those derived from plant survival on agar media. To date, experiments with six other, nonallelic mutants have demonstrated increases in constitutive freezing tolerance ranging from 1.3°C to 5.1°C.

When grown at 22°C under continuous light, *esk1* plants were darker green in color and more compact in stature than wild type (Fig. 2*A*). Measurements of shoot dry weight accumulation under these conditions yielded relative growth rates of 0.338 ± 0.025 and 0.262 ± 0.012 (mean ± SE) for wild type and mutant, respectively. Despite these differences, the mutant retained a normal pattern and chronology of development with germination, flowering, and seed development after similar timing to wild type (Fig. 2*A*). Interestingly, darker color and more compact growth are characteristic of wild-type *Arabidopsis* grown at temperatures (c. 4°C) that induce cold acclimation. Indeed, growth at 4°C produced wild-type and mutant plants that were indistinguishable both in appearance (Fig. 2*B*) and growth rate (0.062 ± 0.004 and 0.064 ± 0.005 for

FIG. 2. Growth habits of wild-type (*Left*) and *esk1* plants. (*A*) Plants grown for 30 days at 22°C under 150 μmol quanta $m^{-2}{\cdot}s^{-1}$ continuous light. (*B*) Plants grown for 7 days at 22°C and then for 60 days at 4°C under 90 μmol quanta $m^{-2}{\cdot}s^{-1}$ continuous light.

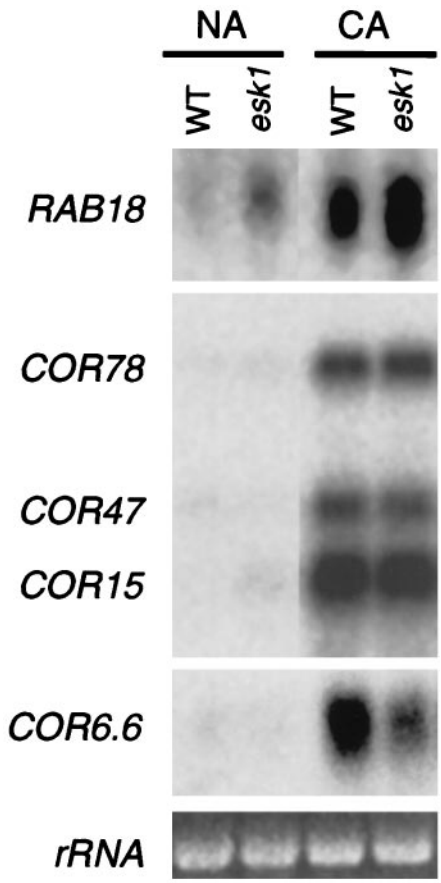

FIG. 3. Gel-blot analysis of transcripts from five cold-regulated genes in wild-type (WT) and *esk1 Arabidopsis.* Plants were grown at 22°C under 150 μmol quanta $m^{-2} \cdot s^{-1}$. Total RNA (10 μg) isolated from leaf tissue of nonacclimated plants (NA) or from plants cold acclimated at 4°C for 2 days (CA) was separated on a 1.2% agarose-formaldehyde gel and probed successively with cDNAs corresponding to the cold-regulated genes indicated. The ribosomal 25S rRNA was visualized on the nylon membrane by using ethidium bromide to demonstrate equal loading. Quantitative comparisons reported in the text are based on PhosphorImager analyses of the blots. The experiment was repeated four times with similar results.

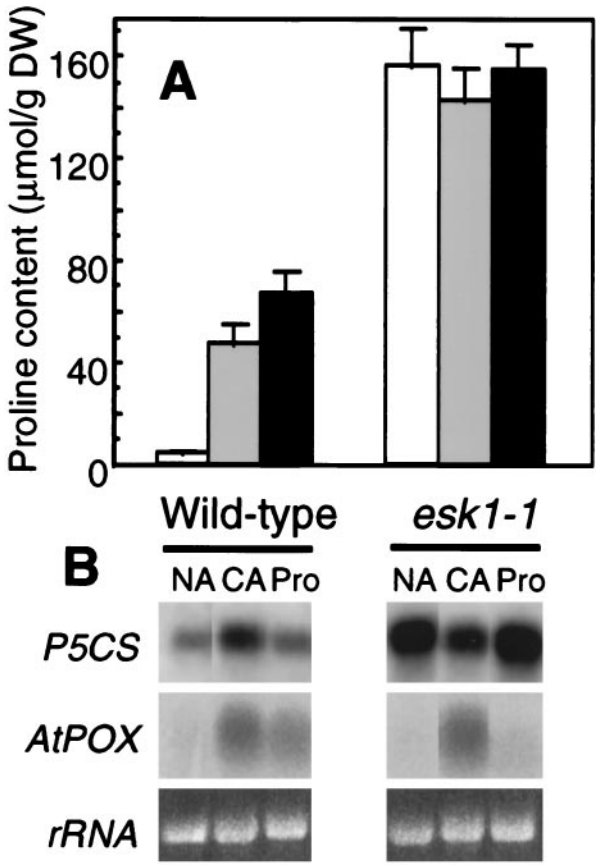

FIG. 4. Proline accumulation and expression of proline metabolism genes in wild-type (WT) and *esk1 Arabidopsis.* (*A*) Proline levels in nonacclimated plants (open bars), in plants cold acclimated at 4°C for 2 days (shaded bars), and in plants 10 hr after application of 100 mM proline (solid bars). Harvested leaf tissue was frozen in liquid nitrogen and lyophilized. Free amino acids were extracted and quantified by using an amino acid analyzer. (*B*) Gel-blot analysis of transcript levels for genes encoding Δ^1-pyrroline-5-carboxylate synthetase (P5CS) and proline oxidase (AtPOX). Methods are those described in Fig. 3.

wild type and mutant, respectively). These morphological features of the *esk1* phenotype cosegregated with freezing tolerance during backcrossing experiments and also were identical in an independently isolated *esk1–2* mutant. We conclude that the *esk1* mutations have pleiotropic effects that may reflect, to some extent, changes that occur in wild-type plants during cold acclimation.

Freezing Tolerance in *esk1* Does Not Depend on Expression of Four *COR* Genes. Differential screening techniques have identified genes that are induced during cold acclimation. The most strongly induced of these cold-regulated genes in *Arabidopsis* include *COR6.6*, *COR15a*, *RAB18*, *COR47*, and *COR78* (9). The precise roles of these genes in cold acclimation remain unknown, and several of them are induced by both drought and abscisic acid, as well as by low temperature (22). This suggests that they may help to protect cells during dehydration, which is a major component of freezing stress. Constitutive expression of the *COR15a* gene in transgenic *Arabidopsis* has been shown to provide some increase in freezing tolerance to chloroplasts and protoplasts derived from nonacclimated plants (14).

To determine whether expression of these cold-regulated genes contributes to the constitutive freezing tolerance of *esk1* plants, mRNA levels corresponding to the five genes were assayed by gel blot analysis. Only one of the genes, *RAB18*, showed significant constitutive expression in *esk1* plants (Fig. 3). The remaining genes showed very strong induction (ranging from 25- to 100-fold) after cold acclimation of either wild-type or *esk1* plants, but transcript levels in nonacclimated *esk1* plants were essentially the same as in wild-type controls. Thus, the extensive freezing tolerance achieved in the *esk1* mutants does not depend on high levels of *COR* gene expression.

Accumulation of Free Proline Is One Aspect of the *esk1* Phenotype. Proline is one of several compounds that act as compatible osmolytes to ameliorate the effect of dehydration that occurs during freezing and drought stress (32). Increases in proline content occur in many plant species during cold acclimation (13). In our experiments, cold acclimation of wild-type plants resulted in a 10-fold increase in free proline from 4.3 to 47.0 μmol·g^{-1} dry wt (Fig. 4*A*), and such data are comparable with those obtained in other studies of cold acclimation (13) and drought stress (33, 34). By contrast, *esk1* plants constitutively maintained proline at more than 150 μmol·g^{-1} dry wt and did not show any increase after cold acclimation. Analyses showed that levels of other amino acids in the mutant remained close to those in wild-type plants, indicating that constitutive accumulation of proline as a compatible solute indeed may be a component of freezing tolerance in the mutant. We also determined the levels of soluble sugars, another class of compatible osmolytes, and the osmotic pressure of leaf cell sap in wild-type and *esk1* plants. The level of soluble sugar was found constitutively in leaf tissue of *esk1* was 5.9 mg·g^{-1} fresh wt compared with only 2.3 mg·g^{-1} fresh wt

for wild-type controls. The osmotic pressure of leaf cell sap was −1.41 MPa in *esk1* compared with −0.80 MPa in wild-type plants.

The *esk1* Mutation Affects the Expression of Genes Controlling Proline Synthesis and Degradation. In plants, proline levels are maintained by transcriptional regulation of both synthesis and degradation (32, 33). The first committing step in proline synthesis is catalyzed by the enzyme Δ^1-pyrroline-5-carboxylate synthetase (P5CS). Consistent with the accumulation of free proline in cold-acclimated wild-type plants, the expression of *P5CS* transcript was increased 3-fold in these plants compared with nonacclimated controls (Fig. 4*B*). Without acclimation *esk1* plants exhibited an 8-fold higher level of transcript than nonacclimated wild type (Fig. 4*B*). After acclimation, the *P5CS* transcript was not increased in *esk1* plants, but instead was slightly decreased.

Proline is degraded through proline oxidase (32, 33), which is encoded by *AtPOX* genes in *Arabidopsis* (34). The *AtPOX* genes are normally induced by proline (34, 35). However, the transcript abundance of *AtPOX* were similar in *esk1* mutants and wild-type plants even though *esk1* plants accumulate free proline to a level 30-fold higher than nonacclimated wild type (Fig. 4). This suggests that the *esk1* mutation acts to prevent the induction of *AtPOX* genes by proline. To test this possibility, soil-grown wild-type and *esk1* plants were supplied with exogenous proline by directly watering plants with 100 mM proline solution. Such treatment increased free proline in wild-type plants to a level about one-third of that as observed in *esk1* plants (Fig. 4*A*) and induced a significant increase in the abundance of *AtPOX* transcript. However, the same treatment only slightly increased *AtPOX* transcript in the *esk1* mutant. We also observed that *AtPOX* transcript was increased during cold acclimation in both wild-type and *esk1* plants.

DISCUSSION

Cold acclimation involves changes in many different cellular processes. To dissect the signaling pathways mediating the complex changes that result in increased freezing tolerance, we have isolated 26 mutants with enhanced freezing tolerance in the absence of cold acclimation. Complementation tests with the first nine mutants identified six separate loci. This suggests that the 26 mutants we have isolated may represent 12–15 different genes, which, when mutated, result in constitutive increases in freezing tolerance. Most mutant lines are represented only by one or two alleles, suggesting that the screen has not been saturated. The freezing assay that we have developed will facilitate the screen of more mutagenized populations to approach saturation. Characterization of the existing 26 mutant lines and isolation of additional mutants should lead eventually to a broader understanding of all aspects of cold acclimation. The particular focus of this investigation was a mutation at the *esk1* locus that provides a constitutive increase in freezing tolerance of 5.1°C, which compares with a 7.1°C increase observed in fully acclimated wild-type plants. When *esk1* plants were cold acclimated, they became 9.3°C more freezing tolerant than nonacclimated wild-type plants.

Free proline increases in plants in response to many stresses (32). However, its role in stress tolerance remains equivocal. Here, we provided evidence that proline is a factor contributing to increased freezing tolerance. Proline content increased in wild type during cold acclimation (Fig. 4*A*), and similar increases have been reported in other plant species (13, 32). In the absence of acclimation, both *esk1–1* and *esk1–2* plants accumulated similar amounts of free proline, indicating that accumulation of proline was caused by *esk1* mutation rather than another closely linked but unrelated mutation. We have measured proline levels in five other, nonallelic lines derived from our mutant screen. Of these, two lines showed constitutive proline levels of 163 and 188 μmol·g^{-1} dry wt, whereas the remaining three lines showed proline levels similar to nonacclimated wild type. These results strongly suggested that proline does play an important role in plant freezing tolerance. However, in the mutants that did not accumulate proline, the levels of constitutive freezing tolerance ranged from 3° to 5°C better than nonacclimated wild type. Thus, although proline accumulation may be a component of constitutive freezing tolerance in some mutants, it is not essential for the considerable freezing tolerance observed in others. For the *esk1* mutation, the accumulation of free proline (150 μmol·g^{-1} dry wt) accounted for about 2% of total dry matter. There appears to be a recognizable penalty associated with the accumulation of free proline and increase in constitutive freezing tolerance in *esk1* mutants. However, several other mutants derived from our screen are similar to wild type in growth and appearance. Apparently, it is possible to achieve an increase in constitutive freezing tolerance without significantly compromising growth at higher temperatures.

The high levels of proline found in *esk1* plants would normally induce the *AtPOX* gene and result in increased breakdown of this amino acid (33, 34). A much lower concentration of proline in wild-type plants supplied with exogenous proline led to a 6-fold increase at *AtPOX* transcript. Thus, the *esk1* mutation leads to the activation of *P5CS* and the accumulation of proline while also preventing the induction of *AtPOX.* The *esk1* mutation does not map to any of the *P5CS* or *AtPOX* loci (34–36), indicating that *ESK1* may be a regulatory component of cold acclimation. We consider it unlikely that proline accumulation is the only process controlled by the signaling pathway involving *ESK1*. The increased *RAB18* transcript levels, accumulation of soluble sugars, and decreased osmotic pressure in the mutant are all consistent with this view. Given our incomplete knowledge of the biochemical processes that contribute to freezing tolerance, it will require considerable effort with the mutants and with other approaches to define all the additional processes involved. However, if the *esk1* mutation is specifically activating a suite of genes contributing to increased freezing tolerance, it is likely that the mutants will facilitate the identification of these genes by differential screening strategies while reducing interference from genes that are induced by low temperature but not involved in freezing tolerance.

The recessive nature of the *esk1* mutation suggests that the wild-type *ESK1* protein normally functions as a negative regulator to repress *P5CS* expression and other responses in the absence of low-temperature signals. Mutations at *esk1* apparently disrupt the repressor activity and result in hyperactivation of proline synthesis and other processes controlled by the *ESK1* pathway. Presumably, presence of active ESK1 protein is also required for proline to induce the *AtPOX* gene at warm temperatures. Cloning of the *ESK1* gene may help elucidate the dual function of the ESK1 protein in repressing *P5CS* and allowing proline-induced activation of the *AtPOX* genes. We currently have no rationale for the low-temperature induction of *AtPOX* transcript in both wild-type and *esk1* plants, but a similar induction has been observed in the early stages of drought stress before any detectable increase in proline (35).

Transcript levels of *RAB18* in nonacclimated *esk1* plants showed a consistent 3- to 4-fold increase compared with wild-type controls. Although cold acclimation of *esk1* led to a further 3-fold induction of *RAB18*, the data nevertheless suggest that increased *RAB18* expression is one aspect of the *esk1* phenotype. By contrast, four other cold-regulated genes (*COR6.6*, *COR15a*, *COR47*, and *COR78*) exhibited no constitutive increase in expression in mutant plants but instead showed normal induction after 2 days at 4°C. Previous work has demonstrated that overexpression of the *COR15a* gene in *Arabidopsis* provides for increased freezing protection of chloroplasts and protoplasts derived from nonacclimated transgenic plants compared with untransformed controls (14). More recently, a transcription factor, CBF1, that binds to cis-acting regulatory elements within the *COR15a* and *COR78* promoters has been cloned by using a yeast "one-hybrid" strategy (37). Overexpression of CBF1 driven by a

strong, constitutive promoter activated *COR6.6*, *COR15a*, *COR47*, and *COR78* at normal temperatures in transgenic *Arabidopsis* plants and resulted in increased freezing tolerance in the absence of cold acclimation (15). These findings indicate that some of the *COR* gene products are important in freezing tolerance. It is likely that increased expression of the *COR* genes contributes to the additional 4.2°C increase in freezing tolerance that occurs upon cold acclimation of *esk1* mutant plants.

Taken together, our results indicate that it is not appropriate to consider cold acclimation as a simple, linear signaling pathway activating the full set of processes required for increased freezing tolerance. Instead, we propose a model for cold acclimation in which parallel or branched signaling pathways activate distinct suites of cold-acclimation responses. Constitutive activation of one of these pathways can result in considerable freezing tolerance without support from other components. Previous studies have shown that the expression of *COR* genes is controlled by both ABA-dependent and ABA-independent signal pathways (21, 22, 38). Because *esk1* plants do not overexpress any of the four major *COR* genes at 22°C, *esk1* apparently defines another signaling pathway of cold acclimation distinct from those that mediate the expression of *COR* genes. Several mutant lines from our screen neither accumulate proline nor activate the *COR* genes (unpublished results); they must define additional pathways that are distinct from the three described above. Hence, the minimum number of separate signaling pathways involved in cold acclimation is four, and further studies of the mutants may identify additional pathways.

The complexity of cold-acclimation signaling that we propose here is consistent with the results of Ishitani *et al.* (38), who identified mutations that influence differentially the effect of ABA, drought, cold, and salt stress on the activity of the *COR78* (=*RD29A*) promoter. Recently, Warren *et al.* (25) isolated seven nonallelic *sfr* (*s*ensitive to *fr*eezing) mutants of *Arabidopsis*, which acquire only partial freezing tolerance after acclimation. Consistent with the notion that multiple signal pathways are involved in cold acclimation, most of the *sfr* mutants retain more than 50% capacity to cold acclimate. Epistasis analysis of these *sfr* mutants to our constitutively freezing tolerant mutants will greatly extend our ability to genetically define the signaling networks controlling cold acclimation.

An intriguing and very pertinent observation from our studies is that cold acclimation of *esk1* produces plants that are more than 2°C more freezing tolerant than acclimated wild type (Fig. 1*B*). It is possible that wild-type *Arabidopsis* could be induced to exhibit higher levels of tolerance by a more extensive acclimation regime. However, studies by us and by others (7, 8, 11, 12) have not provided evidence for such a possibility. Thus, the increased tolerance resulting from cold acclimation of *esk1* is consistent with the concept of multiple signaling pathways discussed above but also suggests that mutations at *ESK1* may hyperactivate the signaling pathway in which this gene is involved. This explanation implies that cold-acclimation signaling may have evolved to modulate the level of freezing tolerance so that it is sufficient for the conditions found within the geographic range of a given ecotype without limiting the plant's competitiveness. Such a hypothesis implies that it may be possible to improve freezing tolerance by increased activation of preexisting pathways of cold acclimation.

At present, it is unclear which genes or biochemical processes are essential to the development of freezing tolerance and which are general responses to low, nonfreezing temperatures but are not required for freezing tolerance. Except for the induction of a few *COR* genes, the signal cascades mediating most aspects of cold acclimation, such as increases in ABA, synthesis of compatible osmolytes, and changes in membrane lipid composition, are unknown. The isolation of a series of constitutively freezing tolerant mutants now opens new routes to study the processes required for freezing tolerance and to identify components of the signaling pathways that mediate these processes.

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