Plant Physiology, June 1999, Vol. 120, pp. 391–399, www.plantphysiol.org © 1999 American Society of Plant Physiologists

Cold-Induced Freezing Tolerance in Arabidopsis[1]

Leslie A. Wanner[2]* and Olavi Junttila

Institute for Biology, University of Tromsø, N-9037 Tromsø, Norway

Changes in the physiology of plant leaves are correlated with enhanced freezing tolerance and include accumulation of compatible solutes, changes in membrane composition and behavior, and altered gene expression. Some of these changes are required for enhanced freezing tolerance, whereas others are merely consequences of low temperature. In this study we demonstrated that a combination of cold and light is required for enhanced freezing tolerance in Arabidopsis leaves, and this combination is associated with the accumulation of soluble sugars and proline. Sugar accumulation was evident within 2 h after a shift to low temperature, which preceded measured changes in freezing tolerance. In contrast, significant freezing tolerance was attained before the accumulation of proline or major changes in the percentage of dry weight were detected. Many mRNAs also rapidly accumulated in response to low temperature. All of the cold-induced mRNAs that we examined accumulated at low temperature even in the absence of light, when there was no enhancement of freezing tolerance. Thus, the accumulation of these mRNAs is insufficient for cold-induced freezing tolerance.

Many important cultivated plants, such as potato, tobacco, and maize, have a limited capacity to survive temperatures below freezing. In contrast, leaves of plants such as cabbage, lettuce, and spinach can develop tolerance to below-freezing temperatures in response to low but above-freezing temperatures. Significant differences in freezing tolerance can be seen after even 1 d at low temperature, and some plants reach maximum tolerance after only a few days (Guy et al., 1985; Gilmour et al., 1988; Kurkela et al., 1988). Enhanced freezing tolerance is also rapidly reversible; it can be lost within 1 to 2 d after plants are returned to a higher temperature. Other forms of osmotic stress, including dehydration (Siminovitch and Cloutier, 1983; Lee and Chen, 1993; Mäntylä et al., 1995) and high salinity (Ryu et al., 1995), can also enhance freezing tolerance, as can the treatment of cells or plants with ABA (Chen and Gusta, 1983; Lee and Chen, 1993).

Numerous physiological changes inside the plant leaf occur in response to low temperature. Calcium ion fluxes have been observed within seconds after the transfer of plants to the cold (Knight et al., 1991, 1996; Polisensky and Braam, 1996), and changes in protein phosphorylation have been observed within minutes (Monroy et al., 1993b). Altered gene expression, including changes in both mRNA accumulation (for summary, see Hughes and Dunn, 1996) and enzyme activity (Guy, 1990; Thomashow, 1990; Holaday et al., 1992; Howarth and Ougham, 1993), was observed within a few hours. Decreased water content and structural changes such as increased leaf thickness occurred within days to weeks (Ristic and Ashworth, 1993; Uemura et al., 1995). It is unclear which alterations are necessary for enhanced freezing tolerance or how individual alterations contribute to freezing tolerance. Insight into the contributions of specific physiological changes to enhanced freezing tolerance can be gained by carefully comparing the time course of the development of enhanced freezing tolerance with the time course of a change in a physiological characteristic.

Arabidopsis acclimates rapidly in response to low temperature; enhanced freezing tolerance has been observed after only 1 d of cold acclimation (Gilmour et al., 1988; Kurkela et al., 1988; Ristic and Ashworth, 1993). Soluble sugars and starch accumulate during cold acclimation (Ristic and Ashworth, 1993; McKown et al., 1996), as does Pro (Xin and Browse, 1998). Changes in the behavior of membranes in Arabidopsis within 6 h after beginning cold treatment have been documented (Ristic and Ashworth, 1993).

Many *COR* (*cold-responsive*) genes have been cloned from Arabidopsis on the basis of induction of expression at low temperature (for summary, see Hughes and Dunn, 1996). Cold-induced mRNAs generally begin to accumulate within a few hours at low temperature and remain at high levels until plants are returned to normal growth temperature. The correlation between the expression times of these genes and enhanced freezing tolerance suggests that *COR* gene products could play a role in freezing tolerance, but roles for individual *COR* gene products have been difficult to establish (Thomashow, 1998). Recently, transgenic plants expressing a transcriptional activator that binds to motifs often found in cold-inducible genes have been constructed. These plants display both constitutively enhanced freezing tolerance and overexpression of a set of *COR* genes (Jaglo-Ottosen et al., 1998; Liu et al., 1998). However, Xin and Browse (1998) also described a constitutively freezing-tolerant mutant that does not express *COR* genes; thus, the role(s) of these genes in freezing tolerance remains unclear.

The goal of this study was to better understand the roles of several cold-induced physiological changes in enhanced freezing tolerance in Arabidopsis. We manipulated the light conditions during low-temperature treatment to ob-

[1] This work was supported in part by the Norwegian Research Council.

[2] Present address: Laboratory for Molecular Plant Biology, P.O. Box 5051, Meieribygget, Agricultural University of Norway, N–1432 Aas, Norway.

* Corresponding author; e-mail leslie.wanner@ikb.nlh.no; fax 47–64–94–14–65.

tain different rates and degrees of enhanced freezing tolerance, and we examined the accumulation of soluble sugars, Pro, and several mRNAs that are encoded by *COR* genes under these different regimes.

MATERIALS AND METHODS

Growth, Cold Acclimation, and Freezing Tests

Arabidopsis ecotype Columbia plants were grown from seeds in glass jars containing sterile 0.5× Murashige and Skoog agar plus 1% Suc. Jars were covered with clear plastic foil or semitransparent plastic lids containing semipermeable membrane gas-exchange ports (SunCaps, Sigma). Plants were grown in temperature- and humidity-controlled rooms at 21°C with a 12-h photoperiod. The PPFD (measured at plant height) of 150 to 175 μmol m^{-2} s^{-1} was provided by natural daylight spectrum fluorescent lamps (model 96, Philips, Eindhoven, The Netherlands). Growing the plants at controlled temperature and humidity in glass jars on sterile medium produced uniform plant material that was not exposed to additional, uncontrolled stresses such as water stress, nutrient depletion, pathogens, or insect pests, any of which could also induce some level of freezing tolerance.

In the standard cold-acclimation regimen, 3-week-old plants were treated at 1°C under the same fluorescent lamps (measured PPFD at plant height was 75–90 μmol m^{-2} s^{-1}) but for different photoperiods of up to 5 d. Some plants were returned to 21°C and 12-h photoperiods for deacclimation. For freezing tests, whole plants were removed from agar, placed between damp paper towels, packed in moist sand in metal boxes, and placed in a temperature-regulated freezer at −1°C to −2°C for 2.5 h for temperature equilibration and ice formation. During this period, both the sand and the paper towels were completely frozen. The temperature was then lowered at a rate of approximately 2.5°C h^{-1}, and individual boxes of plants were removed at temperatures from −4°C to −14°C. Temperatures in the boxes were recorded as the average of two thermocouple probes placed inside. After freezing, plants were allowed to thaw overnight in the sand boxes at 4°C. Plants were then removed from the sand boxes, placed on moist paper towels in clear plastic boxes, and returned to 21°C with a 12-h photoperiod. Plants were watered after 2 d at 21°C with 0.5× Murashige and Skoog medium.

Evaluation of Freezing Tolerance

When the thawed plants were removed from the sand boxes, they lacked turgidity and were visibly water soaked. Plants that survived freezing regained turgidity and lost their water-soaked appearance during the 1st d at 21°C. It was not possible to determine which plants would ultimately regain turgidity and which would remain collapsed until after several hours of recovery at 21°C. We assessed the recovery and survival of plants after freezing by visually monitoring the fate of the frozen leaves and the progress of new root and leaf growth during the next 7 to 10 d at 21°C. Plants could survive freezing if only a few of the youngest leaves survived, even if most of the fully enlarged leaves did not.

DCMU Treatments

Three-week-old plants were transferred to 1°C with a 16-h photoperiod and misted once a day with 0.005% Silwet (OSi Specialties, Sistersville, WV) with or without 50 μM DCMU for 1, 2, or 3 d before the freezing tests. There was no visible damage to plants treated with DCMU for up to 5 d at 1°C with a 16-h photoperiod, in contrast to the discoloration and wilting that was visible after 2 d of DCMU treatment at 21°C.

Soluble Sugar and Pro Measurements

We harvested leaves directly into liquid nitrogen at specific times after the plants were transferred to 1°C under each light period or after the beginning of DCMU treatments. Soluble sugars were extracted from the frozen leaves in 90% ethanol and were quantified using a phenol-sulfuric acid assay with Suc as a standard (Farrar, 1993). Pro content was measured in acidic extracts and quantified spectrophotometrically using acid-ninhydrin reagent with Pro as a standard (Lea and Blackwell, 1993). We repeated the sugar and Pro measurements three to five times on independent batches of plant material at different times between 0 and 5 d of hardening. We plotted mean values; in the figures, error bars indicate the SDs.

Measurement of Dry Weight Ratio

Whole plants were carefully pulled from the agar, the external moisture and agar were blotted away, and 10 to 13 plants with an average total fresh weight of 0.5 to 1.0 g were placed on a moisture analyzer (model MA30, Sartorius, Edgewood, NY). The plants were rapidly dried at 130°C until there were no further changes in weight.

RNA Analysis

We used a phenol-extraction/LiCl precipitation procedure (Wanner et al., 1993) to prepare total RNA from leaves harvested at different times after transfer to 1°C. RNA was separated by size on agarose gels containing 3% formaldehyde, and gels were blotted on Hybond-N nylon membranes (Amersham) using standard methods. The RNA was fixed to filters in a microwave oven at full power (800–1000 W) for 2 min. We repeated all northern blots with RNA preparations from two to four independent sets of plants, and we prepared radioactively labeled probes from isolated inserts of DNA fragments using a kit (Redi-Prime, Amersham). Hybridization was carried out at 65°C in 5× SSC, 1% SDS, 0.5× Denhardt's solution, and 0.1% denatured salmon-sperm DNA. Filters were washed at 65°C in 2× SSC with 0.8% SDS three times for 10, 20, and 30 to 40 min. The damp filters were exposed to Kodak X-OMat R film at −70°C or to phosphor screens and then imaged on a phosphor imager (Molecular Dynamics, Sunnyvale, CA). Blots were stripped of probes in boiling

0.2× SSC with 0.5% SDS and rehybridized with additional probes. The sources of the cloned probes were the 0.7-kb *Eco*RI insert of pLCT10 (*COR15a*; Lin and Thomashow, 1992), the 1.1-kb *Eco*RI insert of pHH7.2 (*COR47*; Hajela et al., 1990), and the 1.5-kb *Eco*RI insert of pHH28 (*COR78*; Hajela et al., 1990); the fourth source of the cloned probes was a PCR product that was amplified from pAC1–6, which is a clone identified by differential display PCR using total RNA from plants during the first hours of 1°C treatment with the primer $T_{(12)}$AC and primers 50-31 (5′-TAGCACAGTC; Genosys, The Woodlands, TX).

RESULTS

Enhanced Freezing Tolerance Is Not Uniform throughout the Plant

The increase in freezing tolerance in Arabidopsis during 3 d of cold acclimation is shown in Figure 1A. Nonacclimated plant leaves did not survive at −5°C, but most acclimated (at 1°C for 1 d) plant leaves survived at −7°C. Enhanced freezing tolerance was not uniform throughout the plant; the youngest leaves in the center of the rosette developed freezing tolerance more rapidly than older leaves. Thus, after 24 h at 1°C with a 16-h photoperiod, the smallest leaves usually survived at −10°C, whereas the largest fully developed leaves collapsed irreversibly at this temperature. Leaves at intermediate stages of expansion showed intermediate degrees of damage. At least 2 or 3 d at 1°C with light were required for the majority of fully enlarged leaves to survive freezing below −10°C. The oldest leaves were also most likely to turn yellow 1 week after their return to 21°C; the lower the freezing temperature, the more likely was early senescence. After cold acclimation for 1 to 2 d, the plant as a whole was able to survive, but there was significant damage to older leaves. The survival of as few as one or two immature leaves allowed the plant to reroot and begin to grow again.

We also observed differences in freezing tolerance between different leaf tissues. The midveins and petioles of expanded leaves appeared to be more susceptible to freezing injury than the rest of the leaf, often not regaining turgidity and becoming yellow/brown during the first days of recovery. If the petiole and midvein were lethally injured by freezing, the remainder of the leaf usually remained turgid and green for several days but eventually senesced. Roots and cotyledons were also less freezing tolerant than other parts of the plant. Cotyledons showed no enhancement of freezing tolerance; they did not survive freezing temperatures of −4°C to −5°C even after 5 d of cold acclimation. The freezing sensitivity of Arabidopsis cotyledons is in contrast to the behavior of spinach cotyledons, which are able to acclimate in the cold and enhance their tolerance to freezing (Guy et al., 1987).

Deacclimation was rapid; within 1 d after plants returned to a normal growth temperature (21°C), they could be killed by subsequent freezing to −7°C (Fig. 1A).

Photosynthesis Is Required for Enhanced Freezing Tolerance

As shown in Figure 1B, freezing tolerance was not enhanced during 3 d at 1°C in darkness. Light intensity was also a factor: we saw no appreciable enhancement in freezing tolerance after cold acclimation for 3 d with a 24-h photoperiod at a low light intensity of 5 μmol m^{-2} s^{-1} (data not shown). However, the requirements for cold temperature and for light could be separated: plants acclimated nearly as well with a 12-h photoperiod at 21°C and a 12-h dark period at 1°C as they did with a 12-h photoperiod at a constant temperature of 1°C. The converse light and temperature regimen, 21°C in dark and 1°C in light, produced little enhancement of freezing tolerance (Fig. 1C).

To establish whether photosynthesis produced the primary effects of light on cold acclimation, plants were cold acclimated with a 16-h photoperiod in the presence of the photosynthesis inhibitor DCMU. Plants thus treated at 1°C failed to harden and were killed by freezing to −8°C (Fig. 1D).

Sugar and Pro Accumulate during Cold Acclimation

The accumulation of total soluble sugars during 5 d at 1°C and during deacclimation at 21°C is shown in Figure 2A. Sugar accumulation was evident within a few hours after the transfer of plants to 1°C, and sugar levels increased steadily during the first 5 d. When we harvested leaf material at close intervals throughout the photoperiod, we could see that sugars accumulated during the light period, but the rate of accumulation decreased during the dark period. Sugar levels declined rapidly after plants were returned to 21°C. The soluble sugars that accumulated were primarily Suc, Glc, and Fru (data not shown). Sugar did not accumulate at all at 1°C in darkness, and accumulation was greatly reduced in the presence of DCMU (Fig. 2A), probably because of the unevenness of application of the inhibitor by surface spraying (Fig. 2A).

Pro accumulation during cold acclimation, which was detectable after 24 h at 1°C and continued over a 5-d period, is shown in Figure 2B. After plants were returned to 21°C, Pro continued to accumulate for a few hours before it began to decline. As with the sugars, Pro did not accumulate in plants at 1°C in darkness or in the presence of DCMU (Fig. 2B).

The accumulation of sugars and Pro was paralleled by changes in the dry weight percentage of plants during cold acclimation (Fig. 2C). Increases in dry weight percentage were discernible between 1 and 2 d after the beginning of cold acclimation and were modest in the first 2 to 3 d. There was a slight loss in dry weight percentage in plants kept at 1°C in darkness, and dry weight percentage also decreased rapidly upon deacclimation (Fig. 2C).

Effect of Different Light Periods on Freezing Tolerance, Sugar, and Pro Accumulation

The kinetics of enhanced freezing tolerance under various light periods is shown in Figure 3A. Less enhancement

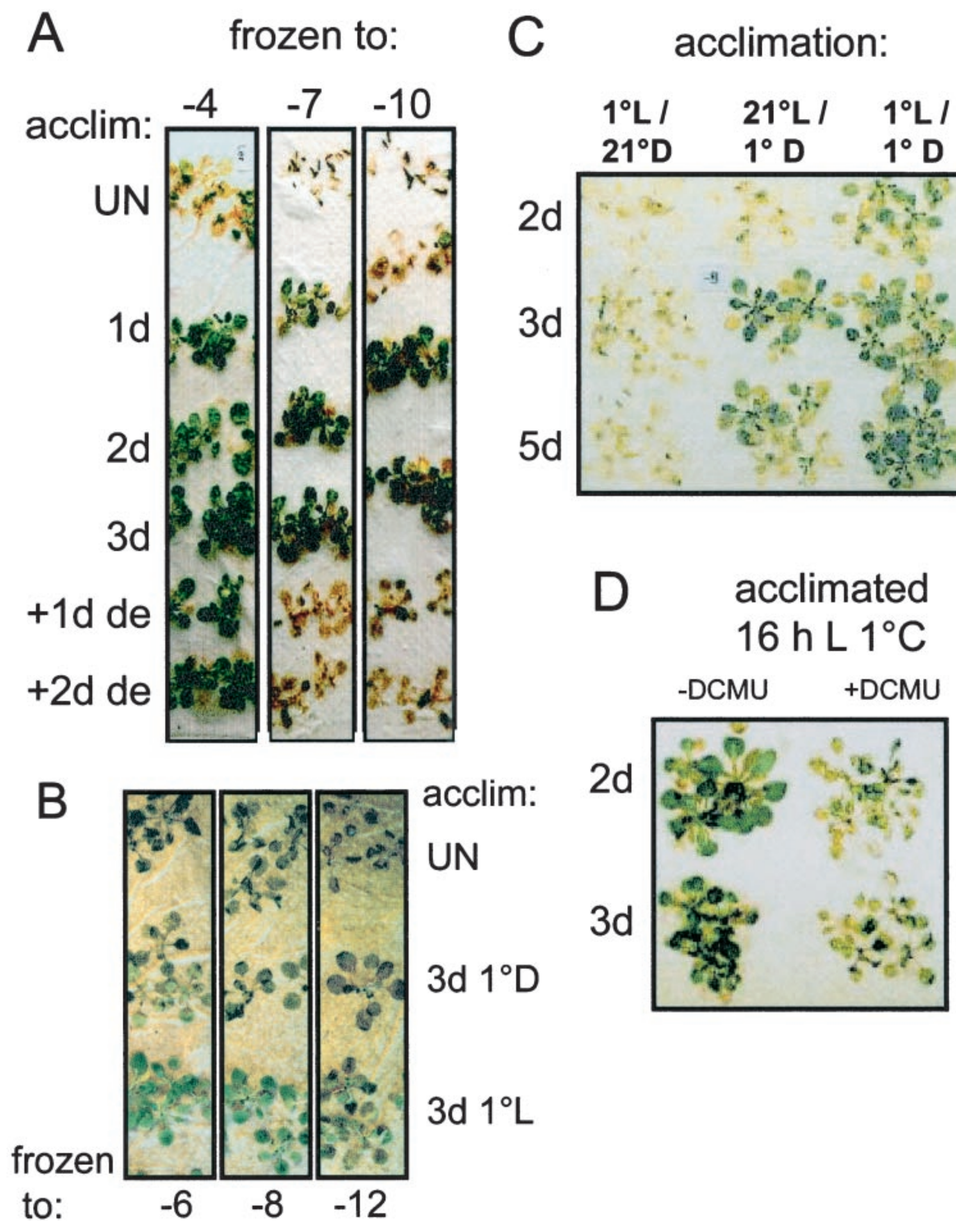

Figure 1. Freezing survival of Arabidopsis after different acclimation (acclim) treatments 1 week after freezing. A, Three-week-old plants were acclimated at 1°C with a 16-h photoperiod for 0 (UN), 1, 2, or 3 d or acclimated for 3 d followed by deacclimation at 21°C for 1 d (+1d de) or 2 d (+2d de). The plants shown were frozen to −4°C (left), −7°C (middle), or −10°C (right). B, Three-week-old plants were not cold acclimated (UN) or kept for 3 d at 1°C in darkness (3d 1°D) or a 16-h photoperiod (3d 1°L) before freezing to −6°C, −8°C, or −12°C. C, Three-week-old plants were acclimated under 12-h light periods with low temperature (1°C) during the light and high temperature (21°C) during the dark (1°L/21°D), with high temperature during the light and low temperature during the dark (21°L/1°D), or at 1°C for both light and dark (1°L/1°D) for 2, 3, or 5 d before freezing to −8°C. D, Three-week-old plants were acclimated at 1°C and 16 h of light for 2 or 3 d in the presence or absence of 50 μM DCMU before freezing to −8°C.

of freezing tolerance was seen in plants cold acclimated under shorter light periods (3 or 6 h of light). Longer light periods resulted in greater freezing tolerance. Plants were near maximal freezing tolerance (−12°C to −14°C) after 2 d at 1°C under long light periods. Plants cold acclimated for 1 d under continuous light had freezing tolerances similar to plants hardened for 3 d with 12-h light periods.

As shown in Figure 3B, both the rate and magnitude of soluble sugar accumulation were altered by the light period during cold acclimation. The longer the light period, the more rapidly sugar accumulated in plant leaves. A comparison of A and B in Figure 3 shows that enhancement of freezing tolerance under each of the light periods was closely correlated with increasing soluble sugar content.

Accumulation of Pro during cold acclimation under different light periods is shown in Figure 3C. Longer light periods during acclimation resulted in more rapid Pro accumulation and higher Pro content: after 5 d at 1°C with a 24-h light period, plants contained 25 times more Pro per

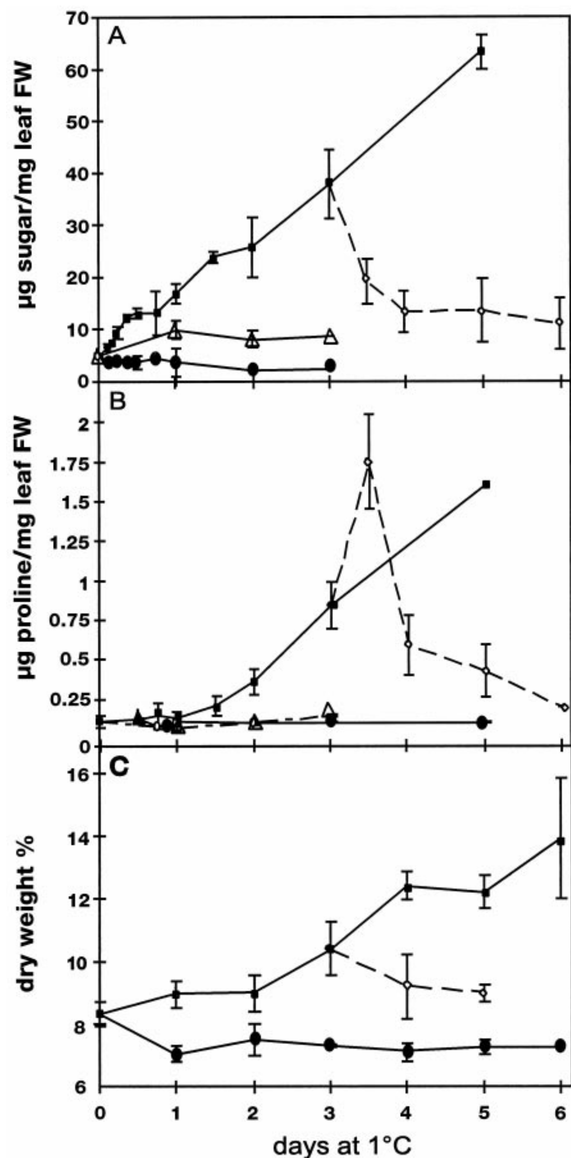

Figure 2. Accumulation of sugar and Pro and dry weight changes during cold acclimation of Arabidopsis. At different times after transfer to 1°C, total soluble sugar (A) or Pro (B) was measured in leaves. Data points in A and B are averages from three to five independent sets of plants acclimated at 1°C with a 16-h photoperiod (■) or deacclimated at 21°C (◇) and from two independent sets of plants acclimated at 1°C with a 16-h photoperiod and treated with 50 μM DCMU (△) or at 1°C in darkness (●). C, Dry weight changes during cold acclimation: Fresh weight (FW) and dry weight were measured in whole plants harvested at different times after transfer to 1°C. Data presented are averages of dry weights as percentages of fresh weights of independent sets of plants acclimated at 1°C with a 16-h photoperiod (■), 3 d of acclimation followed by deacclimation at 21°C (◇) (n = 3), or 1°C in darkness (●) (n = 3). Error bars, which are covered by the data points in some cases, indicate SD.

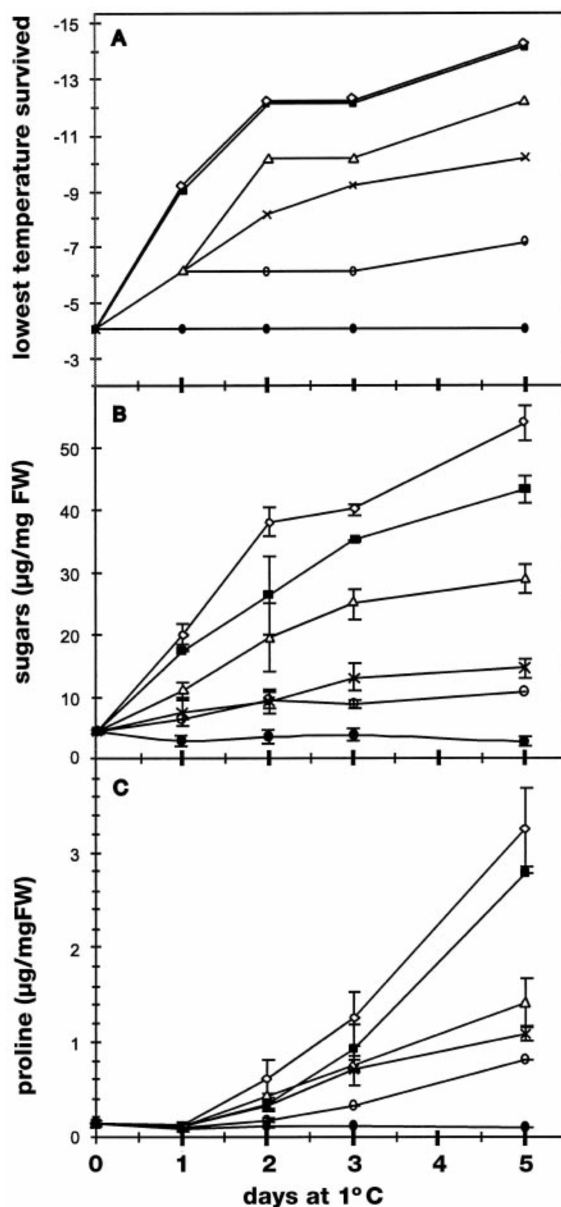

Figure 3. Comparison of freezing survival, sugar accumulation, and Pro accumulation in Arabidopsis plants cold acclimated under different light periods. A, Average lowest freezing temperature survived by plants cold acclimated under different light periods. Sets of plants were grown for 3 weeks at 21°C and acclimated at 1°C for 1, 2, 3, or 5 d. Light periods used were: constant light (◇; n = 3), 18 h (□; n = 3), 12 h (△; n = 6), 6 h (×; n = 3), or 3 h (○; n = 3) of light or darkness (●; n = 3). Sets of plants were then frozen to a series of temperatures indicated on the y axis. After thawing, plants were allowed to recover at 21°C. The lowest temperature survived by most of the plant leaves after a 1-week recovery period is shown. B, Soluble sugar content of Arabidopsis leaves acclimated under different light periods as in A. Total soluble sugar content is expressed as micrograms of soluble sugar per milligram leaf fresh weight (FW). C, Pro accumulation in plants acclimated under different light periods as in A. Pro content is expressed in micrograms per milligram leaf fresh weight. Data are averages from two to six independent sets of plants, with each set representing pools from 8 to 15 plants. Error bars, which are covered by the data points in some cases, indicate SD.

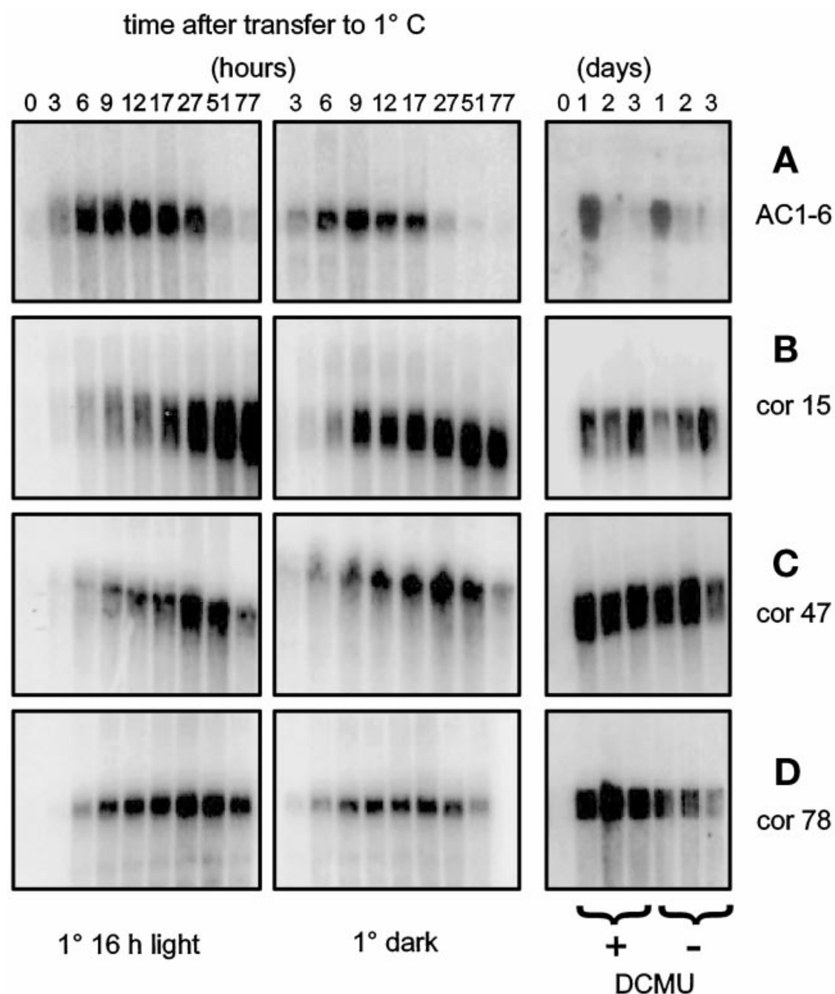

Figure 4. Accumulation of mRNAs at low temperature. Shown here are northern blots of total RNA prepared from leaves harvested at the indicated times after transfer to 1°C under a 16-h light period, in darkness, or under a 16-h photoperiod in the presence (+) or absence (−) of DCMU. Blots in A were hybridized with AC1–6, a probe for a cold-induced transiently expressed mRNA isolated by differential display PCR. Blots in B, C, and D were hybridized to *COR15a*, *COR47*, and *COR78*, respectively, all of which are abundant cold-induced mRNAs (Hajela et al., 1990; Thomashow et al., 1993).

gram fresh weight than unacclimated plants. There was, however, a lag of about 1 d before Pro accumulation was detectable, regardless of light period. Since significant enhancement of freezing tolerance was already detectable after 1 d of cold acclimation under the longer photoperiods, accumulation of Pro was not required for enhanced freezing tolerance.

Expression Patterns of Some Cold-Induced mRNAs

Accumulation of four cold-induced mRNAs at 1°C for a 16-h photoperiod and at 1°C in darkness is shown in Figure 4. All of these mRNAs began to accumulate within the first hours of treatment at 1°C, although differences in their patterns of accumulation could be seen later. For example, AC1–6 mRNA accumulated during the first 24 h of cold treatment but began to decrease during the next 24 h (Fig. 4A), whereas levels of mRNA from *COR15*, *COR47*, and *COR78* remained high during at least 3 d of cold treatment (Fig. 4, B–D; Hajela et al., 1990).

The accumulation of cold-inducible mRNAs was not dependent on light; they accumulated similarly at 1°C in the dark and at 1°C with a 16-h photoperiod. In addition, accumulation of these mRNAs was not inhibited by DCMU (Fig. 4). Thus, these mRNAs accumulated under conditions that did not produce enhanced freezing tolerance.

DISCUSSION

A combination of low temperature and light is required for the enhancement of freezing tolerance in Arabidopsis. Arabidopsis can acclimate to survive freezing at temperatures of at least −8°C within 24 h of acclimation at 1°C and a photoperiod of 16 h or more; after 2 to 3 d of these acclimation conditions, plants can survive freezing temperatures of at least −12°C. The kinetics of acclimation and the degree of freezing tolerance were altered by the duration of the light period at low temperature. Freezing tolerance was not uniformly enhanced throughout the plant; there was a pronounced gradient in leaf survival, with the youngest leaves in the center of the rosette developing the capacity to survive lower freezing temperatures more rapidly than older, fully expanded leaves.

Our results concerning freezing tolerance in Arabidopsis differ slightly from those of other investigators, who have reported more modest and/or slower enhancement of freezing tolerance (Gilmour et al., 1988; Kurkela et al., 1988; Ristic and Ashworth, 1993; Uemura et al., 1995). Differ-

ences in cold-acclimation conditions, including both light quantity and temperature, probably explain the differences reported. Several studies used 4°C or 5°C for acclimation instead of 1°C. Greater enhancement of freezing tolerance caused by lower cold-acclimation temperatures has been widely reported (Levitt, 1980; Monroy et al., 1993a; Dunn et al., 1994; Henriksson, 1995). Short photoperiods during cold acclimation have been used in several studies; we show that short photoperiods resulted in less freezing tolerance. Low light intensity during acclimation, which is a common problem with fluorescent lamps at low temperatures, also reduces freezing tolerance. We saw little if any enhancement in freezing tolerance when plants were acclimated at a measured PPFD of 5 μmol m^{-2} s^{-1}. Although we did not extensively address this issue in our experiments, we assume that enhancement of freezing tolerance is a function of the total number of photons received rather than of the duration of the photoperiod and that higher light intensities during acclimation would result in more rapid and/or greater freezing tolerance within the limits imposed by photoinhibition at high light intensities.

The observed temperatures for freezing survival are also affected by the evaluation methods. In many studies survival after freezing was estimated using an electrolyte-leakage test; if the test includes both younger and older leaves, it will underestimate plant survival, which is determined by the survival of the meristem and the youngest leaves.

Requirement for Light in Enhanced Freezing Tolerance

As reported many years ago (Levitt, 1980), light is essential for enhanced freezing tolerance induced by cold temperature in herbaceous winter annuals and biennials. Light is also required for enhanced freezing tolerance induced by cold in Arabidopsis. The light and cold requirements in cold acclimation are separable, because freezing tolerance is similarly enhanced after acclimation with a high "daytime" temperature and a low "nighttime" temperature, just as it is after continuous low temperature with a photoperiod of 12 h or longer. Complementary but independent effects of low temperature and light on frost hardiness in ivy have been reported (Steponkus, 1971). The most obvious role of light is photosynthetic carbon fixation, which is necessary for the accumulation of Suc and other compatible solutes. The need for photosynthesis for enhanced low-temperature-induced freezing tolerance was supported by the DCMU experiments.

Solute Accumulation during Hardening

Steponkus (1984) suggested that the accumulation of compatible solutes in the cytoplasm contributes to freezing survival by reducing the rate and extent of cellular dehydration, by sequestering toxic ions, and/or by protecting macromolecules against dehydration-induced denaturation. We found that soluble sugar accumulation was correlated with enhanced freezing tolerance, whereas accumulation of another compatible solute, Pro, was not.

Sugar accumulation at cold temperatures has been widely documented in many plants, including Arabidopsis (Ristic and Ashworth, 1993). Results of studies of Arabidopsis mutants are equivocal regarding the importance of sugar accumulation for enhanced freezing tolerance. The *sfr4* mutant was impaired in cold-inducible freezing tolerance and did not accumulate Suc and Glc in response to low temperature (McKown et al., 1996). In addition, a constitutively freezing-tolerant single-gene Arabidopsis mutant, *eskimo1*, was high in both Pro and sugars (Xin and Browse, 1998). Sugar accumulation alone, however, was insufficient for the enhancement of freezing tolerance; several single-gene mutants that accumulated sugars normally in response to low temperature were nevertheless defective in freezing tolerance (McKown et al., 1996).

Studies have correlated Pro accumulation with enhanced freezing tolerance in several plants (Levitt, 1980; Koster and Lynch, 1992; Dörffling et al., 1997); Xin and Browse (1998) described a constitutively freezing-tolerant Arabidopsis mutant that is a Pro overaccumulator. Although we found Pro accumulation to be closely correlated with enhanced freezing tolerance, it lagged behind freezing tolerance by 1 d and is therefore probably a consequence rather a cause of the enhanced freezing tolerance.

Cold-Induced Gene Expression

Numerous mRNAs accumulate during cold acclimation, and it is attractive to think that some of the products of these messages play important roles in enhancing freezing tolerance. Cold-induced genes have been identified and cloned from several plants, including a number of *COR* genes from Arabidopsis (for review, see Hughes and Dunn, 1996). Previously characterized, cold-inducible mRNA accumulation was induced within hours, and the messages remained abundant for the duration of cold treatment. We showed that, in contrast to the enhancement of freezing tolerance and the accumulation of sugars and Pro, the accumulation of cold-inducible mRNA is not dependent on light and photosynthesis. Previously described *COR* genes that show primary regulation at both the transcriptional (*COR15a*) and the posttranscriptional (*COR47* and *COR78*) levels (Hajela et al., 1990), as well as a number of other previously described and newly identified cold-inducible transcripts, show light-independent cold induction (this paper; L. Wanner and O. Junttila, unpublished data). Thus, although expression of *COR* gene mRNAs may be necessary, it is not sufficient for cold-induced enhancement of freezing tolerance. Whether the protein products of these *COR* genes also show light-independent accumulation or activity remains to be determined.

The role of *COR* gene products in freezing tolerance remains unclear. Few data are available regarding the accumulation kinetics of the protein products of *COR* genes. The protein products of two *COR* genes have been detected immunologically after 1 (Mäntylä et al., 1995) to 7 d (Gilmour et al., 1996). Despite a considerable effort (for review, see Thomashow, 1998), no single *COR* gene product has been shown to have more than a minor effect on any aspect of freezing tolerance. However, high levels of expression in

transgenic plants of a transcription factor that binds to upstream elements found in a number of *COR* genes resulted in constitutively enhanced freezing tolerance and the constitutive expression of four *COR* genes (Jaglo-Ottosen et al., 1998; Liu et al., 1998), providing additional correlative evidence for roles that *COR* gene products may play in freezing tolerance. On the other hand, mRNAs from several *COR* genes were not detected in the constitutively freezing-tolerant *eskimo1* mutant (Xin and Browse, 1998), demonstrating that it is possible to have enhanced freezing tolerance without the expression of at least some *COR* genes.

In summary, the level of freezing tolerance and the accumulation of soluble sugars parallel one another under different photoperiods during cold acclimation, indicating that sugar accumulation is a fundamental component of enhanced freezing tolerance. Pro accumulation is also light (photosynthesis) dependent but it begins after approximately 24 h, when plants have already acquired significantly enhanced freezing tolerance. Pro accumulation therefore probably does not play a role in the enhancement of freezing tolerance, although it may be important in longer-term adjustments to low-temperature-induced drought stress. The accumulation of many *COR* mRNAs appears to be strictly low-temperature dependent and does not require light (photosynthesis); therefore, cold-induced accumulation of these mRNAs is insufficient for enhanced freezing tolerance. Because lethal freezing injury is manifested as a collapse of cell membranes, it would be interesting to study changes in membrane composition and behavior at early times under different acclimation conditions, as we have done here for sugars, mRNA, and Pro. Future experiments could address the specific role of sugars in enhancing membrane stability, the kinetics of alterations in membrane lipid and protein composition during cold acclimation, and the kinetics of *COR* gene product accumulation.

ACKNOWLEDGMENTS

The authors thank Dr. Michael Thomashow and his laboratory staff at Michigan State University for the *COR78*-, *COR47*-, and *COR15a*-cloned probes. They are grateful to Rigmor Reiersen, Coby Weber, Stein Lian, and Veronica Jensen for help with growing plants. Special thanks go to Leidulf Lund, Jarle Nielsen, and the staff at the phytotron (University of Tromsø) for providing carefully controlled temperature and growth conditions and help with monitoring the freezers and to Dr. Kent Peters and an anonymous reviewer for useful suggestions concerning the manuscript.

Received December 2, 1998; accepted March 2, 1999.

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