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Low Temperature Induces the Accumulation of Alcohol Dehydrogenase mRNA in *Arabidopsis thaliana*, a Chilling-Tolerant Plant[1]

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mRNA encoding alcohol dehydrogenase (ADH) increases in etiolated seedlings and leaves of *Arabidopsis thaliana* (L.) Heynh. upon exposure to low temperature. The analysis of this response after water stress and abscisic acid (ABA) treatments in *Arabidopsis* wild type and ABA-deficient and -insensitive mutants indicates that cold accumulation of *ADH* mRNA could be induced by both anaerobic metabolism and increase of ABA concentration resulting from low temperature exposure. By using one *Arabidopsis ADH* null mutant, we show that ADH activity is not required for successful development of freezing tolerance in this species.

Many plant species from temperate regions are able to develop freezing tolerance after being exposed to low nonfreezing temperatures for several days. This complex adaptive process is known as CA (see Graham and Patterson, 1982; Guy, 1990, for reviews). CA involves both adaptation to low-temperature survival and development of freezing tolerance and has been correlated with rapid changes in gene expression (Guy et al., 1985; Gilmour et al., 1988). Cold-inducible genes have been cloned in several species, but the function of their protein products and their involvement in chilling and/or freezing tolerance remain to be characterized (Hajela et al., 1990; Kurkela and Franck, 1990; Nordin et al., 1991).

Exogenous application of ABA, which is known to accumulate in response to low temperatures (Chen et al., 1983), promotes an increase in freezing tolerance when applied at nonchilling temperatures (Chen and Gusta, 1983). Consistently, water stress, which causes an increase of endogenous ABA levels, also results in an increased tolerance to low and freezing temperatures (Cloutier and Siminovitch, 1982). These results suggest a role for this hormone in the CA process (Chen and Gusta, 1983). Two recent reports have shown low-temperature induction of *cor* genes in ABA-deficient (*aba*) and ABA-insensitive mutants (*abi1*, *abi2*, *abi3*) of *Arabidopsis*, indicating that high ABA levels are not required for *cor* induction (Gilmour and Thomashow, 1991; Nordin et al., 1991). In addition, *abi1* (Nordin et al., 1991) and, to some extent, *aba* mutants (Gilmour and Thomashow, 1991) develop freezing tolerance in a similar way as the wild type. Taken together, these results suggest that freezing tolerance can be induced independently from ABA.

One of the primary effects of low temperatures in plant cells is the alteration of membrane fluidity properties, which directly affects membrane-bound metabolic processes (Levitt, 1980). Among these processes, respiration is known to be affected in chilling-sensitive species, many of which show clear signs of anaerobic metabolism at low temperatures (Kimmerer and Kozlowski, 1982). This shift toward an anaerobic metabolism has been shown to involve changes in gene expression in two chilling-sensitive species, maize and rice, where low temperature results in a rapid increase of *Adh1* mRNA and protein activity levels in roots of both species and in rice shoots (Christie et al., 1991). Whether these changes are directly due to the lack of mitochondrial function, or whether they are regulated through signal transduction pathways similar to the ones that govern CA, is still unknown.

We show here that in *Arabidopsis*, a chilling-resistant plant, *ADH* mRNA levels increase upon low-temperature exposure, similar to what has been found in rice leaves, roots, and maize roots. This increase takes place not only in etiolated seedlings, but also in leaves. Experiments based on water stress and ABA treatments, and the analysis of the low-temperature responses in *aba* and *abi1* mutants, indicate that both cold-induced anaerobic metabolism and ABA are involved in the cold induction of *ADH* mRNA levels in *Arabidopsis*. Furthermore, we show that, after acclimation, an *ADH* null mutant is able to develop similar levels of freezing tolerance as the wild type, indicating that ADH activity is not required for the acquisition of CA.

MATERIALS AND METHODS

Plant Material

Arabidopsis thaliana (L.) Heynh. plants, ecotype Lansberg *erecta*, were used in this study. Mutants *aba* and *abi1* were kindly provided by Maarten Koornneef (Wageningen, The Netherlands). Plants homozygous for *aba* have leaf ABA levels that correspond to 30% of wild type (Koornneef et al., 1982). Plants homozygous for the *abi1* mutation are insensitive to ABA (Koornneef et al., 1984). The *adh* R002 null

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Abbreviations: ADH, alcohol dehydrogenase; CA, cold acclimation; *cor*, cold regulated.

mutation was used for the acclimation experiments and was isolated in the ecotype Bensheim (Jacobs et al., 1988). The mutant line used in this study was derived from the backcross into Landsberg *erecta* and was kindly provided by Ruth Wilson (East Lansing, MI).

Growth Conditions, Temperature Treatments, and Freezing-Tolerance Determination

Plants were grown at 22 to 24°C, under constant illumination of 100 μmol m^{-2} s^{-1}, in pots containing a mixture of perlite, vermiculite, and sphagnum (1:1:1), and irrigated with mineral nutrient solution (Haughn and Somerville, 1986). Acclimation of 3- to 4-week-old plants was performed at 4°C under constant illumination (50 μmol m^{-2} s^{-1}) for different lengths of time. Seeds were sown, under sterile conditions, in Petri dishes containing mineral nutrient solution (see above) solidified with 0.8% agar, and germinated in the dark. CA of etiolated seedlings was carried out at 4°C, in the dark, for different lengths of time.

Freezing tolerance was estimated as the percentage of plants surviving a specific freezing temperature. After acclimation for 7 d, plants were moved to a dark growth chamber and the temperature was lowered from 4°C at a rate of 2°C per h. The final freezing temperature was maintained for 6 h and then raised again to 4°C at the same rate. After 6 h at 4°C, plants were placed at 22°C under constant light (50 μmol m^{-2} s^{-1}), and survival was measured 2 weeks later as the capacity to resume growth. At least 40 plants were analyzed for each treatment.

ABA, Hydric Stress, and Anaerobiosis Treatments

ABA treatments were performed by spraying 3- to 4-week-old plants with 100 μM ABA. The ABA stock solution was prepared in DMSO, and control treatments were given with water containing the same concentration of the ABA solvent. Plants were sprayed with ABA every 6 h, and the leaves were collected after 24 h of treatment. Hydric stress was induced by placing leaves under a laminar flow hood for different times until they had lost approximately 25 or 50% of their initial fresh weight. Anaerobiosis treatment was given by placing 3- to 4-week-old plants in a nitrogen atmosphere for 24 h.

RNA Extraction and RNA Blot Analysis

Total RNA was extracted from etiolated seedlings following the hot-phenol method (Ausubel et al., 1987) and from leaves by the guanidine hydrochloric acid method (Logeman et al., 1987). RNA was electrophoresed through formaldehyde gels, transferred by capillary action to Hybond N^+ membranes (Amersham) as recommended by the manufacturer, and hybridized with ^{32}P-labeled DNA probes. A 2.5-kb *Sac*I-*Hin*dIII fragment containing part of the *Arabidopsis ADH* gene (Chang and Meyerowitz, 1986) was used as a probe. The probe was labeled by random primer extension, and hybridizations were performed following standard protocols (Ausubel et al., 1987). An rDNA probe from *A. thaliana* (Pruitt and Meyerowitz, 1986) was used as loading control.

RESULTS

ADH mRNA Accumulates during Low-Temperature Exposure

ADH mRNA levels were analyzed in etiolated seedlings and leaves of plants exposed to 4°C for different times. In etiolated seedlings, time-course experiments showed an increase of *ADH* mRNA after 24 h, reaching a maximum after 3 d (Fig. 1S). *ADH* mRNA levels decreased when exposed to low temperatures for additional days and after 24 h at room temperature (Fig. 1S). In leaves, *ADH* mRNA also accumulated during low-temperature exposure (Fig. 1L). This accumulation could already be observed after 12 h, reaching a maximum after 24 h and decreasing after 4 d. Similar to what was observed in etiolated seedlings, *ADH* mRNA decreased after plants were returned to room temperature. The faster kinetics of induction in leaves compared with etiolated seedlings could be the result of using different cold-exposure conditions (see "Materials and Methods"). Three repeats of these time-course experiments showed that the patterns of induction were very consistent.

ABA Is Involved in *ADH* mRNA Accumulation

To determine the involvement of ABA in cold accumulation of *ADH* mRNA, we followed two approaches: (a) we studied the effect of ABA and water stress on *ADH* mRNA level, and (b) we analyzed the cold induction of *ADH* mRNA in *aba* and *abi1* mutants. Both ABA and water stress treatments produced *ADH* mRNA accumulation in wild-type plants (Fig. 2). The *aba* mutant showed similar response to the ABA treatment as the wild type and no detectable response to water stress, whereas almost no effect of ABA and water stress was observed in the *abi1* mutant (Fig. 2). Cold-induced accumulation of *ADH* mRNA was between 3 and 4 times lower in the mutants than in the wild type, whereas anaerobiosis induced similar levels of *ADH* mRNA in all cases (Fig. 2). These results suggest that ABA plays a role in the cold

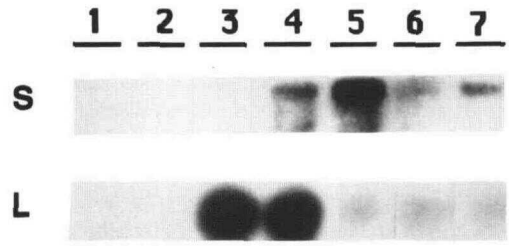

Figure 1. Time course accumulation of *ADH* mRNA during cold stress. Ten micrograms of total RNA from etiolated seedlings (S) and leaves (L) of *Arabidopsis* were fractionated by gel electrophoresis, blotted, and hybridized with an *ADH* probe. S, Lane 1, Etiolated seedlings grown at an ambient temperature of 22°C for 7 d; lanes 2–6, 7-d ambiently grown etiolated seedlings shifted to 4°C for 6 h, 12 h, 24 h, 3 d, and 7 d, respectively; lane 7, 3-d cold-exposed etiolated seedlings returned to 22°C for 1 d. L, Lane 1, Plants grown at an ambient temperature during 3 weeks; lanes 2–6, 3-week-old ambiently grown plants shifted to 4°C for 6 h, 12 h, 24 h, 4 d, and 7 d, respectively; lane 7, 24-h cold-exposed plants returned to 22°C for 24 h.

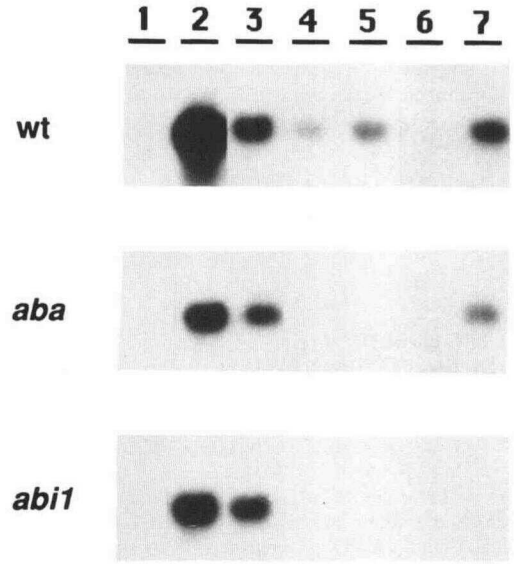

Figure 2. Expression of *ADH* mRNA in wild type (Landsberg *erecta*) and *aba* and *abi1* mutants of *Arabidopsis* in response to low temperatures, anaerobiosis, ABA treatment, and water stress. Ten micrograms of total RNA from leaves of wild type and mutants were fractionated by gel electrophoresis, blotted, and hybridized with an *ADH* probe. Lane 1, Control plants grown at 22°C; lane 2, plants exposed to 4°C for 24 h; lane 3, plants exposed to anaerobic stress for 24 h; lanes 4 and 5, water-stressed plants showing up to a 25 or 50% loss of their initial fresh weight, respectively; lane 6, plants sprayed with water; and lane 7, plants sprayed with 100 μM ABA as described in "Materials and Methods."

induction of *ADH* mRNA, but not in the anaerobic induction. These experiments were repeated three times with consistent results.

ADH Is Not Required for Freezing Tolerance

Because accumulation of *ADH* mRNA levels was correlated with low-temperature exposure, we decided to study whether ADH activity is required for the development of freezing tolerance in *Arabidopsis*. To do this, we characterized the freezing-tolerance properties of the *ADH* null mutant R002. In *Arabidopsis*, there is a single *ADH* gene (Chang and Meyerowitz, 1986) and the R002 mutant plants completely lack any detectable ADH protein or mRNA due to a single base substitution that introduces a stop codon in the reading frame (Dolferus et al., 1990). The survival of acclimated and nonacclimated R002 mutants at freezing temperatures was similar to the survival of the corresponding wild-type plants (Fig. 3). Therefore, the lack of ADH activity seems not to alter the capability to develop freezing tolerance during CA.

DISCUSSION

The effect of low temperatures on membrane physical properties and, as a consequence, on membrane-bound processes like respiration is well known (Levitt, 1980). In chilling-sensitive species, low temperatures cause a shift toward ethanolic fermentation (Levitt, 1980), and in maize and rice these changes have been correlated with changes in gene expression (Christie et al., 1991). Our results (Fig. 1) show that in *Arabidopsis*, a chilling-tolerant species, *ADH* mRNA accumulates after cold exposure in a similar way to what has been described for maize and rice. These results suggest that the shift toward ethanolic fermentation is a general phenomenon at low temperatures.

In spite of these similarities, two important differences between *Arabidopsis* and maize and rice cold responses should be considered. First, the kinetics of mRNA accumulation is faster in maize and rice at 10°C (Christie et al., 1991) than in *Arabidopsis* at 4°C. Second, in *Arabidopsis*, *ADH* expression seems to be only transitionally induced during CA, whereas in maize, *ADH* mRNA levels remain high after at least 10 d of exposure, the maximum time analyzed (Christie et al., 1991). We think that these differences could reflect the distinct chilling tolerance of maize and rice and *Arabidopsis*. The ADH mRNA level could be related to the metabolic stress supported by the plant cells under low temperature. As a consequence, chilling-sensitive plants would show a faster and more prolonged metabolic stress than chilling-tolerant ones.

The results from the ABA and water-stress treatments of *aba*, *abi1*, and wild-type plants indicate that ABA is responsible for a part of the cold-induced *ADH* mRNA accumulation through a signal transduction pathway that could be mediated by the ABI1 product. This signal transduction pathway must not be involved in anaerobiosis-induced accumulation because there is no difference in the anaerobiosis response between mutants and wild type (see Fig. 4 for a schematic representation). Although a similar role of ABA in cold induction of *ADH* mRNA has not been proposed in maize, the effect of ABA treatments on the increase of ADH activity and anaerobiosis tolerance (Hwang and Van Toai, 1991) suggests that *ADH* expression could also be ABA responsive in this

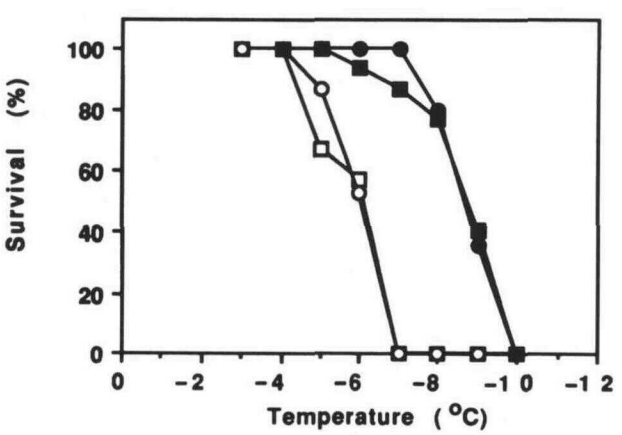

Figure 3. Effect of cold acclimation on the development of freezing tolerance in wild type and *ADH* R002 null mutant. Plants were acclimated and frozen as described in "Materials and Methods." Freezing tolerance was estimated as the percentage of plants surviving a specific freezing temperature. Control nonacclimated plants are represented by open symbols, □ for wild type and ○ for the R002 mutant. Acclimated plants are represented by closed symbols, ■ for wild type and ● for the R002 mutant.

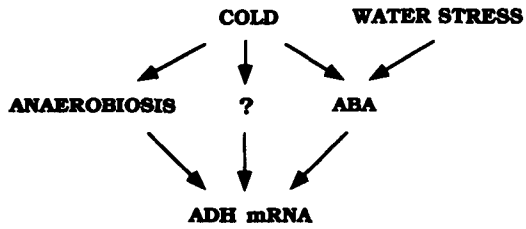

Figure 4. A schematic representation of the interactions between different regulatory pathways in the control of the *ADH* mRNA accumulation. The question mark represents the existence of a cold-specific regulatory pathway, whose effect is neither required nor rejected by the results reported in this work.

species. Consequently, cold induction of *ADH* mRNA levels in *Arabidopsis*, and likely in maize, would be the result of an additive effect of the anaerobic metabolism and the increased ABA levels promoted by low temperatures through two independent signal transduction pathways (Fig. 4).

Additionally, based on the lack of response in *aba* and *abi1* mutants, water-stress induction of *ADH* mRNA would require a strong accumulation of ABA that is not produced in the leaky *aba* mutant. Genes that are induced by low temperature or water stress are generally responsive to ABA applications. This response to ABA has been related to the presence of specific sequences containing G-box-like motifs in their promoters (Guiltinan et al., 1990). The *Arabidopsis ADH* promoter (McKendree et al., 1990), as well as the promoters of *cor* genes (Schindler et al., 1992), also contains G-box-like motifs that could be involved in the ABA response. Recent reports have suggested the existence of additional cold-specific signals that could regulate cold-inducible gene expression independently from ABA (Gilmour and Thomashow, 1991; Nordin et al., 1991). Such signals are not required to explain the cold-induced change in *ADH* mRNA level when anaerobic induction is considered (Fig. 4).

The kinetics of *ADH* mRNA accumulation in *Arabidopsis* under low temperature conditions is slower than that observed for *cor* genes under similar acclimation conditions (Hajela et al., 1990). Increases in *cor* gene mRNA abundance can be observed after 4 to 6 h of exposure to 4°C, whereas changes in *ADH* mRNA levels are observed after 12 h. Furthermore, in opposition to what we have observed for *ADH* mRNA, *cor* gene mRNAs are maintained at a high level as long as the plants are at low temperature. These differences suggest different functions for *ADH* and *cor* genes during CA. By taking advantage of the availability of *ADH* null mutants, we showed that this gene, whose mRNA is activated during cold acclimation, is in fact not required for the acquisition of freezing tolerance.

Two nonexclusive possibilities could explain this result: *Arabidopsis* may use an alternative way to ethanolic fermentation to overcome mitochondrial dysfunction and/or the plant does not suffer a very prolonged metabolic stress when exposed at 4°C because of its CA capability. The use of a similar approach involving mutants will certainly help to verify these possibilities and to elucidate the involvement of other *cor* genes in the development of freezing tolerance.

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