Plant Physiol. (1994) 104: 1341–1349

Alterations in Water Status, Endogenous Abscisic Acid Content, and Expression of *rab18* Gene during the Development of Freezing Tolerance in *Arabidopsis thaliana*[1]

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Treatments as diverse as exposure to low temperature (LT), exogenous abscisic acid (ABA), or drought resulted in a 4 to 5°C increase in freezing tolerance of the annual herbaceous plant *Arabidopsis thaliana*. To correlate the increase in freezing tolerance with the physiological changes that occur in response to these treatments, we studied the alterations in water status, endogenous ABA levels, and accumulation of *rab18* (V. Lång and E.T. Palva [1992] Plant Mol Biol 20: 951–962) mRNA. Exposure to LT and exogenous ABA caused only a minor decline in total water potential (Ψ_w), in contrast to a dramatic decrease in Ψ_w during drought stress. Similarly, the endogenous ABA levels were only slightly and transiently increased in LT-treated plants in contrast to a massive increase in ABA levels in drought-stressed plants. The expression of the ABA-responsive *rab18* gene was low during the LT treatment but could be induced to high levels by exogenous ABA and drought stress. Taken together, these results suggest that the moderate increases in freezing tolerance of *A. thaliana* might be achieved by different mechanisms. However, ABA-deficient and ABA-insensitive mutants of *A. thaliana* have impaired freezing tolerance, suggesting that ABA is, at least indirectly, required for the development of full freezing tolerance.

Plants can exhibit varying degrees of freezing tolerance depending on the environment in which they grow. Tropical plants are usually sensitive to both chilling and freezing temperatures (Levitt, 1980a). In contrast, many plants growing in temperate regions may have to either avoid or tolerate freezing stress (Levitt, 1980a). Annual herbs are often exposed to freezing temperatures during growth, and the main mechanism for surviving subzero temperatures is to tolerate extracellular freezing (Levitt, 1980a). Many herbaceous species show enhanced freezing tolerance following exposure to low, nonfreezing temperatures (LT), a process called cold acclimation (Levitt, 1980a; Sakai and Larcher, 1987). Increased freezing tolerance can also be achieved by exposure of plants to other environmental conditions, e.g. to D (Tyler et al., 1981; Cloutier and Siminovitch, 1982; Guy et al., 1992; Lee and Chen, 1993) or by treatment with the phytohormone ABA (Chen et al., 1983; Chen and Gusta, 1983; Orr et al., 1986; Lång et al., 1989; Lee and Chen, 1993). Adaptation of a plant to survive extracellular ice formation is mainly adaptation to freeze-induced cellular dehydration (Levitt, 1980a; Sakai and Larcher, 1987; Steponkus and Lynch, 1989; Yelenosky and Guy, 1989; Steponkus, 1990). Numerous physiological alterations, including plasma membrane composition, leaf water status, phytohormone level, and gene expression have been correlated with this adaptation process (Levitt, 1980a; Sakai and Larcher, 1987; Steponkus and Lynch, 1989; Guy, 1990; Steponkus, 1990; Thomashow, 1990). However, a clear role for any of these changes in the acquired stress tolerance is yet to be demonstrated.

Alterations in plant water status are well established in higher plants exposed to water deficit. Many plant species adapt to cellular dehydration stress by osmotic adjustment (Levitt, 1980b). This adjustment is achieved by accumulation of compatible osmolytes and results in the retention of turgor and the capacity for cell elongation at low water potential (Levitt, 1980b). Various degrees of osmotic adjustment have been reported in herbaceous plants during cold acclimation (Levitt, 1980a; Chen and Li, 1982; Lalk and Dörffling, 1985; Sakai and Larcher, 1987; Yelenosky and Guy, 1989), but more importantly, cold acclimation-induced osmolytes such as sugars and amino acids might help the plant to survive freeze-induced cellular dehydration stress by acting as noncolligative cryo- or osmoprotectants (Yelenosky and Guy, 1989).

ABA is suggested to mediate plant responses to both LT and water deficit: (a) LT can increase the endogenous ABA levels in chilling-sensitive (Daie and Campbell, 1981; Pan, 1990; Plant et al., 1991), chilling-tolerant annual (Chen et al., 1983; Guy and Haskell, 1988), and overwintering (Wightman, 1979; Lalk and Dörffling, 1985) species. Water deficit induces endogenous ABA levels even more dramatically (Wright, 1978; Walton, 1980; Cornish and Zeevaart, 1984; Bray, 1991; Plant et al., 1991). (b) Application of ABA can induce increased freezing (Chen et al., 1983; Chen and Gusta, 1983; Orr et al., 1986; Lång et al., 1989), chilling (Rikin et al., 1976; Bornman and Jansson, 1980), or D tolerance (Davies

[1] This work was supported by the Swedish Natural Sciences Research Council, the Swedish National Board for Technical Development, and Sarec. A grant from the Nilsson-Ehle Foundation is gratefully acknowledged.

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Abbreviations: D, drought; DA, deacclimation; LT, low temperature; Ψ_p, pressure potential; Ψ_s, osmotic potential; Ψ_w, total water potential; WT, wild type.

and Mansfield, 1983; V. Lång, unpublished results). (c) An ABA-deficient mutant of *Arabidopsis thaliana* (Koornneef et al., 1982) is less tolerant to both freezing temperatures (Heino et al., 1990; Gilmour and Thomashow, 1991) and water deficit (Koornneef et al., 1982). (d) Several LT-induced genes are also responsive to exogenous ABA or D treatment (see Cattivelli and Bartels, 1992). In addition, expression of several ABA-responsive, late embryogenesis-abundant, and dehydration-responsive *rab/lea/dhn* genes (Skriver and Mundy, 1990) can be induced by LT in both freezing-tolerant (Grossi et al., 1992; Hong et al., 1992; Lång and Palva, 1992; Sutton et al., 1992) and chilling-sensitive (Hahn and Walbot, 1989; Skriver and Mundy, 1990; Bray, 1991) species. Taken together, these results suggest that ABA may be a trigger for both cold acclimation and adaptation to water deficit, suggesting a close similarity between these adaptation mechanisms.

The aim of our study was to investigate how a freezing-tolerant annual herb, *A. thaliana*, adapts to subzero temperatures by exposure to LT, exogenous ABA, or D, and how ABA might be involved in these processes. Therefore, we measured adaptation-induced alterations in physiology and gene expression of WT as well as ABA-deficient (Koornneef et al., 1982) and ABA-insensitive (Koornneef et al., 1984) mutants of *A. thaliana*. The results suggest that separate adaptation mechanisms leading to increased freezing tolerance might coexist in *A. thaliana*. The ABA mutant analysis indicated that ABA is, at least indirectly, involved in both LT- and D-induced freezing tolerance.

MATERIALS AND METHODS

Plant Material and Growth Conditions

The *Arabidopsis thaliana* (L.) Heynh. ecotype Landsberg *erecta*, and ABA-deficient (*aba-1*, isolate A26) (Koornneef et al., 1982) and ABA-insensitive (*abi1*, isolate AII) (Koornneef et al., 1984) mutants generated in this ecotype were used in all experiments. Axenic plants growing in 24-well tissue culture plates were used to facilitate the application of exogenous ABA and to achieve uniform water stress (Lång et al., 1989). The RH inside the plates was estimated to be >80%. We used a controlled growth cabinet with 22°C day/20°C night and 16-h photoperiod with a light intensity of 80 to 100 μmol m^{-2} s^{-1} at plant level. LT treatment was done by transferring the 2-week-old plants to a growth cabinet with 4°C day/2°C night and 14-h photoperiod with a light intensity of 30 to 50 μmol m^{-2} s^{-1}. Plants were deacclimated in a 22°C growth cabinet for 1 d. Exposure to water stress (atmosphere of 35% or 60–70% RH) was done by removing the lids of the tissue culture plates in corresponding 22°C growth cabinets and allowing the plants to dry for the times indicated. ABA treatment was done in a 22°C growth cabinet by adding 60 μM ABA (final concentration) to the growth media.

Soil-grown plants were kept in pots in a mixture of soil and perlite and covered with a net to keep the humidity higher for the ABA mutants. Growth conditions were 24°C day/22°C night and 16-h photoperiod with a light intensity of 100 to 150 μmol m^{-2} s^{-1}. One-month-old plants (before bolting) were exposed to LT as described above. For DA, the plants were transferred back to the greenhouse for 1 d. Exposure to extracellular freezing was done in a controlled temperature cabinet by gradually lowering the temperature 2°C h^{-1} from +4°C to −10°C, and keeping plants at −10°C for 4 h. To initiate extracellular freezing, plants were sprayed with tap water at −3°C.

Plant material for freezing tests as well as for Ψ_w and Ψ_s measurements (used directly), ABA extraction (frozen at −80°C), and northern analyses (frozen in liquid N_2) were collected between 9 and 11 AM to eliminate possible fluctuations due to diurnal variation. Exceptions were 3- and 6-h samples, which were collected at noon and at 3 PM, respectively.

Freezing Tolerance Assay

To assess freezing tolerance, in vitro-grown plants (without roots) were frozen in a controlled temperature bath and survival of the material was determined both by visual estimation and by ion leakage (Sukumaran and Weiser, 1972) as described previously (Lång et al., 1989). Freezing tolerance of soil-grown plants was assessed in a controlled-temperature cabinet by gradually lowering the temperature 2°C h^{-1} from +4°C to −10°C. To initiate extracellular freezing, plants were sprayed with tap water at −3°C. Plants were kept at −10°C for 4 h, after which the temperature was gradually increased 2°C h^{-1} to 0°C. Survival of soil-grown plants was visually estimated after 4 d of regrowth at 24°C/22°C.

Measurement of Water Status

Ψ_w was measured with a Wescor HP-115 dew point microvoltmeter connected to C-52 sample chambers (Wescor) according to instructions from the manufacturer. For each measurement, two in vitro-grown plants (without roots) were used. Four parallel measurements were made for each time point and two independent experiments were performed. The Ψ_s was determined by the dew point method from the same material after freezing the tissue in liquid N_2 and thawing. The Ψ_p of the plant material was estimated by subtraction of osmotic potential from leaf water potential ($\Psi_p = \Psi_w - \Psi_s$).

Measurement of ABA

Each sample consisted of leaf and stem (in vitro-grown plants) or leaf (soil-grown plants) material, homogenized with a mortar and a pestle in liquid N_2. One gram of powder was extracted for 1 h with 10 mL of 80% methanol containing 0.02% of the antioxidant sodium diethyldithiocarbamate, and 25 to 100 ng of [$^2H_4$]ABA (1 μg mL^{-1}) was added as an internal standard. Extraction was performed with continuous stirring at 4°C in the dark. After filtration, the extract was reduced to the aqueous phase by rotary evaporation at 40°C, adjusted to pH 2.7, and applied to a Waters C_{18} Sep-Pak cartridge prewashed with 5 mL of methanol and 5 mL of 1% acetic acid. The C_{18} cartridge was washed with 5 mL of 1% acetic acid and then connected with a silica Sep-Pak cartridge prewashed with 5 mL of dichloromethane. The two cartridges were eluted in series with 10 mL of dichloromethane. The

silica Sep-Pak was then washed with 4 mL of heptane followed by 4 mL of 20% ethylacetate in heptane before eluting the sample with 4 mL of 70% ethylacetate in heptane. The organic phase was reduced to dryness under N_2 and subjected to HPLC. The HPLC mobile phase consisted of 45% methanol in 1% acetic acid and was delivered at a flow rate of 1 mL min^{-1} by Waters model 501 pumps. The sample was introduced by a Waters 712 WISP onto a 10 cm × 8 mm i.d. 4-μm Nova-Pak C_{18} cartridge fitted in a RCM 8 × 10 module (Waters Associates AB, Partille, Sweden). Fractions corresponding to the retention volume of standard (+)-*cis,trans* ABA (Sigma) were automatically collected, methylated with ethereal diazomethane (Schlenk and Gellerman, 1960), and quantified by GC-selected ion monitoring-MS. GC-MS was performed using a Hewlett-Packard 5890 gas chromatograph linked by a direct inlet to a Hewlett-Packard 5770 mass selective detector. Volumes of 2 μL were injected splitless at 230°C onto a 25 m × 0.25 mm i.d. SE-30 column with a film thickness of 0.25 μm (Quadrex Co., New Haven, CT). The carrier gas was helium with a flow of 1 mL min^{-1}. The column temperature was initially 60°C, was then programmed at 20°C min^{-1} to 200°C, followed by an increase of 4°C min^{-1} to 216°C, and finally an increase of 10°C min^{-1} up to 250°C. The ionization potential was 70 eV and the interface temperature was held at 250°C. The ratio of m/z 162:166 was used to check peak homogeneity, whereas the ratio of m/z 190:194 was used to calculate the amount of endogenous ABA by reference to a standard curve obtained by analyzing 7.5 ng μL^{-1} methylated ABA together with known amounts of 2.5 to 20 ng μL^{-1} [$^2H_4$]ABA. Two to three independent measurements were done for the most relevant time points (see Tables II and III).

RNA Isolation and Northern Analysis

RNA was isolated from in vitro-grown plants (without roots). The plant material was harvested at time points similar to those used for the freezing tolerance tests and for Ψ_w, Ψ_s, and ABA determinations. RNA extraction and gel electrophoresis, as well as hybridization and washing conditions, were as described previously (Lång and Palva, 1992). For probes we used a fragment containing the coding region of the *rab18* gene (Lång and Palva, 1992) and the partial cDNA clone of *lti78* (Nordin et al., 1991). The filters were quantified by PhosphoImage scanner (Molecular Dynamics). Two to three independent analyses were done for the most relevant time points (data not shown).

RESULTS

Water Status Is Differently Altered in Response to LT, Exogenous ABA, and Water Deficit

LT, exogenous ABA, and D treatments all resulted in a similar 4 to 5°C increase in freezing tolerance of axenically grown WT *A. thaliana* plants, whereas the *aba-1* and the *abi1* mutants were impaired in developing full freezing tolerance in response to LT and D treatments (Table I). Furthermore, the initial level of freezing tolerance of the *aba-1* mutant was lower than that of WT and the *abi1* mutant (Table I).

To determine whether the three treatments influenced plant water status, we measured Ψ_w and Ψ_s as a function of time in plants exposed to LT, exogenous ABA, and water stress. The results showed that LT treatment for 5 d caused about 0.5 MPa reduction in Ψ_w of both the WT and the *aba-1* mutant, whereas subsequent DA for 1 d returned Ψ_w to the noninduced level (Fig. 1, A and C). Similar to the decline in Ψ_w, the Ψ_s of the cell sap was decreased in the WT and the *aba-1* mutant (data not shown), resulting in a positive turgor potential (Ψ_p) throughout the LT treatment. In the *abi1* mutants only a marginal LT-induced reduction in Ψ_w (Fig. 1B) and Ψ_s (data not shown) was observed.

Exposure of the WT and the *aba-1* mutant to exogenous ABA for 3 d caused a decrease in Ψ_w (Fig. 1, A and C) and Ψ_s (data not shown) similar to that caused by LT treatment for 5 d (Fig. 1, A and C). The ABA-induced decline in Ψ_w (Fig. 1B) and Ψ_s (data not shown) was slightly delayed in the *abi1* mutant (Fig. 1B).

In contrast to the results obtained by LT and exogenous ABA treatments, D treatment at 60 to 70% RH for 2 d resulted in a pronounced decrease in both Ψ_w (Fig. 1, A–C) and Ψ_s (data not shown) in the WT plants (Fig. 1A) and especially in the *abi1* and the *aba-1* mutants (Fig. 1, B and C). During this time the WT plants increased in freezing tolerance to the same level as plants exposed to LT for 5 d or plants treated with exogenous ABA for 3 d (Table I). Prolonged exposure of the WT plants to water stress beyond 2 d resulted in wilting and simultaneous loss of freezing tolerance (E. Mäntylä, unpublished data). The *aba-1* increased slightly in freezing tolerance during the 1st d of D treatments (Table I), after which both the *aba-1* and the *abi1* mutant wilted and no further increase in freezing tolerance was observed (Table I).

Endogenous ABA Content Increases Transiently at Low Temperature, but Markedly Less than during Water Stress

Since ABA has been suggested to mediate the responses of plants to both water and freezing stress, we wanted to compare the alterations in endogenous ABA level in LT- and D-treated *A. thaliana* plants. Our results show that the level of ABA in in vitro-grown WT plants was only transiently affected by LT. We detected a 2- to 3-fold transient increase in ABA level during the 1st d of treatment (Table II). To confirm these results obtained with in vitro-grown plants, we also extracted ABA from LT-treated soil-grown WT plants. A similar increase in ABA level was observed in soil-grown WT plants during the 1st d of LT treatment, after which the ABA content gradually declined (Table III).

In the *abi1* mutant the level of ABA also increased 2- to 3-fold after overnight exposure to LT, but in contrast to WT plants, ABA remained at the elevated level throughout the treatment (Table II). As expected, the level of endogenous ABA was low in the *aba-1* mutant and did not change during the LT treatment (Table II). DA had an effect on ABA level only in the *abi1* mutants (Table II), where ABA returned to the noninduced level after 1 d of DA.

In contrast to the modest and transient increase in ABA levels observed after exposure to LT, a severe D stress (35% RH) resulted in a large increase in endogenous ABA content. In 3- and 6-h water-stressed WT plants and the *abi1* mutant the ABA level was significantly higher than in 15-h or 1-d

Table I. *Freezing tolerance of axenically grown A. thaliana WT, ABA-deficient mutant aba-1 (Koornneef et al., 1982), and ABA-insensitive mutant abi1 (Koornneef et al., 1984) exposed to LT, DA, ABA, or D*

The values for the killing temperatures are obtained from a combination of visual estimation and ion leakage determination (Sukumaran and Weiser, 1972) as described previously (Lång et al., 1989). The numbers in parentheses refer to the number of plants tested.

Treatment	Time (d)	Killing Temperature (°C)		
		WT	*abi1*	*aba-1*
Control[a]	0	−3 (36)	−3 (36)	−2 (12)
LT[b]	1	−3.5 (36)	−3.5 (36)	−2 (12)
	5	−7 (36)	−5 (36)	−3 (12)
DA[c]	1	−3 (36)	−3 (36)	−2 (12)
ABA[d]	1	−5.5 (12)	−3.5 (12)	−5 (12)
	3	−8 (12)	−3.5 (12)	−8 (12)
D[e]	1	−5.5 (12)	−3.5 (12)	−3.5 (12)
	2	−7 (12)	−3 (12)	−3 (12)

[a] Grown at 22°C day/20°C night. [b] Days at 4°C day/2°C night. [c] Days at 22°C, after 5 d of LT treatment at 4°C/2°C. [d] Days at 22°C with 60 μM ABA. [e] Days at 22°C with 60 to 70% RH.

LT-treated WT plants and the *abi1* mutant (Table II). The *aba-1* mutant also exhibited a slight increase in endogenous ABA content during D stress, but to a significantly lower level than that observed in the WT plants and the *abi1* mutant (Table II). The WT plants did not show any symptoms of wilting after 6 h at 35% RH, whereas the *aba-1* and *abi1* mutants started to wilt after 3 h of treatment.

Since extracellular freezing causes extensive dehydration stress to a cell (Steponkus, 1990), we asked whether endogenous ABA content was increased during extracellular freezing of cold-acclimated WT plants. For this purpose, 5-d LT-acclimated soil-grown plants were gradually exposed to −10°C, and at that stage ABA was extracted from these plants. The 5-d cold-acclimated plants survived this temperature, whereas the nonacclimated plants were killed (data not shown). We could not detect any significant difference in endogenous ABA content between the cold-acclimated frozen and nonfrozen plants (Table III), indicating that the level of endogenous ABA was not increased during brief extracellular freezing.

Accumulation of *rab18* mRNA Correlates with Endogenous ABA Content in LT- and D-Treated Plants

We have recently shown that the expression of *rab18* gene in response to LT and water stress is ABA mediated (Lång and Palva, 1992). In this study we wanted to correlate the endogenous ABA levels to the accumulation of *rab18* mRNA in treated plants. The results showed that the accumulation of *rab18* mRNA was 6- to 10-fold lower in LT-treated WT plants than in ABA-treated or water-stressed plants (Fig. 2A; Lång and Palva, 1992). These differences in mRNA levels are in accordance with the observed differences in endogenous ABA levels during LT (varying from about 6–24 ng g^{-1}

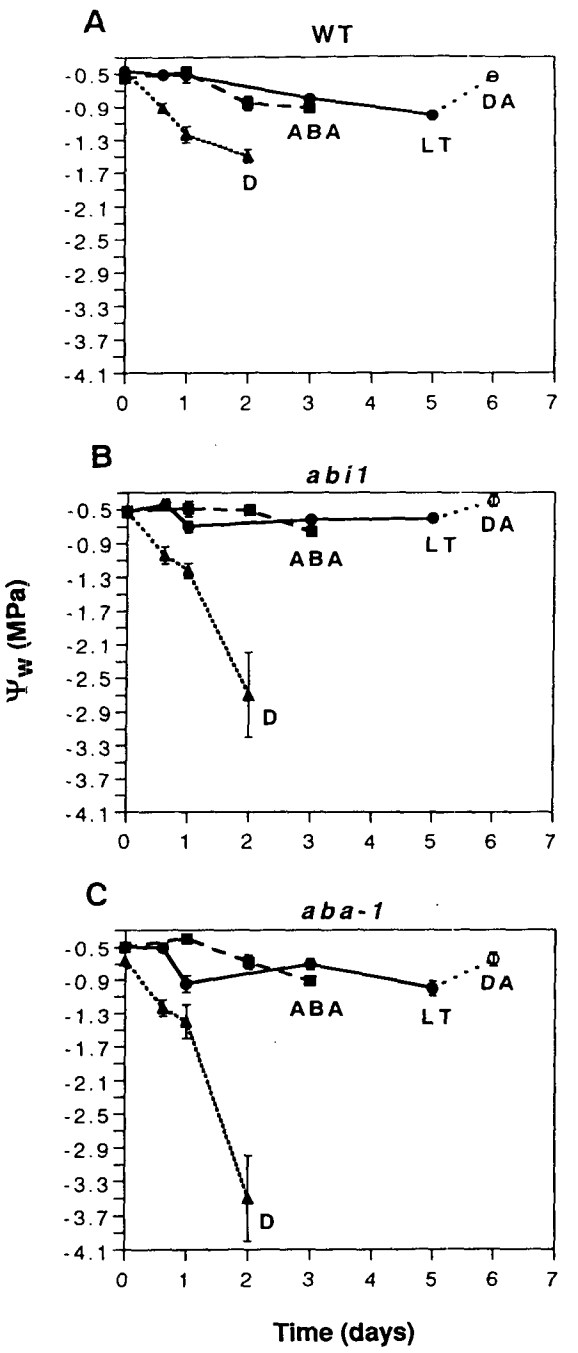

Figure 1. Ψ_w of in vitro-grown *A. thaliana* plants exposed to LT (4°C day/2°C night), DA (22°C), ABA (60 μM), or D (60–70% RH). DA was done by transferring 5-d LT-treated plants to nonacclimating temperatures for 1 d. A, WT; B, ABA-insensitive mutant (*abi1*); C, ABA-deficient mutant (*aba-1*). Each symbol represents the mean ± SD (n = 4). Similar results were obtained from two independent experiments.

fresh weight) and water stress treatments (about 150 ng g^{-1} fresh weight) (Table II). However, the small but reproducible increase in *rab18* mRNA level persisted throughout the cold-acclimation period (Fig. 2A; Lång and Palva, 1992), in contrast to the endogenous ABA level, which decreased to the noninduced level at later stages of cold acclimation (Table II).

In the *abi1* mutant, *rab18* mRNA accumulated markedly less than in the WT during LT, ABA, and D treatment (Fig. 2B; Lång and Palva, 1992) in spite of the elevated endogenous ABA levels (Table II). In accordance with low ABA levels (Table II), *rab18* mRNA was hardly detectable in the *aba-1* mutant during LT and D treatments but was abundantly expressed in response to exogenous ABA (Fig. 2C; Lång and Palva, 1992).

We also wanted to test whether *rab18* mRNA could be induced by extracellular freezing in cold-acclimated soil-grown WT plants. For this purpose, we extracted RNA from the material, which was also used for endogenous ABA determination (Table III). In accordance with the unchanged ABA levels at −10°C (Table III), there was no further accumulation of *rab18* mRNA during extracellular freezing of cold-acclimated plants (data not shown).

Accumulation of *lti78* mRNA (Nordin et al., 1991, 1993) was used as a positive control for different treatments in northern analyses (Fig. 2, D–F). The LT- and D-induced expression of this gene appears not to be ABA mediated (Nordin et al., 1991). In accordance with this, *lti78* mRNA was accumulating both in WT and ABA mutant plants in response to LT and D treatments (Fig. 2, D–F).

Table III. *Content of free ABA in rosette leaves of soil-grown A. thaliana WT exposed to LT, DA, or exogenous freezing (−10°C) treatments*

Where more than one measurement of ABA content was made for a particular time point, the mean is shown with the individual values appearing in parentheses.

Treatment	Time (h)	ABA Content (ng/g fresh weight)
Control[a]	0	3 (2, 3, 4)
LT[b]	6	3.5 (3, 4)
	9	4 (–)
	12	4 (–)
	15	10 (10, 10, 10)
	24	11 (10, 12)
	72	8 (7, 9)
	120	6 (6, 6)
DA[c]	24	5 (–)
LT/−10°C[d]	0	5 (–)
	120	6 (–)

[a] Grown at 24°C day/22°C night. [b] Hours at 4°C day/2°C night. [c] Hours at 24°C, after 5 d of LT treatment at 4°C/2°C. [d] Hours at 4°C/2°C, followed by freezing to −10°C.

DISCUSSION

The freezing tolerance of *A. thaliana* can be moderately (4–5°C) increased by different treatments, i.e. exposure to LT (Gilmour et al., 1988; Kurkela et al., 1988), exogenous ABA (Lång et al., 1989), or water deficit (Table I). Similar, moderate increases in freezing tolerance can be achieved by LT in other annual herbaceous plant species such as potato (aerial parts) (Chen and Li, 1982) and spinach (Yelenosky and Guy, 1989), whereas overwintering plant species increase in freezing tolerance substantially (20–40°C) for the winter period (see Levitt, 1980a; Sakai and Larcher, 1987).

We investigated changes in three physiological parameters that have previously been correlated with the development of freezing tolerance, namely alterations in water status, endogenous ABA level, and ABA-mediated gene expression. Our data with an annual herb *A. thaliana* revealed that (a) exposure to LT, i.e. cold acclimation, resulted in only minor changes in plant water status, endogenous ABA level, and

Table II. *Free ABA content of axenically-grown A. thaliana WT, ABA-insensitive mutant abi1, and ABA-deficient mutant aba-1 exposed to LT, DA, or D treatments*

When more than one measurement of ABA content was made for a particular time point, the mean is shown with the individual values appearing in parentheses.

Treatment	Time (h)	ABA Content (ng/g fresh weight)		
		WT	*abi1*	*aba-1*
Control[a]	0	6.5 (5, 7, 8)	13 (11, 15)	0.7 (0.6, 0.6, 0.9)
LT[b]	6	5.5 (5, 6)	10 (–)	ND
	15	24 (24, 24)	20 (–)	0.8 (0.8)
	24	7.5 (5, 10)	18 (–)	0.5 (–)
	48	7 (5, 9)	17 (–)	0.8 (–)
	72	8 (7.5, 8.5)	20 (–)	0.5 (–)
	96	7.5 (–)	18 (–)	ND
	120	7 (7, 7)	20 (–)	0.6 (–)
DA[c]	24	7 (–)	10 (–)	0.6 (–)
D[d]	3	127 (124, 130)	133 (–)	4 (–)
	6	148 (–)	213 (206, 220)	6.7 (6.6, 6.7)

[a] Grown at 22°C day/20°C night. [b] Hours at 4°C day/2°C night. [c] Hours at 22°C, after 5 d of LT treatment at 4°C/2°C. [d] Hours at 22°C with 35% RH.

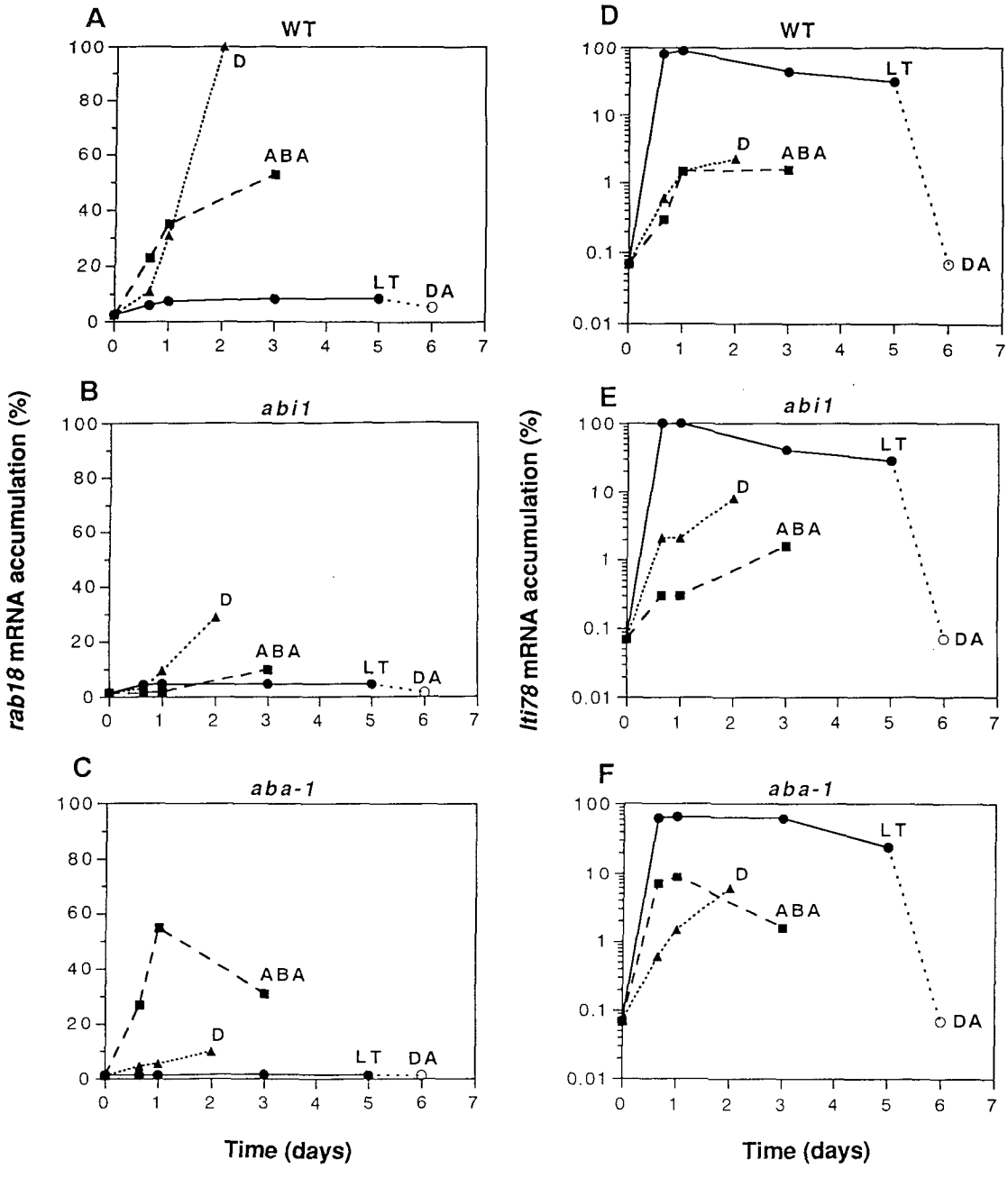

Figure 2. Accumulation of *rab18* (Lång and Palva, 1992) and *lti78* (Nordin et al., 1991) mRNAs of in vitro-grown *A. thaliana*. Ten micrograms of total RNA were separated by formaldehyde gel electrophoresis, transferred to nylon membrane, and probed with ^{32}P-labeled fragments corresponding to *rab18* or *lti78* genes. Radioactivity on the membrane was quantified by a PhosphoImage scanner. The levels of *rab18* and *lti78* mRNAs after LT (4°C day/2°C night), DA (5-d LT-treated plants transferred to 22°C for 1 d), ABA (60 μM), or D (60–70% RH) treatments were expressed as percentages of the maximum levels of respective mRNAs (2-d D-treated WT plants and 1-d LT-treated *abi1* mutant, respectively). A and D, WT; B and E, ABA-insensitive mutant (*abi1*); C and F, ABA-deficient mutant (*aba-1*). Note that the *y* axes in D through F showing *lti78* mRNA accumulation are in logarithmic scale.

expression of the ABA-responsive *rab18* gene, (b) treatment with exogenous ABA led to significant accumulation of *rab18* mRNA but resulted in only minor changes in water status, and (c) water stress resulted in substantial changes in all three parameters.

The marginal but continuous decrease in Ψ_w during LT and ABA treatments was caused mainly by a simultaneous decrease in Ψ_s (Fig. 1, A–C). Similar decreases (about 0.5 MPa) in Ψ_w and Ψ_s have been observed in winter rape exposed to 2°C for 18 d (Kacperska, 1993). A small osmotic adjustment has also been observed in other annual freezing-tolerant herbs but mainly in the beginning of cold acclimation (Chen

and Li, 1982; Yelenosky and Guy, 1989). However, osmotic adjustment is suggested to result in only a minor increase in freezing tolerance of annual herbs, and hence, additional mechanisms such as noncolligative cryoprotection might be involved in the increase of freezing tolerance (Chen and Li, 1982; Yelenosky and Guy, 1989). As expected, in contrast to the results obtained with LT and ABA, water deficit caused a significant decrease in Ψ_w (Fig. 1, A–C) and Ψ_s. In this case, the decline in Ψ_s might be partially caused by a decreased cell volume resulting from cell shrinkage (Levitt, 1980b). The resulting decrease in water content could explain the increased freezing tolerance of water-stressed plants (Levitt, 1980a). In conclusion, the water status of *A. thaliana* plants adapted to freezing temperatures can differ widely, depending on the adaptive treatment.

A mechanistic role of endogenous ABA in cold acclimation of annual freezing-tolerant herbs has not previously been demonstrated. Earlier reports with aerial parts of potato and spinach revealed that the level of endogenous ABA is transiently increased 2- to 3-fold by LT during cold acclimation (Chen et al., 1983; Guy and Haskell, 1988). No such increase was observed in a potato species incapable of cold acclimation (Chen et al., 1983). Our results show that a similar transient increase in endogenous ABA level also occurs in both in vitro- and soil-grown WT *A. thaliana* plants during cold acclimation (Tables II and III). Interestingly, the level of endogenous ABA was similarly increased by LT in the *abi1* mutant, but in contrast to the WT, remained high throughout the treatment (Table II). These results suggest that the *abi1* mutant might lack a negative feedback regulation in some step of ABA metabolism, and that ABA accumulation could be continuously stimulated in LT-treated plants. No increase in endogenous ABA in response to LT was observed in the *aba-1* mutant (Table II). The endogenous ABA content of LT-treated WT *A. thaliana* plants cannot be directly correlated with increased freezing tolerance. Neither was ABA level increased in cold-acclimated WT plants by extracellular freezing (Table III). However, the observed transient increase in ABA that precedes development of freezing tolerance may be needed as a trigger for cold acclimation of *A. thaliana*, as suggested for other annual freezing-tolerant plant species (Chen et al., 1983).

In contrast, it is not known whether the large and continuous increase in ABA levels in water-stressed plants is more than required for enhanced freezing tolerance. In addition, the sensitivity to ABA might be altered in LT- versus D-treated plants, making the interpretation of the significance of free ABA content in freezing tolerance difficult. However, studies with the cold acclimation-impaired *abi1* and *aba-1* mutants indicated that ABA-controlled mechanisms are needed for both types of adaptation. It should be noted, however, that ABA deficiency and ABA insensitivity cause pleiotropic alterations in the mutant plants (Koornneef et al., 1982, 1984), which might indirectly reduce the viability of the ABA mutants in freezing temperatures.

It has been speculated that in addition to a role in D tolerance, the expression of *rab* genes (especially group 2 *lea* homologs, see Dure et al., 1989; Skriver and Mundy, 1990) is involved in the development of freezing tolerance. The differential expression of these genes in response to different stimuli suggests to us that in spite of their extensive sequence homology, they could be divided into two categories: (a) the "classical" group 2 *lea* homologs, which are expressed mainly during late embryogenesis or in response to ABA and D, and (b) the "lti" group 2 *lea* homologs, which are induced mainly by LT. Furthermore, it appears that the induction of genes in the classical group is ABA mediated, whereas the induction of those in the lti group is independent of ABA (Skriver and Mundy, 1990; Gilmour and Thomashow, 1991; Lång and Palva, 1992; Nordin et al., 1993; Palva et al., 1993). Earlier studies with the classical group 2 *lea* homologs showed that their expression was induced to high levels by LT in chilling-sensitive rice and tomato (Hahn and Walbot, 1989; Bray, 1991), apparently by an ABA-mediated mechanism (Pan, 1990; Plant et al., 1991). In *A. thaliana*, LT-induced expression of the classical group 2 *lea* homolog *rab18*, however, was significantly lower than the ABA- and D-induced expression (Lång and Palva, 1992; Fig. 2A). This difference can be correlated with the differences in levels of endogenous ABA (Tables II and III). These results and similar ones obtained with the ABA mutants (Table II, Fig. 2, B and C) indicate that expression of the classical group 2 *lea* genes might be mainly ABA mediated.

In contrast, several recent reports have shown that lti group 2 *lea* homologs are abundantly expressed in LT-treated overwintering (Guo et al., 1992; Houde et al., 1992; Ouellet et al., 1993; Wolfraim et al., 1993) and annual (Gilmour et al., 1992; Neven et al., 1993; Palva et al., 1993) plants. However, expression of this lti group of genes appears to be often absent or low in plants exposed to exogenous ABA or water stress (Gilmour and Thomashow, 1991; Gilmour et al., 1992; Guo et al., 1992; Houde et al., 1992; Palva et al., 1993; Wolfraim et al., 1993). Studies with the ABA mutants of *A. thaliana* support the notion that the expression of lti group 2 *lea* homologs is independent of endogenous ABA (Gilmour and Thomashow, 1991; Palva et al., 1993). Thus, different members of the group 2 *lea* genes seem to be induced in response to either LT or ABA/water stress, and consequently they may be involved in the development of either LT- or ABA/D-induced freezing tolerance, respectively. The reason for this differential expression, however, is unclear, because the functions of the corresponding proteins may or may not be similar. Hence, it will be interesting to see if overproduction polypeptides belonging either to the classical or the lti group would contribute equally well, if at all, to freezing tolerance at nonacclimating temperatures.

Our results suggest that partially different adaptation mechanisms to freezing temperatures might coexist in *A. thaliana*. As a result of these mechanisms, *A. thaliana* can increase its freezing tolerance by 4 to 5°C. Furthermore, ABA has at least an indirect role in acquired freezing tolerance, as shown by the studies with ABA mutants of *A. thaliana*.

ACKNOWLEDGMENTS

We thank Dr. Einar Jensen (University of Tromso, Norway) for his gift of [$^2H_4$]ABA and Dr. Jürgen Denecke for useful discussions and for critical reading of the manuscript. Gunvor Sandman is thanked for preparing the plant material.

Received July 20, 1993; accepted December 7, 1993.
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