Arabidopsis thaliana avoids freezing by supercooling

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Abstract

Arabidopsis thaliana (L.) Heynh. has been described as a freezing-tolerant species based on freezing-resistance assays. Nonetheless, this type of experiment does not discriminate between freezing-tolerance and freezing-avoidance mechanisms. The purpose of this paper was to determine which of these two freezing-resistance mechanisms is responsible for freezing resistance in A. thaliana. This was achieved by comparing the thermal properties (ice-nucleation temperature and the freezing temperature) of leaves and the lethal temperature to 10, 50 and 90% of the plants (LT₁₀, LT₅₀, and LT₉₀, respectively). Two wild-type genotypes were used (Columbia and Ler) and their mutants (esk-1 and frs-1, respectively), which differ in their freezing resistance. This study’s results indicated that the mutant esk-1, described as a freezing-tolerant species showed freezing tolerance only after a cold-acclimation period. The mutant frs-1, described as freezing sensitive, presented freezing avoidance. Both wild genotypes presented LT₅₀ similar to or higher than the ice-nucleation temperature. Thus, the main freezing-resistance mechanism for A. thaliana is avoidance of freezing by supercooling. No injury of the photosynthetic apparatus was shown by measuring the maximal photochemical efficiency (Fᵥ/Fₘ) and pigments (chlorophyll and carotenoid) during cold acclimation in all genotypes. During cold acclimation, Columbia and esk-1 increased total soluble carbohydrates in leaves. esk-1 was the only genotype that presented freezing tolerance after cold acclimation. This feature could be related to an increase in sugar accumulation in the apoplast.

Key words: Apoplastic fluid, Arabidopsis thaliana, chlorophyll fluorescence, cold acclimation, esk-1, freezing avoidance, freezing tolerance, frs-1, sucrose, supercooling, total soluble sugars.

Introduction

Freezing temperature is a major limiting factor of growth and distribution of plants in many areas of the world. Tropical plants are usually sensitive to both chilling and freezing temperatures (Levitt, 1980). Plants growing in temperate regions have evolved several strategies to survive freezing. They can avoid or tolerate freezing. In the first case, tissue freezing can be delayed or prevented. In the second case, ice forms in the extracellular spaces, without damaging cellular structures (Levitt, 1980; Sakai and Larcher, 1987; Alberdi and Corcuera, 1991).

Supercooling, which is the capacity of cell fluids to be cooled down to a temperature lower than the freezing point without immediate freezing, is a frequent avoidance mechanism against freezing injury in plants from habitats subjected to frosts. Supercooling is especially important when frost occurs during periods of high metabolic or developmental activity (Sakai and Larcher, 1987; Larcher, 2003). Freezing tolerance is usually observed in tropical environments at high latitude where subzero temperatures can occur any night of the year, or in zones with a high seasonal climatic variation (Bravo et al., 2001; Larcher, 2003).

Many plants increase their freezing resistance when exposed to low non-freezing temperature (below 10 °C),

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Abbreviations: FT, freezing temperature; LT, lethal temperature; IN, ice nucleation; MDH, malate dehydrogenase; SPS, sucrose phosphate synthase; TSS, total soluble sugars.

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a process known as cold acclimation or cold hardening (Levitt, 1980; Larcher, 2003). *Arabidopsis thaliana* (L.) Heynh., the best suited model plant for genetic, molecular, and physiological studies, has been described as a freezing-tolerant species (Gilmour et al., 1988; Thomashow, 1994, 1998; Knight et al., 1999). In general, freezing resistance in this species increases from $-3^\circ C$ (lethal temperature 50%, $LT_{50}$) to an $LT_{50}$ of about $-6^\circ C$ after 24 h of cold acclimation at $4^\circ C$. Lower $LT_{50}$ values ($-8^\circ C$ to $-10^\circ C$) were obtained after 8–9 d of cold acclimation (Gilmour et al., 1988). The freezing resistance of 26 mutants of *Arabidopsis* ranged from $-6.8^\circ C$ to $-10.6^\circ C$ in the absence of acclimation. One of these *Arabidopsis* mutants has been described as constitutively freezing tolerant (*eskimol: esk-I*) (Xin and Browse, 1998; Thomashow, 2001).

Many biochemical changes occur in plants during cold acclimation and are likely to have roles in freezing resistance. It is documented that the increased levels of proline and sucrose during cold acclimation are effective cryoprotective mechanisms in *Arabidopsis* (McKown et al., 1996; Wanner and Juntila, 1999) and other plants (Guy et al., 1992; Alberdi and Corcuerá, 1991; Livingston III, 1996). Soluble carbohydrates and free proline may be involved in freezing point depression of cell sap, prevention of plasmolysis during cell dehydration caused by freezing, and membranes stabilization (Strauss and Hauser, 1986; Santarius, 1992). Additional changes associated with an increased stability of the photosynthetic apparatus preventing low temperature-induced photoinhibition have been reported during cold acclimation (Sane et al., 2003). Non-acclimated *esk-I* accumulated some compatible solutes like proline (Xin and Browse, 1998). Artus and Thomashow (1992) screened mutants of *Arabidopsis* deficient in freezing resistance. Mutants sensitive to freezing (*sfr*) in this plant species were also reported by Warren et al. (1996) and Knight et al. (1999). One of these, *sfr-4* showed depressed levels of sugars in leaves (McKown et al., 1996; Uemura et al., 2003).

Freezing tolerance implies a capacity to tolerate ice formation in the intracellular spaces. As explained above, *A. thaliana* (Columbia) is referred as a freezing-tolerant species (Gilmour et al., 1988; Thomashow, 1994, 1999; Warren et al., 1996; Xin and Browse, 1998; Knight et al., 1999). Their experiments and conclusions are based on $LT_{50}$ (temperature at which 50% lethality occurred) determinations by exposure to freezing temperature. This type of experiment only shows the temperature at which the plant or tissues die, ignoring at what temperature ice was formed in the tissues. To conclude that a plant is freezing tolerant, it must be shown that is able to survive ice formation (Levitt, 1980; Sakai and Larcher, 1987; Larcher, 2003). This is achieved by a combination of thermal analyses and lethal temperature ($LT$) determinations. Thermal analysis permits the determination of the supercooling capacity and ice nucleation and freezing temperatures of tissues. To the best of our knowledge, these experiments have not been done in *Arabidopsis thaliana*. Thus, it is not known whether its freezing resistance is due to a capacity to tolerate freezing or to avoid ice formation by supercooling. The main purpose of this paper is to determine whether *A. thaliana*, ecotypes Columbia, Landsberg erecta (Ler) and their mutants, *esk-I* (freezing resistant) and *frs-I* (freezing sensitive), respectively, are freezing tolerant or avoid freezing by supercooling under cold-acclimated and non-acclimated conditions. In addition, changes in freezing behaviour of leaves of *A. thaliana* were correlated with the accumulation of sugars in the apoplast.

### Materials and methods

#### Plant material and growth conditions

Wild types of *Arabidopsis thaliana* (L.) Heynh. Columbia WT-2 and *Arabidopsis thaliana Landsberg erecta* (Ler) seeds were obtained from Lehle Seeds (Round Rock, TX, USA). Their mutants *eskimo-I* (*esk-I*) (freezing tolerant) and *frs-I* (freezing sensitive), respectively, were kindly provided by Drs Zhanguo Xin and John Browse (Institute of Biological Chemistry, Washington State University, Pullman, USA) and Julio Salinas (INIA, Madrid, Spain), respectively. Both mutants have been described elsewhere (Xin and Browse, 1998; Llorente et al., 2000). Surface-sterilized seeds were sown in plastic pots in a 1:1 mixture of vermiculite:soil. Pots were maintained at 21–23 °C with 16/8 h light/dark day length and a photosynthetic photon flux density (PPFD) of about 150 μmol photons m$^{-2}$ s$^{-1}$. The light source consisted of cool-white fluorescent tubes F40CW (General Electric, Charlotte, NC, USA). Plants were watered twice a week and fertilized with Phostrogen® (Solaris, Buckinghamshire, UK) using 0.12 g l$^{-1}$, once every 2 weeks. Relative humidity was around 60–70%. These plants were referred as non-acclimated or controls.

#### Cold acclimation treatment

For cold acclimation, plants at the vegetative stage (basal or rosette leaves with approximately 10–15 leaves, about 2-week-old plants) were used. Plants were transferred to a growth chamber set at 4 °C with the other conditions the same as those mentioned for controls at different times. Cold acclimation was performed only for 1, 7, 14, and 21 d. At each time of cold acclimation, whole plants were used to determine freezing resistance ($LT_{50}$) and leaves from the first or second basal node of the rosette were removed for determinations of thermal analyses, carbohydrates, and pigments (chlorophyll and carotenoids). Plant material was collected 2 h after the beginning of the photoperiod.

#### Thermal analysis

Leaves (first or second basal node of the rosette) from five different plants were removed and attached to a thermocouple (Gauge 30 Copper-constantan thermocouples; Cole Palmer Instruments Co., Vernon Hills, Illinois, USA), and immediately enclosed in small, tightly closed cryotubes to avoid changes in tissue water content. Temperature was continuously monitored (1 measurement s$^{-1}$) with a ACjr data acquisition board connected to a multi-channel temperature terminal panel (Strawberry Tree Inc. Sunnyvale, California, USA). The tubes were placed in a cryostat and the temperature was lowered from 0 °C to $-17^\circ C$ at a rate of approximately 2 °C h$^{-1}$ (Bravo et al., 2001). Temperature records were made and the freezing exotherms were analysed. The temperature at the initiation of the exotherm corresponds to the ice nucleation temperature, while the
highest point of the exotherm represents the freezing temperature of water in the apoplast (including symplastic water driven outwards by the water potential difference caused by apoplastic ice formation) (Larcher, 2003). Thermal analyses experiments were executed without adding an ice nucleating agent. When AgI was added, no second low temperature exotherm was observed in these plants down to –15 °C.

Freezing resistance

This was evaluated as survival of plants after frost at various temperatures. Whole plants in their pots (30 for each frost temperature) were subjected for 2 h at temperatures between 0 °C and –15 °C in a sealed metallic cell submerged in a cryostat. At each temperature, plants were removed and thawed overnight at 4 °C and then introduced into a growth chamber set at 23 °C for survival evaluation by monitoring the fate of the frozen leaves and the progress of new leaf growth over the next 10 d. Plants that produced at least one new leaf were considered as survivors. LT was determined essentially as described by Neuner and Bannister (1995), which consisted of plotting the percentage of survival against frost treatment temperature. Temperature that kills 10% (LT10), 50% (LT50), and 90% (LT90) of the exposed plants were obtained from the curve and used as indexes of incipient, medium, and severe damage, respectively.

Freezing resistance mechanism

From the comparison of the temperatures that cause injury or significantly depress plant survival (LT) and the ice-nucleating temperatures, the mechanism of freezing resistance can be established (Levitt, 1980; Sakai and Larcher, 1987; Squeo et al., 1991). If the ice nucleation temperature is lower than the temperature that causes incipient damage (LT10), the freezing-resistance mechanism is freezing avoidance. Conversely, when the ice-nucleating temperature is significantly higher than the damaging temperature (LT10), the tissue is able to tolerate ice formation.

Apoplastic fluid extraction

Extracts were undertaken as described by Livingston and Hanson (1998), 4 g of non-acclimated (control) and cold-acclimated fresh leaves from first or second node of the rosette of Arabidopsis thaliana were cut in pieces of around 0.5 cm and submerged in distilled water to avoid dessication, and then dried with filter paper for fluid extraction. For extraction, the plant material was placed in the bottom 3 cm of a 50 ml syringe barrel. A 4 ml HPLC insert vial was placed on the syringe tip to collect apoplastic solution. The syringe barrel containing the leaf tissue with the attached 4 ml vial was placed in a 50 ml centrifuge tube and centrifuged at 2000 g for 1 h at 4 °C. To evaluate contamination of the apoplastic fluid, the activity of MDH (malate dehydrogenase), as a cytoplasmic marker, was determined by the NADH oxidation oxalacetate-dependent reaction, according to Duke et al. (1975). The extinction was spectrophotometrically measured in a Shimadzu UV-1203 spectrophotometer at 310 nm. Levels of cytoplasmic contamination in the apoplastic fluid were 2%, which is within the range reported by Husted and Schjoerring (1995).

Soluble carbohydrates determinations

They were measured in leaf tissue and apoplastic fluid. Soluble carbohydrate was extracted from fresh leaf tissue (0.1 g) in 86% v/v ethanol with overnight agitation and then centrifuged at 12 000 g for 10 min. The supernatant was depigmented with chloroform in a mixture 1:3 v/v (extract:chloroform). The supernatant was freeze-dried overnight. The dry residue was resuspended in 500 μl of methanol. Total soluble sugars (TSS) content was determined spectrophotometrically by the Resorcinol method (Roe, 1934), at 520 nm, using sucrose as standard.

Sucrose content was determined by high performance thin layer chromatography (HPTLC) using a silica gel plate (60 F254, Merck) pretreated with 0.1 M methanolic potassium phosphate solution. After loading and running the samples, the plates were developed five times in aceitonitrile:water (85:15 v/v). For calibration, a standard solution containing 0.1 mg ml⁻¹ of sucrose, glucose, and fructose was used. The position of individual carbohydrates was visualized by spraying with diphenylamine/aniline reagent and then heated to 105 °C (Lee et al., 1979). The image was scanned at 520 nm and the amounts were expressed in ng of carbohydrate per area. These values were then converted to mg sugar g⁻¹ dry weight.

Chlorophyll and carotenoid determinations

Chlorophyll and carotenoids were extracted with 80% acetone at 0 °C and spectrophotometrically determined at 663, 646, and 470 nm according to Lichtenthaler and Wellburn (1983).

Dry weight determinations

Samples of leaves from the first or second node of the rosette were removed from plants at the stage of vegetative phase. This tissue was weighed and then dried at 60 °C for about 2 weeks until constant weight was obtained. Similar tissue was used at the same time to determine sugars and pigments content. Water content was determined and values of sugars were expressed on dry weight bases. Pigment contents were expressed on fresh weight bases.

Fluorescence measurements

Room temperature fluorescence signals were generated by a pulse-amplitude modulated fluorometer (FMS 1, Hansatech Instruments Ltd., U.K.). Fully developed leaves (attached to the plant) from the first or second internodes of cold-acclimated and non-acclimated plants were dark adapted for 30 min (to obtain open centres) with leaf-clips provided by a mobile shutter plate. Then the fibre-optic and its adapter were fixed to a ring located over the clip at about 10 mm from the sample and the different light pulses (see below) were applied following the standard routines programmed within the machine. Signal recording and calculations were performed on a personal computer using the data analyses and control software (Hansatech, Instruments Ltd. UK). Minimal fluorescence Fmin (terminology according to Van Kooten and Snel, 1990) was determined applying a weak modulated light (0.4 μmol m⁻² s⁻¹) and maximal fluorescence was induced by a short pulse (0.8 s) of more than 9000 μmol m⁻² s⁻¹. The following fluorescence parameters were measured as indicators of the functionality of the photosynthetic apparatus: the potential maximal PSII quantum yield (Fv/Fm=variable fluorescence/maximal fluorescence), where Fv=Fm−Fmin (maximal fluorescence−minimal fluorescence).

Statistics

The reported values correspond to the means of three different experiments, with three replicates each. Differences between the values (P <0.05) were determined by a two-way ANOVA. A Tukey test was used to identify those values with significant differences. Sigma Stat 2.0 software (SPSS® Inc., Chicago, IL, USA) was used for both analyses.

Results

Freezing resistance

Ice nucleation temperature (IN), the temperature at which ice crystals are initiated, corresponds to the temperature at the beginning of the freezing exotherm. The highest
temperature reached after all apoplastic available water has frozen is the freezing temperature of the apoplastic fluid (FT) (Fig. 1). These results showed that all *A. thaliana* genotypes studied decrease their IN and FT after 21 d of cold acclimation (Fig. 1). IN of cold-acclimated leaves of *A. thaliana* genotypes fluctuated from −6 °C to −11 °C, corresponding to the lowest temperature for *esk-1* (Fig. 1B). The non-acclimated wild genotypes (Columbia and Ler) exhibited similar values of freezing temperature (FT) (−1.3 °C and −1.6 °C, respectively). The mutants *esk-1* and *frs-1* presented lower FT values than their wild types (−2.7 °C and −2.9 °C, respectively). During cold acclimation, the genotype described as freezing tolerant exhibited the lowest FT value after 21 d of cold acclimation (−6.8 °C). This variation with respect to the non-acclimated control represents twice the variation observed in its wild type Columbia. The genotype described as freezing sensitive *frs-1* showed a small variation in FT value during cold acclimation (at 21 d it was −7.8 °C). Its wild genotype Ler exhibited a similar variation with an FT of −7.9 °C at 21 d (Fig. 1C). These results suggest two interesting phenomena: first that supercooling capacity can increase about 4 °C in the *esk-1* mutant after cold acclimation and only about 1–1.5 °C in the other *A. thaliana* genotypes studied. Second, a higher solute accumulation in *esk-1* mutant during cold acclimation could be predicted from the high freezing point depression caused by cold acclimation in this mutant.

The range of temperature associated with 10–90% damage was determined in the *A. thaliana* genotypes.

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**Fig. 1.** Thermal analyses performed in non-acclimated and cold-acclimated genotypes of *A. thaliana*. Leaves of *A. thaliana* cv. Columbia (A), mutant *esk-1* (B), cv. Ler (C), and its mutant *frs-1* (D) were attached to fine thermocouples and cooled down in a programmable cryostat at about 2 °C h⁻¹. Leaf temperatures were recorded twice per second and freezing exotherms were studied to determine ice-nucleation temperatures (IN) and freezing temperatures (FT). Single representative temperature recordings are shown for each treatment. Temperatures (IN and FT) in the boxes represent the average of five independent assays.
studied (Fig. 2). \(LT_{10}\) and \(LT_{90}\) oscillated closely and almost symmetrically around \(LT_{50}\) with a variation ranging from 1–2 °C in most of the genotypes studied. The exception was the ecotype Ler which exhibited in the non-acclimated state and also after 1 d of cold acclimation a high \(LT_{10}\) (about −1.5°C) compared with its \(LT_{50}\), which oscillated around −7.5 °C and \(LT_{90}\) around −8.5 °C (Fig. 2C). Columbia and its mutant (esk-1) exhibited a similar \(LT_{50}\) in the non-acclimated state (Fig. 2A, B). The genotype that presented the lowest \(LT_{50}\) in the non-acclimated state was Ler (\(LT_{50}\) −7.2±0.09 °C, Fig. 2C). Its mutant, described as freezing sensitive (frs-1), showed the highest \(LT_{50}\) value (\(LT_{50}\) −4.6 °C, Fig. 2D) in the non-acclimated state. \(LT_{50}\) decreased significantly (\(P \leq 0.05\)) in Columbia, esk-1, and frs-1 under cold acclimation, reaching the lowest values in esk-1 after 21 d (\(LT_{50}\)−12.2 °C, Fig. 2B). This contrasted with Ler, which maintained its \(LT_{50}\) around −7.5 °C (Fig. 2C). Cold acclimation only caused a significant reduction in \(LT_{10}\) from −1.5 °C to −6.0 °C (Fig. 2C).

Freezing-resistance mechanisms can be established, contrasting the IN temperature with temperatures at which plants are killed or damaged (Fig. 2). With the exception of esk-1 after 7 d of cold acclimation, in which plants injured by the frost treatment were observed at a lower temperature than IN, most of the genotypes at most of the acclimation stages were unable to tolerate ice formation without a significant number of plants being killed (Fig. 2). These results indicate that the main mechanism for surviving frost in \(A.\) \textit{thaliana} is avoidance of freezing by moderate supercooling. Nonetheless, esk-1 is able to tolerate freezing after 7 d of cold acclimation (Fig. 2B).

**Fig. 2.** Freezing resistance of \(A.\) \textit{thaliana} cv. Columbia (A) and its mutant esk-1 (B) and cv. Ler (C) and its mutant frs-1 (D). Plants were subjected to cold acclimation (4 °C), 16/8 h light/dark day length and a photon flux density of 150 \(\mu\)mol m\(^{-2}\) s\(^{-1}\), for different times. 0=start of experiment with non-acclimated plants (23 °C) and the same conditions as above. Bars represent the range of lethal temperatures (\(LT\)) that caused 10–90% plant mortality, \(LT_{10}\) (upper edge of the white portion of each bar) \(LT_{50}\) (division line between white and grey portion of each bar) and \(LT_{90}\) (lower edge of the grey portion of each bar). Values correspond to the average of three \(LT\) determinations using 10 plants per temperature treatment in each \(LT\) determination (\(n=30 \pm SE\)). Thermal analyses were performed using five plants from each acclimation treatment, ice-nucleation temperature (IN) values are the average of \(n=5 \pm SE\).
Accumulation of sugars during cold acclimation

Total soluble sugar accumulation (TSS) was evident within 7 d of cold acclimation \((P \leq 0.05)\) in leaves of three genotypes (Columbia, esk-1, and frs-1) (Fig. 3). TSS increased around 3-fold in Columbia and around 2-fold in frs-1. This genotype exhibited no further accumulation of TSS after 7 d of cold acclimation while Columbia and esk-1 exhibited a significant increase from 7–21 d of cold acclimation. The highest TSS accumulation was observed in esk-1 at 21 d \((63.3 \pm 0.6 \text{ mg g}^{-1} \text{ DW})\). No significant differences in sugar content were observed in Ler during cold acclimation (Fig. 3). Sucrose, glucose, and fructose increased during cold acclimation. However, the content of most of them tended to decrease towards the third week of cold acclimation, except glucose, which was the most abundant sugar in leaves. esk-1 exhibited a sustained accumulation at 21 d of cold acclimation reaching 23.1 \pm 1.7 \text{ mg g}^{-1} \text{ DW} (Fig. 4). Sucrose is the main sugar accumulating in leaf apoplastic fluid during cold acclimation in esk-1, and to a lesser extent, Columbia, but not in the other two genotypes. esk-1 presented an increase of about 4-fold in sucrose concentration at the end of cold acclimation with respect to zero time. Although esk-1 was also the genotype that presented the highest values of glucose and fructose concentration in apoplastic fluid, the difference between it and Columbia and frs-1 is not statistically significant (Table 1).

Fluorescence measurements and pigments determinations

As an indicator of the physiological status of the plants during cold acclimation, the state of the photosynthetic apparatus was evaluated by determining a fluorescence parameter and pigments. Maximum photochemical efficiency \((F_{v}/F_{m}, \text{ variable fluorescence over maximal fluorescence})\) remained around normal values during cold acclimation. In the mutants genotypes, \(F_{v}/F_{m}\) values decreased slightly to 0.79 after 7 d of cold acclimation, recovering progressively to the initial values after 21 d. The two wild-type genotypes showed a similar decrease (around 0.79), but without recovering by the end of the cold acclimation period. These normal values show that the photosynthetic apparatus was functional in all genotypes during the cold treatment (Fig. 5A). This is consistent with a slight increase in total chlorophyll content observed in both mutants after 2 weeks of cold acclimation (Fig. 5B). Carotenoid contents were also slightly higher in both mutants \((esk-1, 0.25–0.29 \text{ mg g}^{-1} \text{ FW}; frs-1, 0.19–0.42 \text{ mg g}^{-1} \text{ FW})\) compared with their respective genetic...
Discussion

The combination of thermal analysis and LT has been widely used to describe frost resistance strategies in plants (Neuner and Bannister, 1995; Pearce, 2001). Determinations of IN temperature are essential to establish if plant tissues can withstand ice crystals without significant injury (freezing tolerance). The description of Arabidopsis thaliana ecotype Columbia as a freezing-tolerant species (Thomashow, 1990, 1999; Lang et al., 1994; Warren et al., 1996; Gilmour et al., 1998, 2000) is not supported by this study's results. The ecotype Columbia is not a suitable genotype to study freezing-tolerance mechanisms because, when the ice is formed in the extracellular spaces (at about -2°C), nearly 50% of the plants have died in the non-acclimated state and a significant number of plants (more than 10%) have been seriously injured at higher temperatures even in cold backgrounds (Columbia, 0.19–0.22 mg g⁻¹ FW; Ler, 0.16–0.17 mg g⁻¹ FW).

Table 1. Apoplastic sugar concentration of four genotypes of Arabidopsis thaliana

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<th>Time of acclimation</th>
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<th>Fruc (µg ml⁻¹)</th>
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Fig. 5. Changes in Fv/Fm (A) and total chlorophyll (B) of non-acclimated (23°C, time zero) and cold-acclimated (4°C) plants of Arabidopsis thaliana. Chlorophyll fluorescence and content were analysed in plants of cv. Columbia, Landsberg erecta (Ler) and their respective mutants esk-1 and frs-1, at different times of cold acclimation.
acclimated state with respect to the non-acclimated wild. The constitutively freezing-tolerant mutant (esk-1) (Xin and Browse, 1998) was used for comparison in this work. Nevertheless, thermal analyses demonstrated that this mutant can tolerate freezing only after 7 d of cold acclimation. In the non-acclimated state it avoids freezing. That means that this genotype is not constitutively freezing tolerant. The Ler mutant frs-1, described as freezing sensitive (Llorentes et al., 2000), has an IN temperature equal to its LT50 even after 21 d of cold acclimation. Furthermore, 10% of plants were killed at about 2 °C higher than the beginning of IN, indicating that this genotype is seriously injured and at least 10–49% of the plants have died prior to ice formation in its extracellular spaces. Since this mutant, cannot withstand apoplastic dehydration. Sugar accumulation at cold temperature has been shown to be essential for cold acclimation. Steponkus (1984) suggested that the accumulation of sugars (especially sucrose) in the apoplast is essential for increasing freezing resistance. It is also possible that sucrose participates in apoplastic cryoprotection non-colligatively in cold-acclimated esk-1 (Strauss and Hauser, 1986). When chilling temperatures reduce the rate of CO2 fixation, thus lowering the overall rate of photosynthesis, light may exceed the amount that can be utilized in photosynthetic electron transport (Demmig-Adams and Adams, 1992). If this excess of energy is not dissipated, the photosynthetic apparatus can be damaged, resulting in a decrease of photosystem II activity (Huner et al., 1993). The behaviour of this photosystem within chilling conditions was studied in all genotypes. Fluorescence
measurements in these genotypes showed that the maximal photochemical quantum yield ($F_v/F_m$) remained under the normal values of healthy plants (Krause and Weiss, 1991), suggesting that no significant perturbation of the photosynthetic apparatus during cold acclimation at the photon fluxes densities used in this work occurred. The maximal photochemical quantum yield ($F_v/F_m$) is used as a good indicator of the photosynthetic apparatus and plant status (Demmig-Adams and Adams, 1992). It has been suggested that values of $F_v/F_m$ close to 0.80 are typical for non-stressed plants (Krause and Weis, 1991; Larcher, 2003). Similarly to the maintained $F_v/F_m$ values during cold acclimation, chlorophyll and carotenoid contents hardly changed at all during cold exposure, indicating that the photosynthetic apparatus remained without significant alteration. Carotenoid increases are frequently associated with mechanisms that protect against high irradiance and low temperatures, avoiding photo-inhibition (Demmig-Adams, 1990).

In summary, these results indicate for the first time that Arabidopsis thaliana avoids freezing by supercooling. The mutant esk-1 showed freezing tolerance only after cold acclimation, which appears to be related to sugar accumulation in the leaves and apoplastic fluid.

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