Water Content during Abscisic Acid Induced Freezing Tolerance in Bromegrass Cells[1]

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ABSTRACT

Changes in water content and dry weight were determined in control cells and those induced to cold harden in response to abscisic acid (ABA) treatment (7.5×10^{-5} molar). Bromegrass (*Bromus inermis* Leyss cv Manchar) cells grown in suspension culture at room temperature (23°C) for 7 days acclimated to −28°C (LT_{50}) when treated with ABA, or to −5°C when untreated. ABA significantly reduced cell growth rates at 5 and 7 days after treatment. Growth reduction was due to a decrease in cell number rather than cell size. When the cell water content was expressed as percent water (percent H_2O) or as grams water per gram dry weight (gram H_2O/gram dry weight [g DW]), the water content of hardy, ABA-treated cells decreased from 85% to 77% or from 6.4 to 3.3 g H_2O/g DW in 7 days. Control cell water content remained static at approximately 87% and 7.5 g H_2O/g DW. However, cell water content, expressed as milligrams water per million cells (milligram $H_2O/10^6$ cells), did not differ in ABA-treated or control cells. The dry matter content of ABA-treated cells, expressed as milligram $DW/10^6$ cells increased to 3.3 milligram/10^6 cells in 7 days, whereas the dry weight of the control cells remained between 1.4 to 2.1 milligrams/10^6 cells. The osmotic potential of ABA-treated cells decreased by the fifth day while that of control cells increased significantly and then decreased by day 7. Elevated osmotic potentials were not associated with increased ion uptake. In contrast to much published literature, these results suggest that cell water content does not decrease in ABA-treated cells during the induction of freezing tolerance, rather the dry matter mass per cell increased. Cell water content may be more accurately expressed as a function of cell number when accompanying changes to dry cell matter occur.

During cold acclimation plants become resistant to freezing stress. Freezing injury is influenced by the site, rate, and extent of ice formation (11). Thus, a clear knowledge of the water status within the plant is important to understanding how plants acclimate. Many studies have shown that the water content of tissues declines as plants acclimate to low temperatures (3, 4, 10, 17). Also, plants accumulate dry matter under cold acclimating conditions (4, 5, 7). Despite the possibility that both water content and dry matter are changing during acclimation, technical difficulties particularly in whole plant measurements result in expression of water content that is not independent of dry matter accumulation: % H_2O:

([FW^3 − DW]/FW) × 100% (3, 10, 17); or g H_2O/g DW: (FW − DW)/DW (6, 10). Erroneous interpretations can occur when two changing variables are presented as dependent functions.

Although measurements of RWC and water deficit have been developed to account for this problem (17), changes associated with cold acclimation may alter the ability of cells to take up water to achieve full turgor. Confounding effects may result if full turgor is not attained.

ABA-induction of cold acclimation in cell cultures provides a useful tool for examining water content during frost acclimation: (a) ABA can substitute for low temperature in inducing cold acclimation and thus avoid plant responses to cold that are not related to acclimation (2, 8, 12–16); (b) ABA induction of hardiness is rapid and occurs in days rather than the weeks required for low temperature induced acclimation (2, 7, 8); (c) cell cultures offer a relatively homogeneous system where barriers to hormone uptake are removed; and (d) cell numbers can be readily estimated so data can be expressed as a function of cell number.

This study compared three methods of expressing cell water content in bromegrass suspension cultures and characterized the dry matter status and growth of these cells during ABA-induced cold acclimation.

MATERIALS AND METHODS

Cell Culture

A bromegrass (*Bromus inermis*) cell suspension culture (BG-970) was grown in a modified Eriksson's medium containing 0.5 mg/L 2,4-D (2). To reduce variability in all subsequent subcultures, similar inoculum sizes (1.75 mL packed cell volume following 20 min of settling in a graduated centrifuge tube) were transferred every 2 weeks into 50 mL media in 250 mL Erlenmeyer flasks and maintained on an Eberbach reciprocating shaker at 110 rpm at 23°C in the dark. (±)-ABA (Calbiochem) at 7.5×10^{-5} M was added to the medium and autoclaved at 121°C for 15 min. Cells were sampled and examined at 1, 3, 5, and 7 d after ABA treatment.

Freeze Test

Cells were harvested and then washed with 200 mL (about 20 volumes) dH_2O to remove the possible cryoprotective

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[3] Abbreviations: FW, fresh weight; DW, dry weight; RWC, relative water content; LT_{50}, lethal temperature at which 50% of the cells were killed; PCV, packed cell volume; TTC, 2,3,5-triphenyltetrazolium chloride.
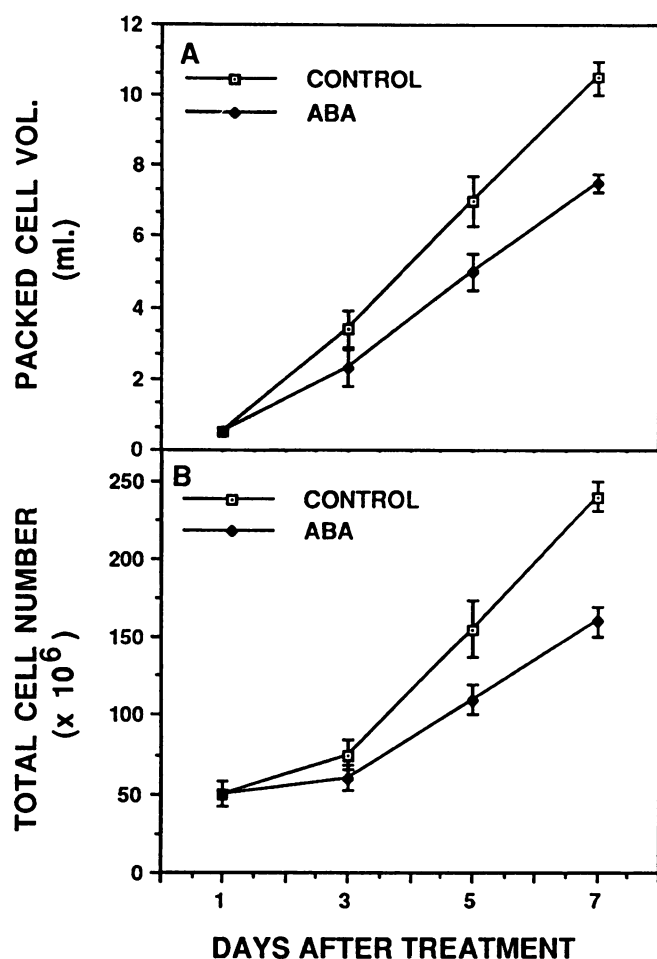
Figure 1. Growth of bromegrass cell suspension cultures with and without 7.5 × 10⁻⁵ m ABA treatment. A, Total packed cell volume (mL). Values represent means ± SD of five separate experiments. B, Total cell numbers in the packed cell volumes. Cell number values represent the means ± SD of two experiments, three replications per sampling date for each treatment and each experiment.

effects of the medium during freeze tests. Cells were frozen in 10 × 75 mm glass culture tubes. Preliminary tests indicated that excess extracellular water within cell clumps significantly affects freeze test results. To standardize the hydration level prior to freeze tests, excess extracellular water was removed by centrifuging cells in plastic 5 mL syringe barrels plugged with 2.5 cm² pieces of double-layered Kimwipe. Syringe barrels containing cells were suspended in 50 mL round bottom centrifuge tubes and spun at 2000 rpm (313g) at 4°C in a Beckman JA-20 rotor in a model J2-21M centrifuge. Cells were spun until no further extracellular water was removed (10 min).

For the freeze test, at least 200 mg cells FW were placed in culture tubes, equilibrated in a Neslab LT-50 low temperature bath at −1°C for 30 min and nucleated with ice crystals. Nucleated cells were held overnight at −1°C. The next day the temperature was lowered at a rate of 2°C/h to −10°C and at a rate of 3°C/h thereafter. At each freezing temperature, cells were sampled and placed at 4°C, for 24 h.

Viability Test

Cell samples (50 mg FW) were weighed into shell vials and cell viability was estimated by the TTC reduction method (19). All samples were sealed with a serum stopper and incubated in the dark for 24 h. TTC solution was then removed with a Pasteur pipet placed flat on the bottom of the shell vial. The reduced TTC was extracted with 3 mL of 95% ethanol per vial for at least 3 d. After extraction, 300 μL samples were transferred to ELISA plates and absorbance read at 495 nm with a Biotek Microplate Autoreader EL 309. Three samples were analyzed for each temperature and treatment. Each experiment was repeated six times. Viability was expressed as LT_{50}, the temperature at which 50% of the cells were killed as determined by the TTC test.

Cell Number Determination

Cells occurring in clumps were separated by suspending samples of 0.2 mL of packed cell volume (PCV) in 3 mL 2 N HCl solution and incubated at 80°C for 45 to 90 min followed by vigorous vortexing. Treatment temperatures and times were selected to minimize cell lysis. ABA treated cells required longer incubation times (90 min) to disperse than control (45 min) cells. Cells were counted on a hemacytometer. Three replications were analyzed for each treatment and each experiment was performed twice.

Fresh Weight/Dry Weight

Fresh samples of 0.7 to 1.0 mL PCV were weighed after centrifugation for 5 min at 2000 rpm as described for the freeze tests. Samples were weighed after 24 h of drying at 80°C. No weight loss was observed after 24 h of drying. Percent water was calculated as: ([FW-DW]/FW) × 100%. Grams of water/g DW was calculated as: (FW-DW)/DW.
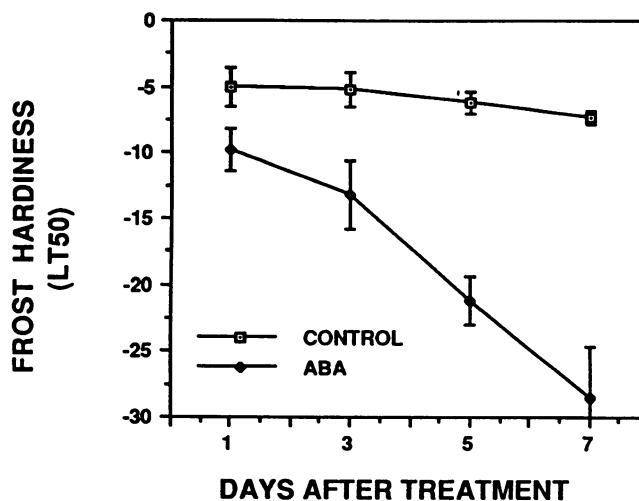

Figure 2. Development of freezing resistance (LT_{50}) in bromegrass cells with and without 7.5 × 10⁻⁵ m ABA treatment, estimated by TTC reduction. Means ± SD represent results of six separate experiments.
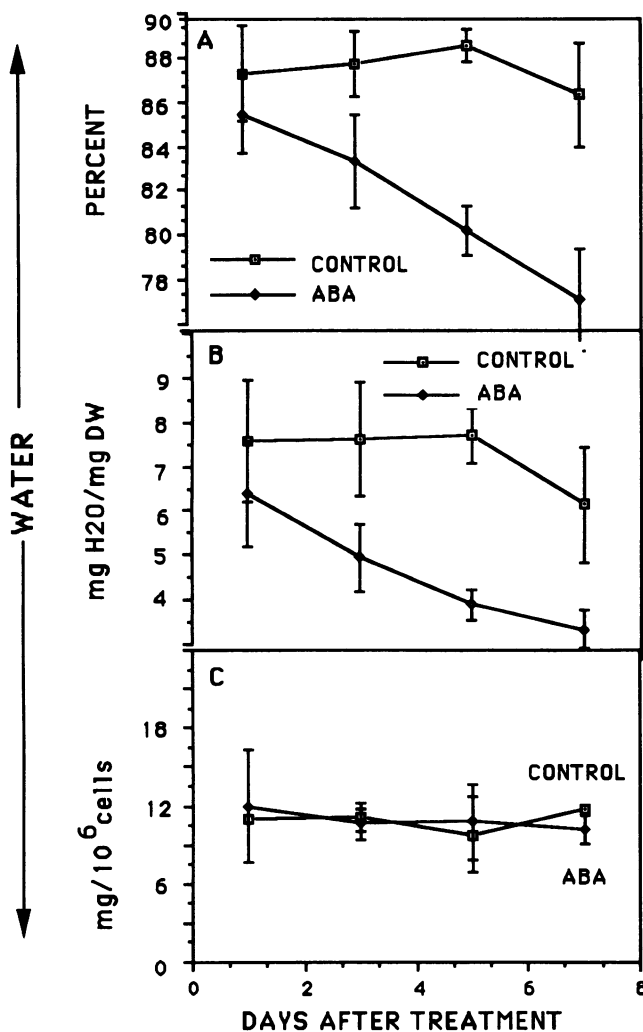
volume, by d 5 and 7 (Fig. 1A). Because there was no significant difference in cell number per mL packed cell volume (Fig. 1B), the growth reduction was apparently not due to a reduction in cell size, but rather to a decrease in cell number.

ABA (7.5×10^{-5} M) treatment induced significant frost hardiness in bromegrass cells compared to controls, after 1, 3 , 5, and 7 d of treatment (Fig. 2). Control cell hardiness remained constant with an LT_{50} of about −5°C, while ABA induced hardiness to −10°C after 1 d of treatment, and to −28°C by 7 d. Between 3 and 7 d of treatment, the rate of increase induced by ABA was 3.8°C/d.

Water Status

When water content was expressed as a function of FW, the water content of control cells remained unchanged throughout the 7-d study period at 87%; and as function of DW at 7.5 g H_2O/g DW. In contrast, the water content of ABA-treated cells decreased significantly during the treatment period, from 86 to 77% (Fig. 3A), and from 6.4 to 3.3 g H_2O/ g DW (Fig. 3B) when calculations were based on fresh and dry weights, respectively.

When water content was expressed on a per cell basis, however, treatments did not differ (Fig. 3C). Both control and ABA cells maintained an average water content of 11 mg/10^6 cells throughout the seven day monitoring period.

Dry Matter Accumulation

Control cell DW remained between 1.4 and 2.1 mg/10^6 cells. The DW of ABA-treated cells increased after treatment (Fig. 4) and by 7 d the DW reached 3.3 mg/10^6 cells.
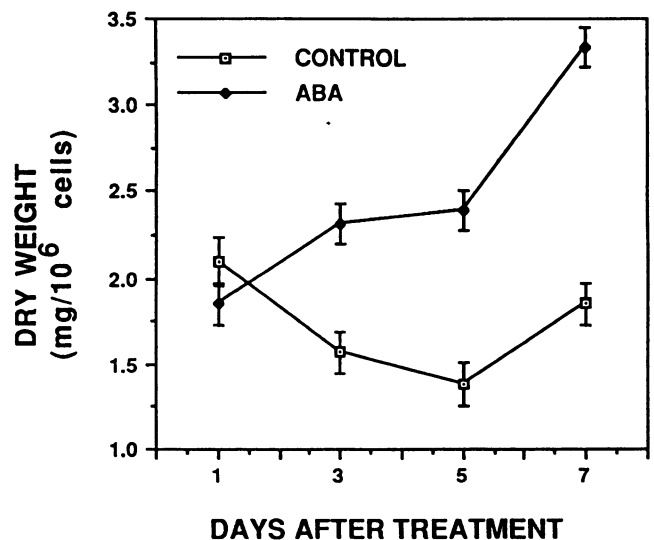
Figure 3. Cell water content of bromegrass cells with and without 7.5×10^{-5} M ABA treatment. A, Water content expressed as % H_2O: ([FW-DW]/FW) × 100%. Means ± SD represent analysis for six separate experiments. B, Water content expressed as mg H_2O/mg DW: (FW-DW)/DW. Means ± SD represent analysis for six separate experiments. C, Water content expressed as mg H_2O/10^6 cells. Cell numbers estimated for two separate experiments, three replications for each treatment in each experiment.

Osmotic Potential

Osmotic potential was measured with a Wescor osmometer after excess intercellular water was removed by centrifugation as described for the freeze tests. Sap was obtained by manually squashing cells in flexible Teflon tubes of 3 mm diameter sealed at one end (1).

RESULTS

Growth and Hardiness

Both ABA-treated cells and control cells continued to grow over the 7-d test period, but ABA-treated cells had significantly reduced rates of growth, measured as packed cell

Figure 4. Dry matter accumulation in bromegrass cells with and without 7.5×10^{-5} M ABA treatment. Means ± SD represent results of six separate experiments.
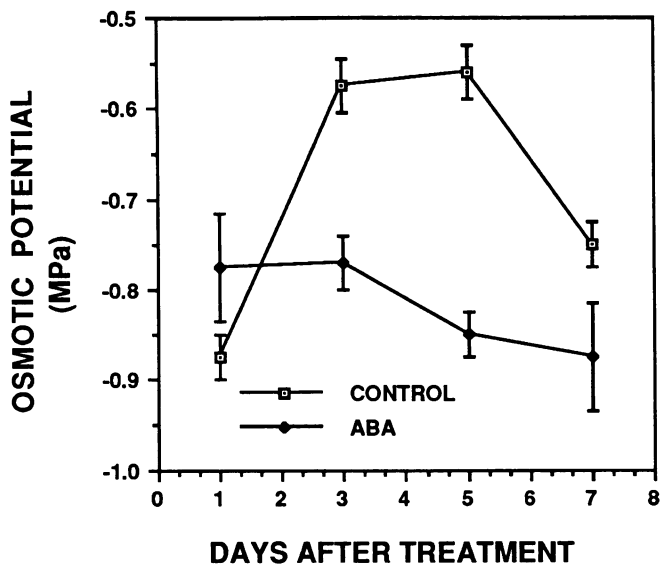
Figure 5. Cell osmotic potential with and without 7.5×10^{-5} M ABA treatment. Means ± SD represent results of two separate experiments.

Osmotic Potential

The osmotic potential of ABA-treated cells was lower than that of control cells (Fig. 5). At d 1, control cell osmotic potential (−0.89 MPa) was higher than that of ABA cells, but subsequently increased to about −0.57 MPa on d 4 and 5 before decreasing to −0.73 MPa on d 7. The osmotic potential of ABA-treated cells decreased slightly, but steadily from −0.78 MPa on d 1 to −0.87 MPa on d 7.

DISCUSSION

Results of this study show that conventional methods for expressing cell hydration, in terms of FW and DW measurements, may be inappropriate and misleading.

Two consistent plant responses during cold acclimation are dry matter accumulation (5, 7) and a decrease in H_2O content expressed as % H_2O (3, 10) or g H_2O/g DW (6, 10, 17). Water content is routinely expressed as either a fraction of the FW or DW of the sample. Since DW is often changing during acclimation, % H_2O and g H_2O/g DW are not appropriate methods to express the water status of the plants or plant cells. In this study, dry matter accumulation was 69% in ABA-treated cells and 12% in control cells. Any method that expresses two interdependent and changing variables as a function of the other will yield misleading results.

Cellular water content of control and ABA treatments did not differ when water content was expressed as a function of cell number. The observed decrease in percent water content thus reflects an increase in cell dry matter rather than a decrease in water content.

Chen and Gusta (4) found that water potentials of wheat crown cells did not change during acclimation and suggested that the reduction in crown water content might be attributed to an exclusion of water from the cell by accumulation of dry

matter and not merely a result of restricted water uptake by hardy crowns. Reaney and Gusta (13) reported that the ratio of DW to FW increased in ABA treated bromegrass cells over time. If water content remained constant, this increase could be attributable to dry matter accumulation. Robertson et al. (16) using the same bromegrass cultures, also found an increase in DW with ABA treatment, but the increase was less than in control cells, and may have reflected an ABA-induced reduction in growth. Fowler et al. (5) reported that DW was among the few factors correlating with field survival in winter wheat. We found that both dry matter accumulation and increased levels of hardiness induced by ABA exhibited similar patterns throughout the 7-d treatment period.

In terms of osmotic potential, our results showed a significant difference between control and ABA-treated cells. ABA-treated cells appear to osmotically adjust as has been observed in ABA-induced cell adaption to salt stress (9). Osmotic adjustment did not appear to be mediated by ion movement since media analysis on 15 elements did not reveal differences between controls and the ABA treatment over time, while pH measurements indicated that the ABA treatment inhibited H^+ extrusion from cells into the media (18). Total sugar accumulation paralleled the increase in osmotic potential.

The observed drop in osmotic potential of control cells (Fig. 5) has been reported in bromegrass cell cultures (14). The absence of a corresponding increase in water content (Fig. 3) implies that osmotically active agents in cells were the primary factors regulating osmotic potential. Similarly, if cell water content is constant, as this study suggests, then the increase in osmotic potential induced by ABA in 7 d cannot be accounted for by water loss. Since dry matter accumulation did occur in this study, it is possible that the increased osmotic potential was due to a decreased volume of space within the cells available for free water.

Tissues containing a larger fraction of free water are highly susceptible to freezing injury (3, 4, 10). The regulation of water content by whole plants may be different than the regulation of water content by cultured cells. However, water content does not appear to be changing during ABA-induced frost acclimation in cell cultures.

Results of this study show that: (a) water content expressed in terms of cell numbers does not vary during ABA treatment of bromegrass cell cultures, (b) changes in cell dry matter during ABA treatment introduces a variable that may preclude using % H_2O or g H_2O/g DW to express cell hydration, and (c) ABA significantly reduces growth rates of cells and reduced growth appeared to be associated with a decrease in cell number rather than with cell size.

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