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Using *Arabidopsis thaliana* as a model to study subzero acclimation in small grains ☆

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Abstract

The suitability of using *Arabidopsis* as a model plant to investigate freezing tolerance was evaluated by observing similarities to winter cereals in tissue damage following controlled freezing and determining the extent to which *Arabidopsis* undergoes subzero-acclimation. Plants were grown and frozen under controlled conditions and percent survival was evaluated by observing re-growth after freezing. Paraffin embedded sections of plants were triple stained and observed under light microscopy. Histological observations of plants taken 1 week after freezing showed damage analogous to winter cereals in the vascular tissue of roots and leaf axels but no damage to meristematic regions. The LT_{50} of non-acclimated *Arabidopsis* decreased from about −6 °C to a minimum of about −13 °C after 7 days of cold-acclimation at 3 °C. After exposing cold-acclimated plants to −3 °C for 3 days (subzero-acclimation) the LT_{50} was lowered an additional 3°C. Defining the underlying mechanisms of subzero-acclimation in *Arabidopsis* may provide an experimental platform to help understand winter hardiness in economically important crop species. However, distinctive histological differences in crown anatomy between *Arabidopsis* and winter cereals must be taken into account to avoid misleading conclusions on the nature of winter hardiness in winter cereals.

Keywords: *Arabidopsis*; Cold-acclimation; Freezing tolerance; Winter hardiness; Gene regulation; Subzero-acclimation; Nuclear pycnosis; Nucleolus; Phloem; Oat (*Avena sativa*); Winter cereals

Freezing tolerance is a crucial component of the winter survival of plants in temperate regions. The freezing tolerance of most plants increases upon exposure to low, non-freezing temperatures over a period of days or weeks. This phenomenon is known as cold-acclimation. Biochemical changes that occur during cold-acclimation include an increase in concentration of fructans, soluble sugars, cryoprotective and antifreeze proteins, amino acids, and organic acids. Physiological and genetic changes that have been documented during cold-acclimation of plants include modification of membrane lipid compositions; protein phosphorylation; calcium ion fluxes; altered expression of genes encoding lipid transfer proteins, late-embryogenesis-abundant proteins, alcohol dehydrogenase, translation elongation factor and expression of other genes of unknown function [8,9,13,21,35,36,40,44,46–49,57]. Cytological changes resulting in an altered appearance of cold-acclimated cells have also been described [31]. These cold-induced changes underscore the complex nature of winter hardiness and dictate that a causal association between metabolism, physiology and/or genetics "should only be expected when it is the limiting factor of the system" [31].

Freezing tolerance beyond that resulting from exposure to low, above-freezing temperature is conferred on plants by exposure of cold-acclimated plants to temperatures

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slightly below freezing but before freezing injury occurs. This supplementary acclimation was first documented by Trunova in 1935 (Cited by Tumanov [51]) in wheat (*Triticum aestivum*) and was called second-phase hardening; we are calling this additional acclimation "subzero-acclimation." Subzero-acclimation is clearly a crucial aspect of crop survival in temperate regions where soils commonly remain at or just below freezing for extended periods during winter. The increased hardiness attained during subzero-acclimation may provide the critical margin for overwintering survival of economically important crops such as wheat, barley, oats and rye.

Due to the extensive characterization of the *Arabidopsis thaliana* (L., Heynh.) genome, it has been used as a model plant that provides a basic understanding of genetic and physiological regulatory mechanisms in higher plants. This has been particularly true with regard to cold acclimation [6,14,16,18,27,42,47,48]. However, the suitability of *Arabidopsis* to provide an understanding of species-specific adaptations is questionable, so the purpose of this research was to compare differences between *Arabidopsis* and winter cereal crops using oat as a representative. Since *Arabidopsis* has been shown to undergo cold acclimation similar to that in winter cereals we wanted to extend the understanding of acclimation to freezing in *Arabidopsis* to include subzero acclimation. While this is not an exhaustive comparison of winter cereals with *Arabidopsis*, we hoped to provide a foundation for ongoing research in the genetics of a specific component of freezing tolerance, namely, subzero-acclimation using the vast genetic resources available from the *Arabidopsis* community.

Materials and methods

Plant material and growth conditions

Arabidopsis plants used in this study were ecotype Columbia (Col-0) . The seeds purchased from Lehle Seeds (Round Rock, TX) were sown in pots (Lehle AS-03) containing potting mixture (Lehle PM-05), wetted with nutrient solution. After three days in the dark at 6 °C under high humidity (achieved by filling the tray of pots with water and wrapping the tray with cellophane), pots were transferred to the growth chamber. The seeds were germinated and grown, at 70% relative humidity under a diurnal regime that included 10 h of illumination at 200–250 μmol quanta $m^{-2} s^{-1}$ (cool white fluorescent lamps) at 22 °C and 14 h dark at 20 °C. When the seedlings germinated (3–4 days), they were thinned to a final density of 3–4 plants per pot and growth was continued for 21 days. The plants were sub-irrigated with water and mineral nutrient solution alternatively twice a week. These plants were then subjected to cold-acclimation.

Oat plants (cv. Wintok) used in the histological observations were planted in individual tubes and were grown at 13 °C under light and nutrient conditions previously described [26].

Cold- and subzero-acclimation

The 3-week-old *Arabidopsis* plants and 5-week-old oat plants, were shifted to a chamber, at 3 °C with a photoperiod of 10 h at 235 μmol $m^{-2} s^{-1}$, supplied by a mixture of cool fluorescent (80%) and incandescent (20%) lights. Pots with plants that had been cold-acclimated were placed in plastic bags and were loosely sealed to help prevent desiccation. To achieve uniform freezing, and prevent supercooling ice shavings were placed in pots prior to the freeze test. The pots were then placed in programmable freezers at −3 °C with thermocouples in the soil to monitor temperatures. It took about 15 h for the soil to completely freeze and come to equilibrium with the freezer temperature at −3 °C. For subzero-acclimation, plants that had been cold-acclimated for 7 days were kept at −3 °C for 1, 3 and 5 days. To optimize the temperature for subzero-acclimation, 7 days cold-acclimated plants were frozen at −1, −3 and −5 °C.

Oat plants were removed from planting tubes, washed and placed in slits cut in circular sponges [26]. From this point on they were treated identically to *Arabidopsis* plants.

Freeze test and analysis of freezing tolerance

After complete freezing of all the water in the soil or sponges (in the case of oats), the temperature in the freezers was lowered to the target temperature at −1 °C h^{-1}. The final freezing temperature was maintained for 3 h and the temperature was then raised to 4 °C at 2 °C h^{-1}. After 36 h at 4 °C, the pots were put back in the growth chamber and survival was measured 1 week later. The plants were rated visually (Fig. 1) on a 0–5 scale, 0 being dead and 5 identical to unfrozen controls. Percent survival (Figs. 2 and 3) was calculated as the number of plants which survived a particular temperature divided by the total number of plants frozen.

Sample preparation for histological observations

Samples were dehydrated according to the procedures outlined by Johansen [15] using a series of ethanol and tertiary butyl alcohol solutions. Fully infiltrated tissues were embedded in Parapast Plus paraffin (Fisher Scientific, Pittsburgh, PA). Embedded samples were kept in a refrigerator until they were sectioned.

The embedded sample blocks were sectioned in a rotary microtome at a thickness of 15 μm. The resulting paraffin ribbon containing serial sections was placed on a glass slide coated with Haupt's adhesive [15], flooded with 3% formaldehyde, and transferred to a slide warmer at 41 °C. Dried slides were stored at room temperature until stained.

The slides were left overnight in dishes containing xylene to remove paraffin before sections were stained. A triple stain with Safranin, Fast Green and Orange G (Fisher Scientific, Pittsburgh, PA) was used as described by Johansen [15]. Safranin stains brilliant red in nuclei, chromosomes,