

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 LT₅₀ 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 LT₅₀ 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.

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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 $\mu\text{mol quanta m}^{-2}\text{s}^{-1}$ (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 $\mu\text{mol m}^{-2}\text{s}^{-1}$, 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 Paraplast 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,

and lignified and cutinized cell walls. Fast Green stains cellulose cell walls and also cytoplasm and mostly appears as blue to bluish-green. It also serves to remove reddish tinge of Safranin from tissues where its presence is undesirable. Orange-G was used as a counter stain to differentiate between Safranin and Fast Green but, occasionally the cytoplasm stained orange. A cover-glass was added to slides with one or two drops of Permount adhesive (Fisher Scientific, Pittsburgh, PA).

Stained sections were viewed with a Zeiss photomicroscope III to observe differences among the samples. Representative sections were viewed under a Wild Heerbrugg (Gais, Switzerland) wide angle dissecting microscope with bottom lighting. Photographs were taken with a Sony DSC707 digital camera attached to the microscope.

Results and discussion

Cold-acclimation

A significant increase in freezing tolerance was observed as early as 2 days after plants were placed in cold-acclimation (Fig. 1). Enhanced freezing tolerance has been reported in *Arabidopsis* after only 1 day of cold-acclimation, with maximum freezing tolerance reported in as little as 2 days and up to 7 days [7,19,39,53,54,57].

In spinach, maximum freezing tolerance was achieved after 1 to 3 weeks of low temperature exposure [10] whereas for the winter cereals barley, oat and rye, 3 weeks of exposure to 3 °C was needed [23]. Under our conditions, after 7 days, *Arabidopsis* plants that had been frozen at −18 °C were not significantly ($P=0.05$) hardier even when exposed to cold-acclimation conditions for 28 days (Fig. 1). In fact, plants were slightly less hardy at 28 days (non-significant difference) than they were at 21 days (Fig. 1). Several

researchers reported no increase in hardness of *Arabidopsis* after 25 days of cold-acclimation [7,19,53] while Tsenov (cited by [31]) reported that freezing tolerance was reduced in wheat when cold-acclimation was extended beyond 45 days. Fowler et al. [5] cites several studies showing that the ability to tolerate freezing is lost in winter cereals when cold acclimation is extended.

Subzero-acclimation

Despite extending the time of exposure to cold-acclimation, the level of freezing tolerance attained after 3 days at −3 °C (subzero-acclimation) was significantly higher than even 28 days of cold-acclimation (Fig. 1). In several freeze tests at −20 °C, subzero-acclimated plants were the only ones to survive (not shown). This demonstrates that the extent of hardness attained by subzero-acclimation was not duplicated by lengthening the time plants were exposed to cold-acclimation. It is possible that desiccation-induced acclimation, which undoubtedly occurred as water froze in the plant, contributed to the additional freezing tolerance at −3 °C [17]. Experiments are underway to determine how desiccation-induced acclimation might be a component of subzero-acclimation.

Krasavtsev [17] suggested that a “new order of submicroscopic protoplasm structure was produced” in plants that were subzero-acclimated. Yoshida et al. [58] described a change in the physical state of water in wheat crown tissue at −3 °C that was signified by a decrease in spin–lattice and spin–spin relaxation times of protons in plants. This change resulted in a decrease in the amount of weakly bound water as well as an increase in water binding strength which is critical for freezing survival of plants [58]. Olien [30] reported that rye (*Secale cereale*) and barley (*Hordeum vulgare*) exhibited subzero-acclimation and

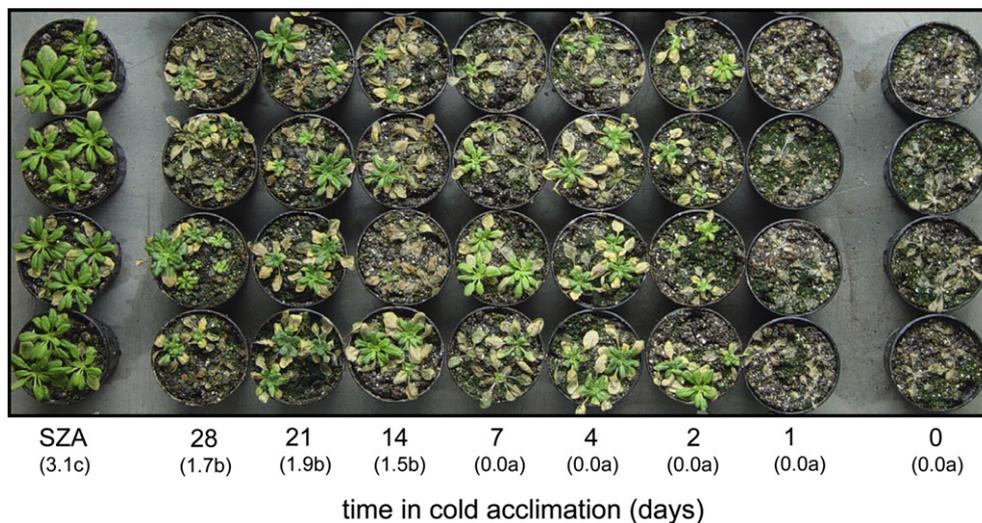


Fig. 1. Recovering *Arabidopsis* plants 7 days after freezing at −15 °C for 3 h, grown under varying duration of cold-acclimation at 3 °C. Zero days cold-acclimated are non-acclimated plants. Subzero-acclimation (SZA) consisted of a 3 days exposure of 7 days cold-acclimated plants to −3 °C in the dark. Note that after about 4 days of cold-acclimation, little increase in freezing tolerance was observed. Numbers in parentheses are the visual survival ratings of plants that had been frozen at −18 °C. Survival ratings with the same letters were not significantly different from each other at $P=0.05$ according to Fisher's protected LSD.

attributed this increase in hardiness to higher concentrations of apoplastic sugars which may help prevent destructive adhesions to cell walls and membranes. These studies were extended to oat (*Avena sativa*) by Livingston [22], who reported a reduction in LT_{50} in a cold-acclimated oat cultivar of 5°C after subzero-acclimation. This increased freezing tolerance of oat, after subzero-acclimation, was correlated with an increase in levels of fructan, sucrose, glucose and higher activities of invertase and fructan exohydrolase in apoplast fluid of crown tissue [23]. However, these compounds were negatively correlated with the capability of two oat genotypes to subzero acclimate [24], underscoring the complexity of the physiology of subzero-acclimation.

In a single replicate of a microarray analysis of *Arabidopsis* the greatest increase among 629 up-regulated and 112 down-regulated genes during subzero-acclimation were genes of unknown function (unpublished data). Examples of known genes included proteins involved in carbohydrate/glycan metabolism, arabinogalactan protein1 (AGP1), and DREB1 and 2 transcription factors. The up and down-regulation of numerous genes points to large-scale restructuring during freezing temperatures and indicates that the acquisition of a few degrees Celsius of additional freezing tolerance is a very complex process. While clearly more research is necessary to confirm and accurately quantitate these results, parallel studies using this approach with wheat [11] indicate that there are overlapping manifestations of subzero-acclimation between *Arabidopsis* and wheat. Even with only a partial set of genes identified by changes in cDNA, the research in wheat has provided candidate genes for forward and reverse genetic analysis of subzero-acclimation. However, if anatomical/morphological characteristics of plants are important in the freezing process as suggested [26,31,37,43,45] then it will be important to characterize genomic changes within specific tissue that is crucial for whole plant survival.

A series of separate tests comparing non-acclimated, cold-acclimated and subzero-acclimated plants indicated a difference in LT_{50} of about 7.2°C between non- and cold-acclimated plants and 2.3°C between cold- and subzero-acclimated plants (Fig. 2). Although the absolute freezing tolerance of subzero-acclimated plants varied slightly from experiment to experiment, a difference in LT_{50} of 2–4°C between subzero-acclimated and cold-acclimated plants was always observed. The conditions in specific experiments which led to a difference in LT_{50} as high as 4°C between cold and subzero-acclimation are not known.

These results confirm that *Arabidopsis* does indeed undergo subzero-acclimation to a similar extent as reported in winter cereals [22,30,51] as well as other species [3,17].

Damage observable under light microscopy in subzero-acclimated plants was identical to that in cold-acclimated plants but was generally less extensive (not shown). Using our staining protocol and with light microscopy, no obvious relationship between subzero-acclimation and the histology of specific tissue recovering from freezing was found

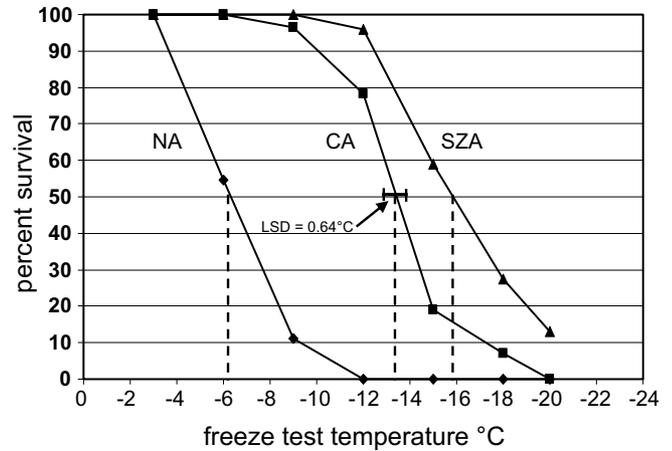


Fig. 2. Percent survival of non-acclimated (NA), cold-acclimated (CA) and subzero-acclimated (SZA) *Arabidopsis* plants frozen at varying temperatures. The vertical broken-line is the estimated LT_{50} of the treatment. Each data point is the mean of seven replicates from three separate experiments. The difference between the LT_{50} of cold-acclimated and subzero-acclimated treatments in individual experiments varied from 2 to 5°C. “LSD” is the least significant difference ($P=0.05$) between the LT_{50} 's of the three treatments according to Fisher's protected LSD.

either here or previously in oats [26]. This suggests that subzero-acclimation is not primarily an effect on a specific region of the crown as was demonstrated for cold-acclimation [26,45]. Those studies indicated that the upper portion of the crown, containing the apical meristems, was less freezing tolerant before cold-acclimation but became more freezing tolerant than the lower portion of the crown (crown core) during cold-acclimation. It is possible that subzero-acclimation is a result of changes within cells that are not visible under light microscopy. Using Electron Microscopy, several changes in cellular ultrastructure were documented in wheat plants that were subzero-acclimated but not subjected to a freeze test. These included changes in

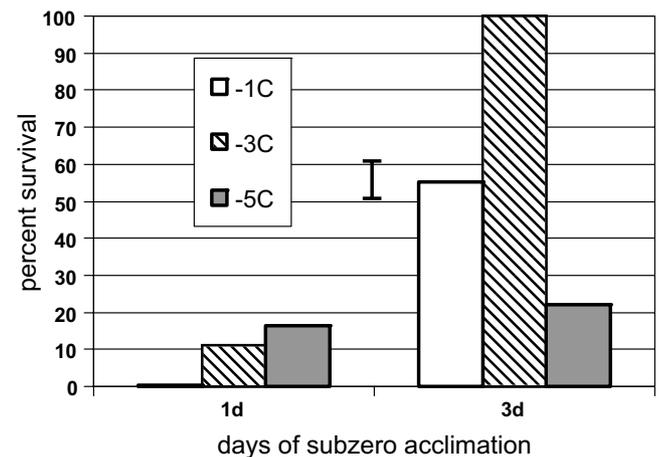


Fig. 3. The effect of time (1 day versus 3 day) and acclimation temperature on the percent survival of *Arabidopsis* plants. Plants were frozen at -18°C as described in materials and methods. The vertical bar in the center is the standard error of the experiment. These data suggest that under our conditions, the parameters which resulted in the maximum level of subzero-acclimation were 3 days at -3°C .

the morphology of the endoplasmic reticulum and other changes in vesicles associated with the golgi [11]. At this time it is not known how/if these ultrastructural changes affect subzero hardening.

Optimizing subzero-acclimation

Taking into consideration the difference in absolute survival between tests, one day at -3 or -1 °C (Fig. 3) most closely resembled results attained by plants which had only been cold-acclimated (Fig. 2). Lowering the temperature of exposure to -5 °C for 1 day made the plants more freezing tolerant but this treatment was not significantly different than those exposed to -3 °C for 1 day (Fig. 3).

A clear increase in freezing tolerance was observed at all 3 temperatures when subzero-acclimation was extended to 3 days (Fig. 3). While the biggest increase in freezing tolerance of 2 oat and 2 barley cultivars was observed in the first day of subzero-acclimation, their freezing tolerance continued to increase even after 7 days at -3 °C [22]. In the present study with *Arabidopsis*, exposure times were not extended beyond 3 days because of difficulties maintaining -3 °C in freezers without a defrost cycle. Because the intent of this study was to determine if *Arabidopsis* could be con-

sistently acclimated beyond cold-acclimation when exposed to freezing temperatures (which was confirmed in Figs. 1–3), we did not feel it necessary to extend subzero-acclimation beyond 3 days.

Similarities and difference between *Arabidopsis* and oat

The ability of *Arabidopsis* to regenerate new roots as well as functional leaf tissue (Fig. 4A and B) from meristematic regions in the area of the plant that is similar to the crown of grasses (Fig. 4C) suggests that this species may be a good model plant for grasses, more specifically the economically important winter cereals such as rye, wheat, barley and oats. However, vascular tissue in the tap-root [2] of *Arabidopsis* leads upwards to the base of the leaves and near the top of the root vascular tissue separates (Fig. 5A and D) into a somewhat symmetrical region that contains mostly parenchyma cells without any vessels (Fig. 5C); this region is referred to as the pith of the stem [2]. From a positional standpoint the pith would be analogous to the crown core of the cereal crown [26] but in cereal crops the crown core is decidedly non-symmetrical and consists mostly of intertwining vessel elements in a seemingly unorganized arrangement, interspersed with parenchyma cells (Fig. 5B

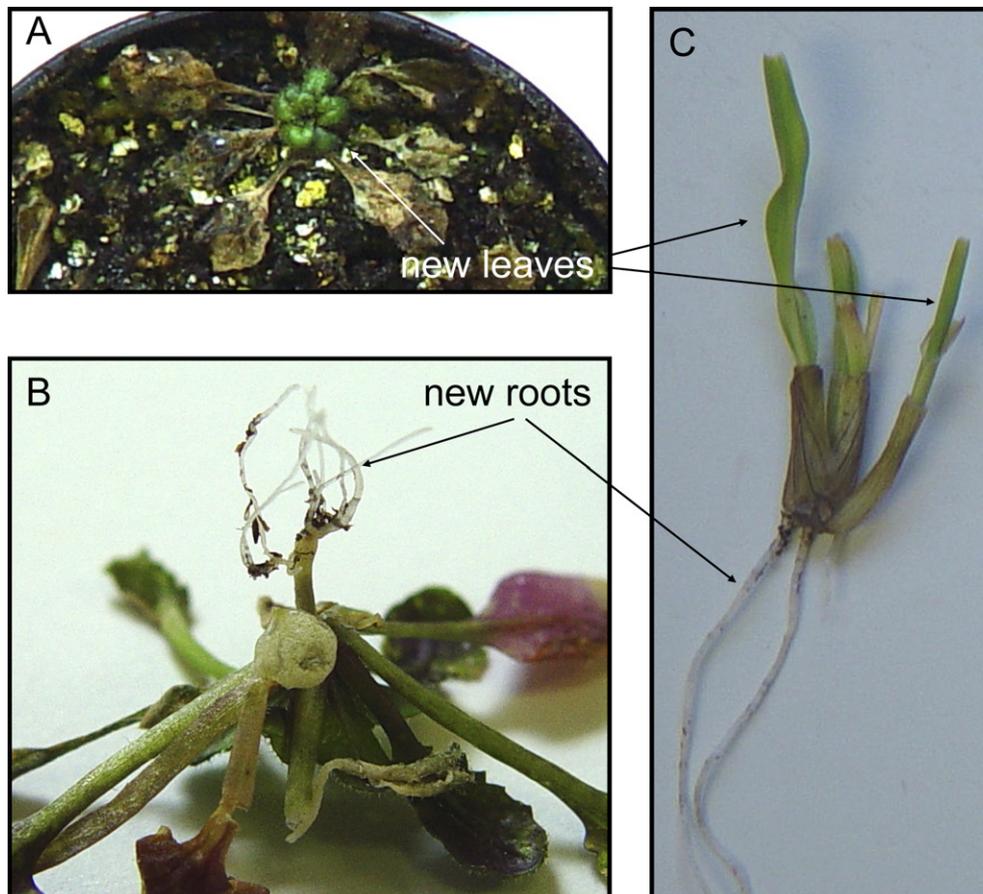


Fig. 4. (A) *Arabidopsis* plant 7 days after freezing at -15 °C showing new leaf growth from meristematic regions shown in Fig. 6. (B) *Arabidopsis* root showing new growth after main root was trimmed. This plant had not been frozen. (C) Oat plant 2 weeks after freezing at -12 °C showing new root and leaf growth. Note the positional similarity in tissue that regenerated following freezing despite one being a dicot (A and B) and the other a monocot (C).

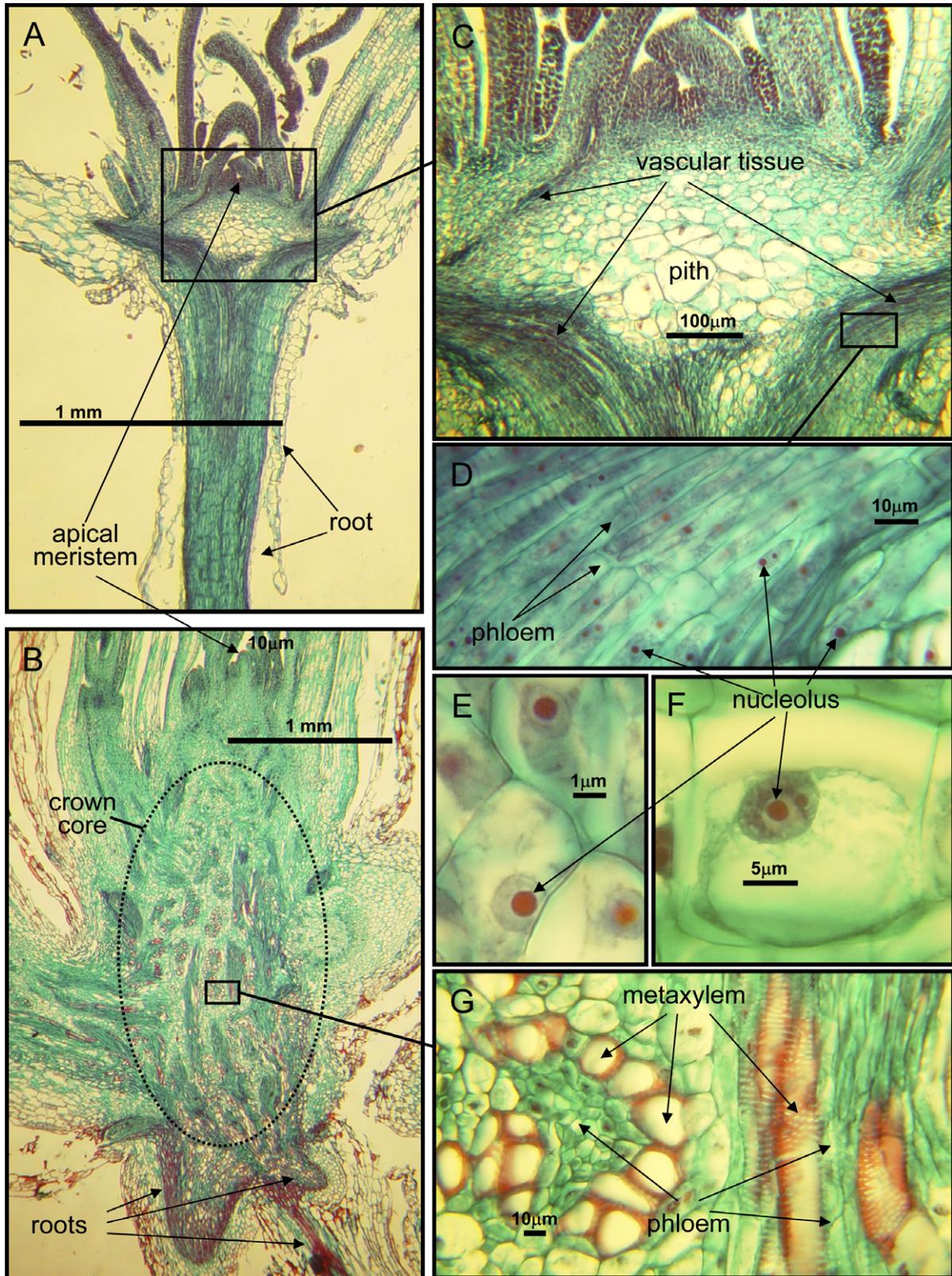


Fig. 5. Longitudinal sections of the crown of *Arabidopsis* (A) and oat (B) where new growth originated following freezing and thawing. Paraffin-embedded sections were triple stained with Safranin, Fast Green and Orange G. All frozen tissue was compared to these controls to assess tissue damage. Note the considerable difference between *Arabidopsis* and oat in the center portion of the crown region referred to as the “crown core” in (B). (C) Closer view of the upper portion of the *Arabidopsis* crown showing an extensive region in the center referred to as the pith. This region is positionally analogous to the crown core (B) of oat. (D) Phloem vessels from the vascular tissue extending through the root and subtending the pith region. (E) Nucleus of a parenchyma cell from the pith region of *Arabidopsis*. Note the prominent nucleolus and lightly staining nucleoplasm as compared to that of oat (F). (F) Nucleus of a parenchyma cell from oat. Note the darker staining nucleoplasm making the nucleolus somewhat more difficult to find. (G) Vascular tissue within the crown core of oat. Notice xylem and phloem sectioned vertically and in cross-section directly adjacent to each other as compared to the more linear and symmetrically arranged vascular tissue of *Arabidopsis* (A, C, and D).

and F, [26]). The crown core of cereals is therefore compositionally different (with regard to cell types) from the pith region of *Arabidopsis* even though it occupies a similar position in the plant.

The nucleus of *Arabidopsis* cells was also somewhat different from that of oat (Fig. 5E and F) and other winter cereals (not shown) under the staining protocol we used. In *Arabidopsis* the nucleolus was the region of the nucleus that stained the darkest (Fig. 5E) and the nucleoplasm was barely visible. This was not the case with oat in which the nucleolus was visible in only rare cases (Fig. 5F) and was surrounded by a much darker nucleoplasm. In addition to differences in staining, nuclei in *Arabidopsis* were 3–5 times smaller than those of oats (compare Fig. 5E with F). It is possible that the smaller genome of *Arabidopsis* contains less chromatin, which absorbs safranin, and therefore appears smaller and lighter in color than the nucleoplasm of oat. Freezing damage to the nucleus has been described in small grains [26,52] as well as other species [38,42]. While certain kinds of nuclear abnormalities may or may not be lethal, these differences may be an important consideration when using *Arabidopsis* as a model for winter cereals.

It is possible that while these anatomical/histological differences between species are visually obvious, their differences from a functional standpoint with regard to freezing tolerance are irrelevant. As more research focuses on the role of specific tissues/cells within the crown in the freezing tolerance of the whole plant, the significance of these differences between species will become more apparent.

Histology of plants recovering from freezing

Histological observations were made 7 days after freezing because while damage at this stage of recovery is not the primary damage event caused by freezing, injuries observed at this stage are effects that will likely result (or already have resulted) in death of the whole plant. Cold tolerant plants can recover from many primary injuries and in some cases recovery from a particular primary injury can enable the whole plant to survive. One such freeze-induced injury from which plants can recover is an increase in membrane permeability [34]. Assaying for this type of injury using conductivity measurements (see [55] for one example) has been a common means of determining freezing tolerance. However, while this kind of assay can determine the extent of immediate damage it will not necessarily predict the ability of the whole plant to survive freezing [34].

Several abnormalities (as compared to unfrozen controls in Fig. 5) were observed in the histology of *Arabidopsis* plants that had been frozen (Fig. 6). The most prominent was what appeared to be vessel plugging that spread throughout the vascular system in the root (Fig. 6A1–A4) and extended into the lower part of the pith (Fig. 6B). In some cases the alleged plugging was discontinuous within vessels; darkly staining material appeared to advance up to the sieve plate in the single phloem vessel shown in Fig. 6C but was not present in the adjoining vessel. This discontinu-

ous plugging in vascular tissue was observed in numerous sections from all plants with visual freeze damage.

Vessel plugging was also observed in vascular tissue of oat recovering from freezing where it was suggested that the darkly staining material was bacterial in nature [26]. Olien and Smith [32] identified three of the most frequent types of colonies isolated from frozen barley roots as bacteria belonging to the Genus *Pseudomonas*. They also stated that these organisms move into the crown during recovery from freezing and were likely responsible for the death of specific tissue within the crown core region [32]. They also present evidence for the proliferation of various fungal species in plants recovering from freezing. Sattlemacher [41] reviews literature that considers the apoplast as a “habitat for microorganisms” and using electron microscopy demonstrated the colonization of maize apoplast with endophytic bacteria. Marshall [29] attributed the death of oats to the presence of *Pseudomonas syringae* on leaf surfaces that only caused infection if the plant had been frozen first.

The darkly staining region within the leaf axel (Fig. 6D) is likely necrotic tissue which may have been caused by the proliferation of microbes extending from the vascular tissue. This may be the cause of leaf death in severely damaged plants such as those shown in Fig. 1 and the plant in Fig. 4A. However, we will need to culture and identify whatever organisms are present in specific tissue before confirming this possibility.

Another abnormality seen in severely damaged plants was complete deterioration of the pith. Fig. 6B is a representative section of the type of damage seen in all 8 plants that were frozen at -15°C (as well as below -15°C) and was particularly pronounced in plants which did not survive. A similar type of damage was seen in the crown core region of frozen oats [26]. In that study the more deterioration within the crown core, the less likely was the plant to survive. In those plants the meristematic regions appeared undamaged and the plant may have survived if vessels providing material support to the meristems had survived [26]. The same appears to be the case in *Arabidopsis*. No damage was observed in the meristematic region of the eight replicates that were severely frozen. Even in non-surviving plants no damage was apparent in the meristematic region. This suggests that the meristematic region of *Arabidopsis* is more freezing tolerant than the lower portion of the stem (the pith, in *Arabidopsis*) as was found in oats [25] and wheat [45].

An abnormality in *Arabidopsis* plants recovering from freezing which was not seen in oats was what appeared to be “frost plasmolysis” (Fig. 6E, compare to normal protoplasm in Fig. 5E). This effect of freezing has been described [20, p. 83] as occurring during extracellular ice formation as the cell contracts. When the cell dies (presumably from severe dehydration or intracellular freezing) the protoplast then lacks the ability to reabsorb water and expand during thawing. Becquerel (cited by Luyet and Gehenio [28]) said this effect was not caused by dehydration but by coagulation of the cytoplasm. Alleged frost plasmolysis was seen in

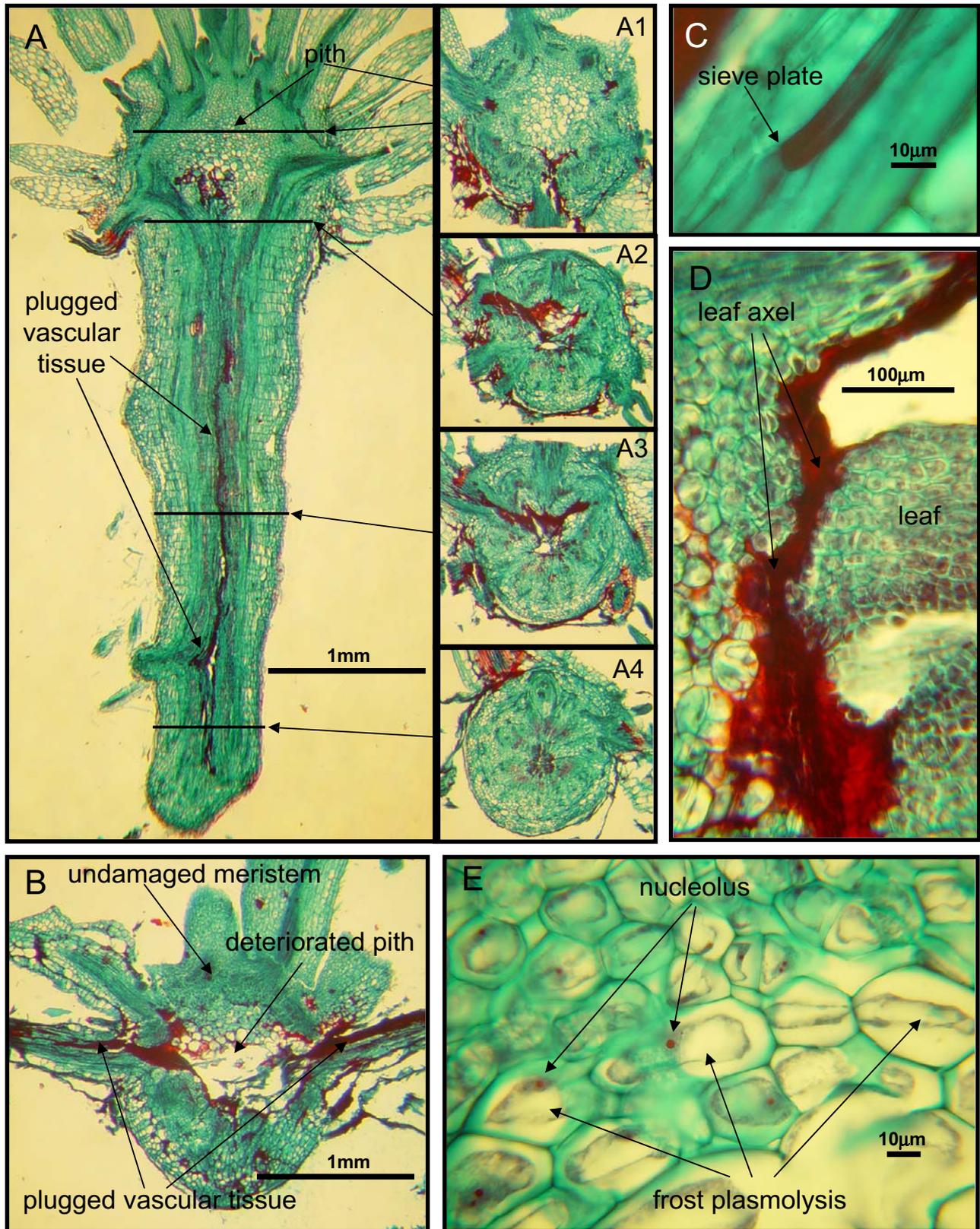


Fig. 6. (A) Longitudinal section of *Arabidopsis* plants frozen at -15°C and allowed to recover for 7 days. Note the plugged vascular tissue running along the center of the root, up into the pith region of the crown. (A1–A4) Cross-sections of regions of the root showing plugging becoming more extensive along the root until the mid to upper portion of the pith region. The 1 mm bar in A applies to these 4 views as well. (B) Upper part of *Arabidopsis* plant frozen at -18°C . Note the near complete deterioration of the pith region (compare to the control plant in Fig. 5) and plugging extending along vascular tissue into leaves. Also note the undamaged meristematic region. (C) Representative view of plugging of vascular tissue in roots showing the inability of the plugging to penetrate the sieve plate of a phloem vessel. (D) Dead tissue within the leaf axel resulting in dead leaves shown in Fig. 1. (E) Frost plasmolysis in the pith region of plants frozen at -9 and -15°C . Note nucleolus of a seemingly undamaged nucleus. No pycnotic nuclei (as described in oats, Livingston et al. 2005, 2006) were found in any frozen *Arabidopsis* plant.

all recovering plants (five replicates) which had been frozen at -9°C . All plants frozen at this temperature survived so while this effect may have damaged individual cells, it was clearly not lethal to the whole plant, at least at this level of damage. Williams [56] suggests that this effect can be prevented osmotically by sugar accumulation in affected tissues.

An abnormality in grasses recovering from freezing that was not found in recovering *Arabidopsis* was nuclear pycnosis. Nuclear pycnosis was first described in freeze-damaged orchardgrass by Shibata and Shimata [43] and was recently described in oats that were recovering from freezing [25,26]. Freeze-induced nuclear damage was verbally described in frozen wheat by Tzinger and Petrovskaya-Baranova [52], and illustrated by electron microscopy in Tall Fescue (*Festuca arundinacea* Schreb.) by Pearce and McDonald [38]. Their descriptions resembled that of nuclear pycnosis but neither authors used the term. Nuclear abnormalities including pycnosis have been reported in mammalian tissue exposed to various sources of radiation, high temperatures, severe dehydration, anesthetics and different carcinogens [4,50]. It is difficult to imagine how cells could function normally with pycnotic nuclei but our observations do not necessarily prove that nuclear pycnosis results in death of the cell. Other authors cite nuclear damage, such as condensed chromatin as a precursor to programmed cell death resulting from ethylene or hypoxia [1] hydrogen peroxide [12] and a decrease in nuclear ABA resulting from water stress [33]. However, it should be mentioned that the term “condensed chromatin” does not always imply nuclear pycnosis. In many cases “condensed chromatin” refers to the condensation of chromosomes as occurs prior to mitosis or meiosis. This results in numerous distinct regions of the nucleus that are more darkly stained. Under light microscopy and our staining protocol, nuclear pycnosis involved the entire nucleus giving it a uniformly stained, deep red appearance and in some cases seemed to produce globules of nuclear material within cells [26]. We are investigating the possibility that nuclear pycnosis is caused by freeze-induced dehydration. However, the apparent lack of pycnosis in *Arabidopsis* will prevent us from using it as a model for this particular manifestation of freezing stress.

Conclusions

Acclimation at subzero temperatures has been demonstrated for 75 years in winter cereals and other plant species. We have shown here that subzero-acclimation imparts freezing tolerance to *Arabidopsis* plants that cannot be replicated by extending the duration of cold-acclimation. Subzero-acclimation in *Arabidopsis* appears to be a discrete physiological event that likely builds on the foundation of changes induced during cold-acclimation, similar to that in winter cereals. However, *Arabidopsis* differs from winter cereals from a histological perspective which should be taken into account when attempting to extrapolate the

genetics of freezing tolerance from *Arabidopsis* to winter cereals. If anatomical/morphological characteristics of plants prove to be important in freezing survival of cereal crops, then the validity of using *Arabidopsis* as a model crop may need to be reconsidered. However, provided these differences are kept in mind, confirming subzero acclimation in *Arabidopsis* provides researchers with a model to study the genetics of this important aspect of freezing tolerance using the vast array of resources that are currently unmatched in other plant systems.

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