

Carbohydrate partitioning between upper and lower regions of the crown in oat and rye during cold acclimation and freezing [☆]

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Abstract

Carbohydrates have long been recognized as an important aspect of freezing tolerance in plants but the association between these two factors is often ambiguous. To help clarify the relationship, the allocation of carbohydrates between specific tissues within the over wintering organ (crown) of winter cereals was measured. A winter-hardy and non-winter-hardy oat (*Avena sativa* L.), and a rye (*Secale cereale* L.) cultivar were grown and frozen under controlled conditions. Crown tissue was fractionated into an upper portion, called the apical region, and a lower portion, called the lower crown. These tissues were ground in liquid N and extracted with water. Extracts were analyzed by HPLC for the simple sugars, sucrose, glucose, fructose, and for fructan of various size classes. After 3 weeks of cold acclimation at 3 °C, carbohydrates accounted for approximately 40% of the dry weight of oats and 60% of the dry weight of rye. The apical region, which is the tissue within the crown that acclimates to the greatest extent, was generally 10% higher in total carbohydrates than the lower crown. During a mild freeze, various carbohydrates were allocated differently between specific tissues in the three genotypes. When frozen, fructan generally decreased to a greater extent in the lower crown than in the apical region but sugars increased more in the apical region than in the lower crown. Results suggest that to understand how carbohydrates relate to freezing tolerance, regions of the crown that endure freezing stress differently should be compared.

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Of all the physiological/biochemical mechanisms implicated in the protection of plants from freezing injury, carbohydrates have arguably had the longest history and the most controversy. As early as 1936 Levitt and Scarth [17] reviewed the carbohydrate/freezing tolerance literature that was current up to

that point. Later reviews include those of Steponkus [35], Santerius [33], Alden and Hermann [1], and Olien and Lester [28]. More recently studies on the stabilizing effect on various membranes by trehalose [5,6], raffinose [10] sucrose [8,29] as well as fructo and gluco oligosaccharides [9] have suggested several cause and effect relationships between carbohydrates and freezing tolerance. In addition to direct membrane stabilization, a regulatory effect of sucrose has been demonstrated for cold-induced

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expression of non-specific lipid transfer protein and dehydrin in barley (*Hordeum vulgare* L.) cell cultures [36]. While precise mechanisms may still be open to discussion, clearly carbohydrates are important in the ability of plants to withstand freezing stress.

In winter cereals it has been shown that whole-plant survival is a result of the survival of specific tissue within the crown [23,27,37]. The crown contains meristematic regions from which apical meristems originate. Provided this region is not damaged beyond a threshold level, new roots and shoots will regrow and produce a fully functional plant. Some studies have shown that the upper portion of the crown, the apical region, is hardier than the lower part of the crown after cold acclimation [22,37]. In orchardgrass (*Dactylis glomerata* L.), Shibata and Shimada [34] found apical meristems to be less hardy than the base of the crown. The differential survival of specific tissue within the crown may be the result of localized effects such as regions where carbohydrates accumulate (called “sumps” [4]) acting as barriers to ice propagation, or proteins in the apoplast [7], or pectin-containing barriers which enable specific tissue to supercool [13], or some as yet undiscovered mechanism. Pearce et al. [31] localized two cold up-regulated genes to the cell layers surrounding the vascular tissue of barley crowns. Koroleva et al. [15] found low concentrations of glucose, fructose, sucrose, and fructan in epidermal cells of cold acclimated barley leaves with much higher concentrations in mesophyll and bundle sheath cells. Heterogeneity between cell types in barley was reported [16] for sugars as an explanation for differences in transcription control and enzyme activity. Ball et al. [3] report a high degree of spatial dependence on water content within leaves of *Eucalyptus* which affected the rate of freezing. These studies seem to support the hypothesis that whole-plant freezing survival may be more related to the water content, sugar concentration, and/or various cryoprotectants associated with individual cells or regions surrounding cells, rather than that of whole plants.

Livingston et al. [25] found that carbohydrates were allocated differently in different regions of oat (*Avena sativa* L.) crowns. The tissue sampled was that of the lower stem and the crown meristem (CM) complex. A more refined fractionation of the crown has since been developed which enabled us to separate the CM complex into two regions that have clear differences in the capacity to acclimate [22,23]. In this study we wanted to see how carbohydrates

were partitioned between the two regions during cold acclimation and freezing, and to compare partitioning between a hardy and non-hardy oat to the most winter-hardy cereal, rye (*Secale cereale* L.).

Materials and methods

Plant culture

Seeds of oat (cv. ‘Wintok’ a winter-hardy oat and ‘Fulghum’ a non-hardy oat) and rye (cv. ‘Rosen’) were grown in Scotts Metromix 510 (Scotts-Sierra Horticultural Products, Marysville, OH) in plastic tubes (2.5 cm diameter × 16 cm height) with holes in the bottom to allow drainage. The tubes were suspended in a grid which held 100 tubes. Plants were watered three times weekly with a complete nutrient solution [18] and flushed three times weekly with tap water. Non-acclimated plants were produced by growing them for 5 weeks at 13 °C day and 11 °C night temperatures with a 16 h photoperiod at 250 μmol m⁻² s⁻¹ PAR (80% cool fluorescent and 20% incandescent).

After the five week growth period, plants were transferred to a chamber at 3 °C with a 10 h photoperiod at 300 μmol m⁻² s⁻¹. Plants receiving the 21 day treatment constituted fully cold acclimated (CA) plants.

Freeze testing

After the respective growth treatments, crowns were separated and removed from each plant by trimming roots and shoots resulting in a 2.5 cm portion of the base of the stem. Crowns were placed in slits cut into damp circular sponges. Sponges were placed into plastic bags and inserted over a pipe-flange assembly to promote thermostabilization. Ice shavings were added to each sponge to promote nucleation and prevent supercooling and the plastic bags were loosely sealed. Sponges with plants were placed in freezers at -3 °C that were programmed to reach a target temperature at a rate of -1 °C/h. Plants were held at the target temperature for 3 h and then brought to 3 °C at 2 °C/h. Roots were trimmed from crowns and then crowns were transplanted into soil mix to allow regrowth. After 3 weeks plants were rated on a scale of 0–5 where 0 was dead and 5 was undamaged. Percent survival was calculated by summing the number of plants that had been rated 1 and above and dividing by the total number of plants frozen at that temperature.

LT_{50} s were estimated by extrapolation and are given as the temperature at which survival was 50% in a particular treatment.

Tissue fractionation

Plants were taken for fractionation just prior to CA, and at 5 and 21 days of CA. They were washed free of soil and roots, and were trimmed to about 5 cm. All subsequent fractionation was performed in

a cold-room at 3 °C. Under 7× magnification, plants were lightly scored all the way around the stem with a razor blade 1–3 mm above the region of the crown where the roots protrude (Fig. 1). With one hand tightly holding the roots and the other hand holding the lower stem, a slight twisting motion with a firm, slow pull allowed nearly a perfect separation of the stem and exposed the crown meristem (CM) complex [23]. About 1–2 mm of tissue was trimmed from the top of the exposed CM complex and was called

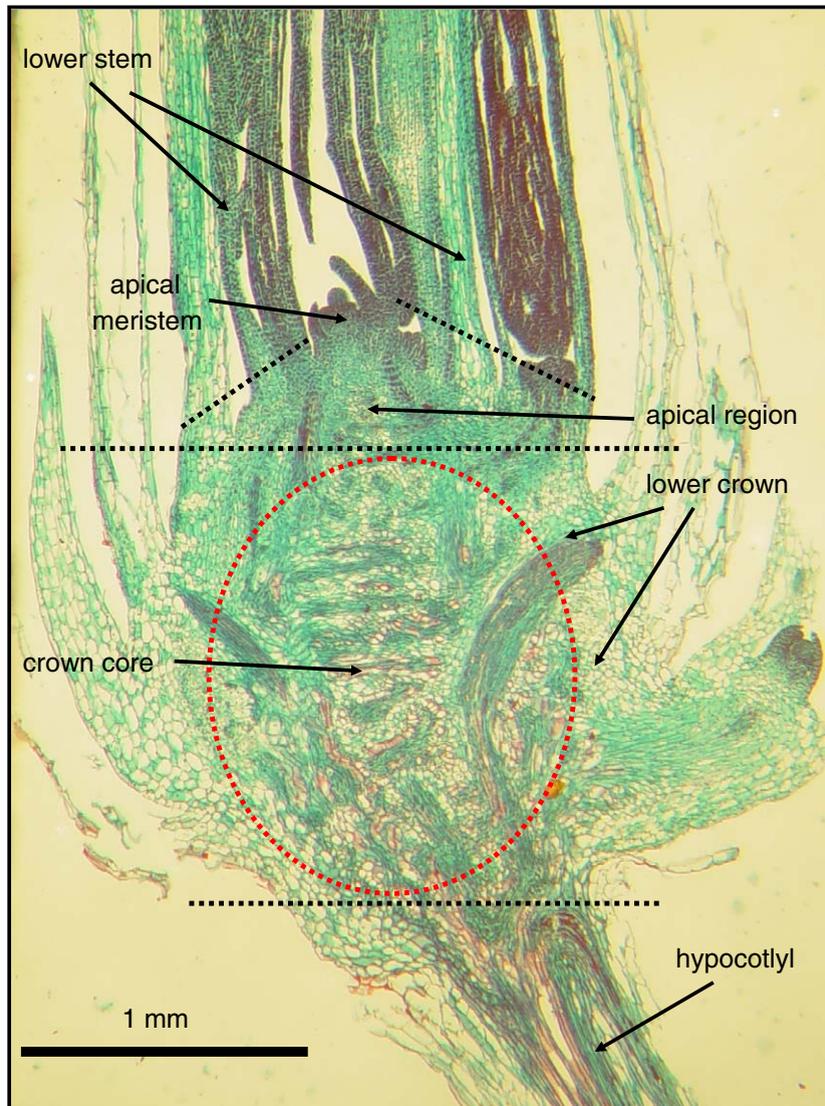


Fig. 1. Longitudinal section stained with Safranin, Fast Green, and Orange G, of a cold acclimated oat plant grown for 5 weeks at 13 °C under controlled conditions and 3 weeks at 3 °C. Crowns of rye were similar enough to oats that it would have been redundant to show them. The dashed lines at an angle show the approximate region where the lower stem was separated from the crown meristem complex by pulling with one hand while twisting with the other after scoring the outside of the stem with a razor. The vertical dashed line near the upper portion of the crown shows where the apical region was separated from the lower crown by a razor blade. The dashed line at the base of the crown is approximately where the roots were cut off with a razor. See [23] for detailed description of various cell types and tissues within the crown region.

the apical region (Fig. 1). The basal portion of the CM complex, the lower crown (Fig. 1), was trimmed of roots, as close to the root–shoot junction as possible [2], although it was not possible to perfectly remove all root tissue from the lower crown. When tissue from 10 plants had been fractionated and placed into a mortar containing liquid N₂, tissue was ground to a powder with a pestle and transferred to a tarred 100 mL beaker. Fresh weight was recorded and 4 mL water/g tissue was added to each beaker. The beaker was heated on a water bath at 95 °C for 10 min to inactivate enzymes. This procedure was repeated three times. All data are means of three replications and each replicate consisted of 10 plants.

Carbohydrate analysis

Ground and heated tissue was filtered through a 0.45 µm filter and injected into a Bio-Rad 42A HPLC column. Fructans were separated according to size class using a modified Bio-Rad (Richmond, CA) Aminex HPX-42A (silver based) analytical HPLC column (7.8 × 300 mm). This column was permanently modified by passing 0.5 M NaNO₃, at a rate of 2 mL min⁻¹, through the column for approximately 18 h. This treatment eliminated on-column hydrolysis of smaller fructans and sucrose, and improved the resolution of smaller sugars. Resolution of larger (DP5 and DP6) fructans, however, was slightly reduced in the modified column. Samples were not desalted prior to injection therefore, a cation and anion exchange guard-column was used immediately preceding the analytical column to prevent co-elution of small ionic compounds with carbohydrates. The mobile phase was HPLC-grade water at a flow rate of 0.4 mL min⁻¹.

Statistical analysis

The experiment was conducted as a randomized complete block design with three replicates and with hardening treatment as the main plot and tissue as sub plot. An analysis of variance was calculated using MSTAT (Michigan State University, East Lansing, MI).

Histology

The lower 2 cm of the stem of plants that had been cold acclimated for 3 weeks was placed in fixative containing 18:1:1 parts of 70% ethyl alcohol/

glacial acetic acid/formaldehyde. Crowns were kept at room temperature for 48 h and transferred to 70% alcohol and kept at 4 °C until they were processed for dehydration and embedding.

Dehydration, infiltration, embedding, and staining

Crowns were dehydrated according to procedures detailed by Johansen [12] using a series of ethanol and tertiary butyl alcohol solutions. Fully infiltrated tissues were embedded in Paraplast Plus and kept refrigerated until sectioned. Embedded sample blocks were sectioned in a Reichert-Jung 2050 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 [12], flooded with 3% formalin, 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 was used as described by Johansen [12]. A cover-glass was added to slides with one or two drops of Permout.

Observation of sections

Sections were viewed under a Wild Heerbrug wide angle dissecting microscope with bottom lighting and on a Zeiss photomicroscope III. Photographs were taken with a Sony DSC848 digital camera attached to the microscope.

Results and discussion

Freezing tolerance

Rye has long been recognized as the most freezing tolerant of the winter cereals so a lower LT₅₀ than oats was not unexpected (Table 1). A difference in winter hardiness between Wintok and Fulghum

Table 1
The temperature at which 50% of plants survived (LT₅₀) a freeze test at varying lengths of time under cold acclimation

	Non-acclimated	Cold acclimated	
		5 days	21 days
Fulghum oat	-3.2	-6.5	-9.0
Wintok oat	-4.9	-8.8	-10.7
Rosen rye	-10.0	-14.4	-16.5

oat was documented in 65 years of testing in the field [20] and was confirmed here in controlled freeze tests (Table 1).

The LT_{50} of both oat cultivars changed by the same amount during CA (Table 1), suggesting that differences between cultivars in freezing tolerance were present prior to CA and that CA induced freeze-protection mechanisms to the same extent in both cultivars. This suggests that where differences between cultivars are concerned, freezing tolerance mechanisms developed during the growth phase may have been more important than those which accumulated during CA. While rye was considerably more freezing tolerant before CA, its freezing tolerance increased during CA to a greater extent than oats (Table 1). This indicates that rye not only has more freezing tolerance before CA but also has a greater capacity to take advantage of CA than either oat cultivar.

Carbohydrates

Differences in carbohydrate concentrations between stem tissue and tissue subjacent to the stem called the crown meristem (CM) complex were described previously [25]. Using a more refined fractionation procedure we found differences between areas within the CM complex in dry weight and the amount of water freezing at -2°C [23]. In this study, we wanted to see how carbohydrates were allocated during CA between the upper part of the CM complex called the apical region and the lower part called the lower crown. The specific tissue under consideration is shown in Fig. 1 and has been described in detail elsewhere [23]. Due to differences in moisture content between the tissues (not shown, see [23]) carbohydrates were expressed on a per gram dry weight basis.

Simple sugars (*glucose, fructose, and sucrose*)

No significant differences for simple sugars were found between tissue from non-acclimated plants in any of the three genotypes (Fig. 2). However, simple sugars rapidly increased during CA in both tissues. In rye, simple sugars increased significantly faster in the apical region than in the lower crown within the first 5 days of CA. While simple sugars also increased rapidly within the first 5 days in oats the difference between the apical region and lower crown was barely significant (Fig. 2).

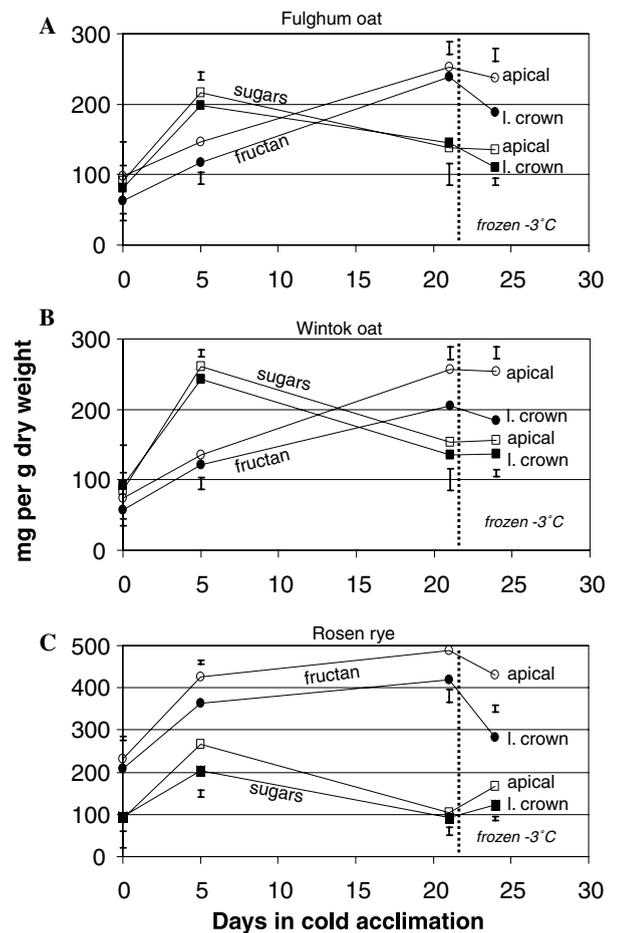


Fig. 2. Change in fructan (DP >2) and simple sugars (sucrose, glucose, and fructose) in the two fractions of the oat crown illustrated in Fig. 1 during cold acclimation. (A) Non-hardy oat cultivar 'Fulghum', (B) winter hardy oat cultivar 'Wintok' and (C) very winter hardy rye cultivar 'Rosen'. To the right of the broken line is the change in carbohydrates after a 3 day freeze at -3°C showing the rapid re-allocation of carbohydrates despite the presence of ice in the plant. The bar above each data point is the standard error.

Simple sugars reached a maximum at day 5 (Fig. 2) and decreased thereafter during CA while freezing tolerance increased steadily throughout CA (Table 1). The lack of a positive correlation between the change in simple sugars and changes in freezing tolerance after day 5 suggests that either simple sugar is not the limiting factor determining freezing tolerance under our conditions or despite fractionation of the crown we are still not able to sample the precise region that is crucial for whole-plant survival. For example, putative barriers found in frozen plants [22,23] may be localized regions of high sugar concentrations described by Canny [4] that were

averaged with the sugar concentration of whole tissue.

The existence of plant species such as sugar beet that are extremely high in sugar, yet have no tolerance to freezing [17], underscore the contention that either proper localization of sugars is the important factor in understanding cause and effect between freezing tolerance and sugars, or another factor with which sugars interact [9] is necessary to produce freezing tolerant tissue.

Glucose was the only simple sugar that was significantly higher in the apical region than the lower crown in all three genotypes and at all stages of CA (Figs. 2–4). Sucrose and fructose concentrations were not present in any clear pattern between tissue or genotypes. In Fulghum at 21 days sucrose as well as small DP fructan was higher in the lower crown than in the apical region (Fig. 3). In some cases (see

Fig. 4, Wintok day 5 and Fig. 2, Fulghum day 5 and 21) it appeared that the small DP fructans more closely resembled the distribution between tissues seen in sucrose than that of the large DP fructan.

Fructan

Oat is unique among winter cereals in that during cold acclimation it tends to accumulate small fructans and simple sugars rather than high DP (>DP 5) fructan. Livingston et al. [19] found that out of 273 oat genotypes, only one had high DP fructan levels that were the same as barley. The concentrations of DP >5 in oat (Figs. 3 and 4) as compared to DP 3, 4, and 5 in rye (Fig. 5) illustrate the difference between oat and other winter cereals.

Differences between tissue for total fructan were not significant in non-acclimated Wintok oat and

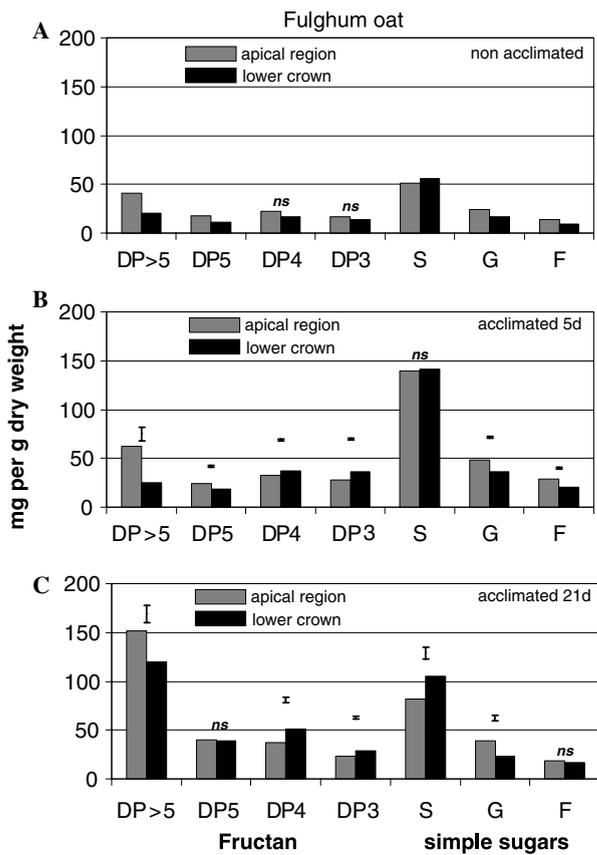


Fig. 3. Details of carbohydrates in Fulghum oat before (A), after 5 days (B), and after 3 weeks (C) of cold acclimation at 3 °C. Various carbohydrates are compared in the two fractions shown in Fig. 1. The bar above each carbohydrate is the standard error. Fractions that did not differ significantly from each other are designated “ns.”

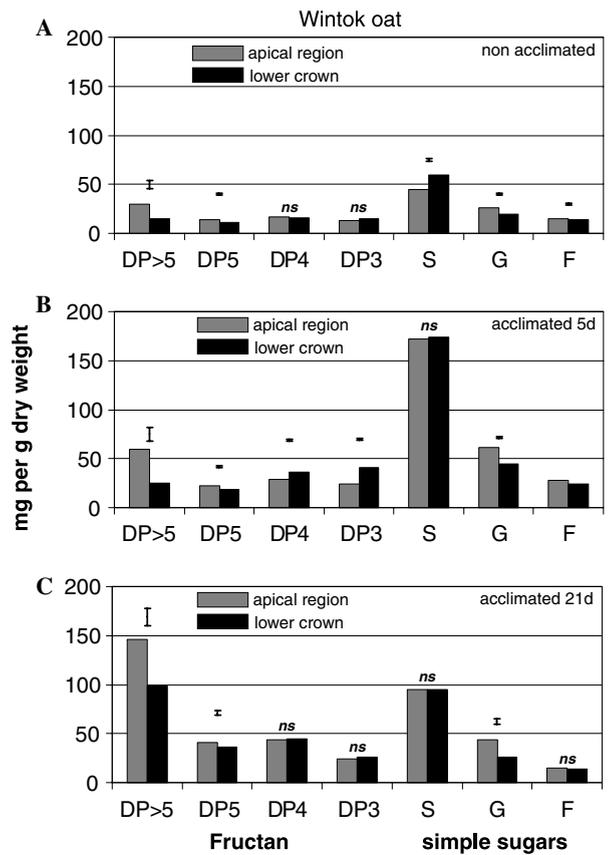


Fig. 4. Details of carbohydrates in Wintok oat before (A), after 5 days (B), and after 3 weeks (C) of cold acclimation at 3 °C. Various carbohydrates are compared in the two fractions shown in Fig. 1. The bar above each carbohydrate is the standard error. Fractions that did not differ significantly from each other are designated “ns.”

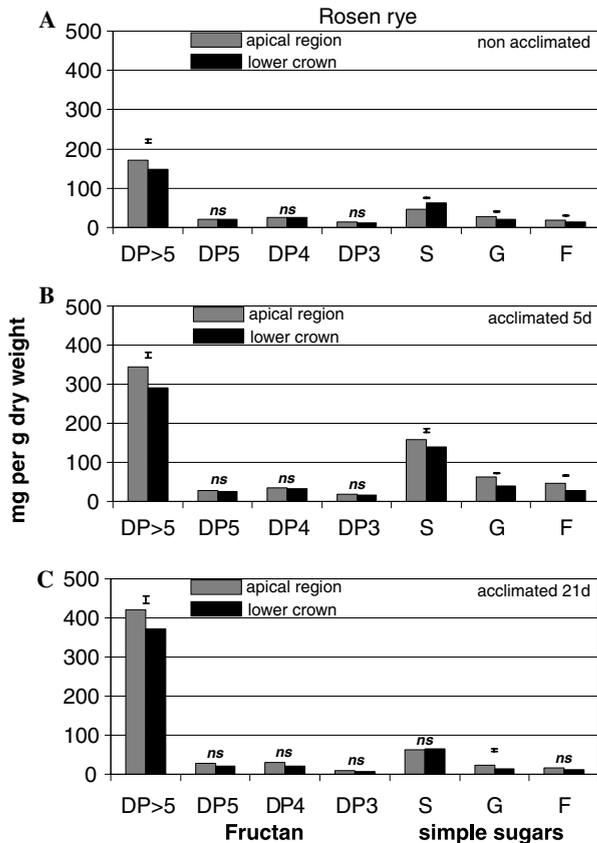


Fig. 5. Details of carbohydrates in Rosen rye before (A), after 5 days (B), and after 3 weeks (C) of CA at 3 °C. Various carbohydrates are compared in the two fractions shown in Fig. 1. The bar above each carbohydrate is the standard error. Fractions that did not differ significantly from each other are designated “ns.”

rye plants (Fig. 2) but during CA the apical region accumulated fructan more quickly than the lower crown, except in Fulghum the least freezing tolerant genotype. In wheat (*Triticum aestivum* L.) [37] and Wintok oat [22] the apical region became more freezing tolerant during CA than other regions of the crown. It is tempting to suggest that the increase in fructan concentration in the apical region is related to its increase in freezing tolerance during CA. Indeed, the fructan concentration of the very freezing tolerant rye was nearly double than that of the two oat cultivars.

While fructan in the apical region was correlated to the difference in freezing tolerance between species (Table 1, Fig. 2) it was not related to the difference in freezing tolerance between the two oat cultivars (Table 1, Fig. 2) even though it may have contributed to the acquisition of freezing tolerance during CA in both cultivars (Table 1, Fig. 2). The

concentration of fructan in the apical region of both oats was not significantly different but, the proportion of total fructan allocated to the apical region was higher in Wintok (Fig. 2), the more freezing tolerant cultivar (Table 1). Since we were not able to localize the fructan beyond a region of tissue, it would not be possible to speculate on differences in localization within the apical region and lower crown which may explain the difference in freezing tolerance between the cultivars. However, fructan has been shown to protect membranes against damage from various forms of abiotic stress [8,9] and may have been an important factor in the increase in freezing tolerance during cold acclimation (Table 1).

Trunova [38] may have been the first to demonstrate a relationship between fructan and freezing tolerance and countless studies have been published since, many (but not all) with positive correlations between fructan concentrations and freezing tolerance. Tobacco plants transformed to accumulate fructan were more freezing tolerant [14] as well as more drought tolerant [32]. If the concentration of high DP fructan (Figs. 3–5) is related to the freezing tolerance of individual cells or tissue then the concentration of high DP fructan in the apical region of rye at least partially explains the higher freezing tolerance of this region of the crown after CA [37]. However, until fructans and simple sugars and their interactions can be correlated with survival of specific tissues and possibly even specific cells, the precise relationship between fructan concentration and whole-plant survival will likely remain elusive.

During a mild freeze, fructan decreased to a greater extent in the lower crown than in the apical region and simple sugars increased in the apical region to a greater extent than in the lower crown in Fulghum oat and rye (Fig. 2). This was a relatively rapid change in carbohydrate allocation despite the presence of ice in the plant. Livingston and Henson [21] previously reported that this rapid change in carbohydrates was reflected in apoplastic fluid and may explain the increase in freezing tolerance during an acclimation period at below freezing temperatures. In a subsequent study however, Livingston and Premakumar [24] reported that the level of carbohydrates in the apoplast was not correlated to the difference in freezing tolerance between Wintok and Fulghum oats after subzero acclimation. It was suggested that the technique used for extracting apoplastic fluid likely resulted in a measurement of carbohydrate in leaf bases rather than the crown core or the apical meristems. This prompted an

initial crown fractionation study [25]. In that study there was no significant difference in the change in fructan between the CM complex and lower stem, while here (Fig. 2) the decrease in fructan was greater in the lower crown in all three genotypes (statistically significant at $P=0.03$). In this study, we have essentially separated the CM complex into two separate regions which were previously considered together [25]. The difference in these two studies illustrates the importance of measuring changes in specific tissue. Admittedly, further fractionation, such as has been achieved in the measurement of metabolites in individual cells of barley leaves [15] would likely reveal differences that we did not find here.

Conclusions

Winter hardiness in plants has been studied for over 100 years but this effort has resulted in little progress in terms of establishing cause and effect relationships between changes in particular metabolites during CA and survival of specific tissue that results in whole-plant survival. Part of the difficulty has been the inability to resolve specific metabolic/genetic changes to the cellular level as well as a dearth of studies on cellular/subcellular freezing damage.

In this study, fructan (primarily the DP >5 fraction) increased to a greater extent in the hardier tissue (apical region) of the more winter-hardy Wintok oat and rye than in the less winter-hardy Fulghum oat. However, simple sugars, which have long been considered to be cryoprotectants, decreased in both regions of the crown of all three winter cereals during the time when freezing tolerance increased. During the period when subzero acclimation has been shown to occur [21], simple sugars increased to a greater extent in the more hardy apical region in all three varieties.

While some results were correlated with the freezing tolerance of the apical region, the change in simple sugars during CA was not. This suggests that to establish cause and effect relationships between metabolites and whole-plant survival, the localization of a particular cold-activated compound or gene and how it (or its product) interacts with tissue and/or water is a more important issue than its abundance in bulk tissue. With some exceptions [3,11,13,30, to cite a few] most studies on freezing tolerance in plants have been carried out using whole plants or parts of plants. Many of these parts,

such as leaves, do not survive freezing and their relationship to whole-plant recovery following freezing is arguable [26,27]. It will be important to show that cryoprotective compounds are either present in regions or within cells that are crucial for survival, or that they directly impact crucial tissue in some way, if one is to decisively conclude that these compounds are important for survival of the whole plant.

In the interim, it should be recognized that numerous mechanisms for protection from freezing exist in winter cereal crowns and that those mechanisms are closely associated with millions of cells, all interacting with each other and with various cold-responsive compounds in ways we may not determine for some time. These interactions would certainly result in a redundancy of cell/tissue systems, which could permit the plant to bypass freeze-damaged areas of tissue and ensure whole-plant survival.

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