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Carbohydrate Changes in Winter Oat Crowns during Recovery from Freezing

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ABSTRACT

Fructan is an important cryoprotectant in plants but its exact mode of action is controversial. Much of the difficulty in identifying the mode of action is related to the lack of localization of fructan within tissues that are important for survival of the whole plant and a lack of studies on changes in fructan concentration after freezing, prior to the formation of new leaves. During recovery after freezing, fructan could ostensibly act as an energy source as well as a source for precursor molecules involved in cell wall synthesis/repair. We dissected 4 zones of the crown and quantified fructan and simple sugars. Winter oat plants which had been frozen and were in recovery contained about ½ the amount of total carbohydrate of those which had not been frozen, suggesting considerable utilization by the plant in the recovery process. The zone in which freezing had the biggest effect was the lowermost zone, above where the roots attach to the crown. The percentage of DP>5 fructan in this zone was significantly higher than unfrozen controls while the percentage of DP3 fructan and sucrose was significantly lower in plants recovering from freezing. Percentages of glucose and fructose were in many cases double what they were in unfrozen controls. These results suggest that carbohydrate re-allocation during recovery from freezing is an important part of overall winter hardiness. In addition, the biggest changes in CHO occurred in the first 3 days after freezing, suggesting that to better understand the metabolism of plants recovering from freezing, analysis should concentrate on the period just after freezing, prior to when new growth emerges.

Keywords: cold acclimation, freezing tolerance, fructan, histology, HPLC, shoot apex

INTRODUCTION

Yields are significantly higher in crops such as wheat (*Triticum aestivum*), barley (*Hordeum vulgare*), oats (*Avena sativa*) and rye (*Secale cereale*) when they are planted in the fall, than when they are planted in the spring. This is because normally hot and dry conditions in the summer are avoided in fall-planted crops. In addition, in some regions growers can harvest a fall-planted crop early enough to plant a second crop after the fall crop is harvested.

The only constraint to planting crops in the fall is their ability to withstand winter temperatures. Of the four winter cereals rye is the most hardy with the temperature at which 50% of the plants survived (LT_{50}) under controlled conditions of about -17°C, then wheat at -15°C, barley at -13°C and oats at -11°C (Livingston *et al*. 2006). The LT_{50} of plants tested under controlled conditions can vary considerably depending on conditions used and cultivars tested. For example, there are very winter hardy oats that can withstand freezing better than some non-hardy winter barley.

The ability to survive winter involves complex biological interactions with ice, within specific tissues of the over wintering organ called the crown (Houde 1995; Hudson and Idle 1962; Livingston *et al.* 2005, 2006; Olien 1981; Pearce *et al.* 1998; Shibata and Shimata 1986; Tanino and Mckersie 1985). In non-acclimated wheat (Tanino and Mckersie 1985) oat (Livingston *et al.* 2006) and barley (Olien 1981) the apical meristem was identified as the tissue within the crown that was most susceptible to freezing stress. After 3 weeks of cold-acclimation at 3°C whole plant survival in oat increased dramatically, primarily due to an increase in the freezing tolerance of the apical meristem (Livingston *et al*. 2006). In fact, after cold-acclimation the apical meristem appeared undamaged while the lower portion of the crown, called the crown core, degenerated completely due to freezing (Livingston *et al.* 2005, 2006).

The effect of freezing on specific tissues within organs of other grass species has also been documented. In maize, mesophyll cells had collapsed when frozen but, bundle sheath and epidermal cells were apparently undamaged (Ashworth and Pearce 2002). Changes in the ultrastructure of meristematic cells of tall fescue (*Festuca arundinacea*) that were frozen and thawed included "swelling and disruption of organelles, accumulation of osmophilic material and contraction of the nucleus" (Pearce and McDonald 1977). In orchardgrass (*Dactylis glomerata* L.), the apical meristem was the most freezing-tender part of the crown in plants that had been cold acclimated (Shibata and Shimata 1985).

Considerable research has been published on metabolic changes during cold acclimation (Pearce 2004; Livingston *et al*. 2007) but very little attention has been devoted to biochemical and histological adaptation that plants undergo during recovery from freezing. This approach would be analogous to infecting a plant with a disease and monitoring its response to the disease.

Carbohydrates are the main source of energy in plant as well the source for various compounds involved in plant components such as cell walls (Salisbury and Ross 1992). In an ongoing effort to understand why oats are the least freezing tolerant winter cereal we conducted a series of experiments to see how carbohydrates change during recovery from freezing in 4 regions of the crown (**Fig. 1**). Carbohydrate changes over time, in plants that had been frozen, were compared to unfrozen plants, grown under the same conditions in which the recovering plants were growing. Differences between the 2 treatments presumably reflect the effect of freezing on carbohydrate metabolism in various regions of the crown.

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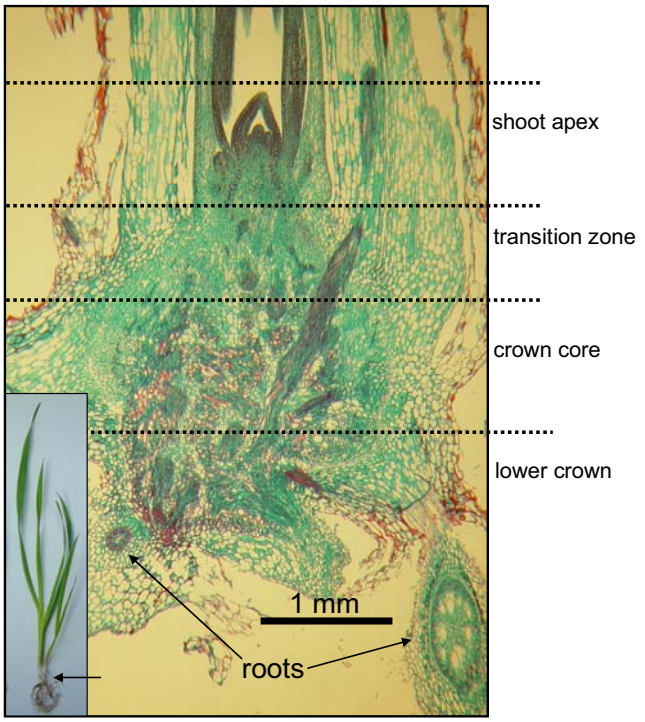

Fig. 1 Longitudinal section of an unfrozen oat crown prepared as described by Livingston *et al.* (2009). The four zones that were analyzed separately for carbohydrates are listed on the right side. The inset shows an intact plant with the arrow pointing to the crown from which this section was taken.

MATERIALS AND METHODS

Plant materials

Oat is the least winter hardy cereal crop and therefore stands to gain the most from any potential mechanism to improve freezing tolerance. The cultivar 'Wintok' has been used as a winter hardy oat check for over 40 years in winter cereal nurseries worldwide; its seeds were germinated in conical plastic tubes (2.5 cm diameter by 16 cm high) with holes in the bottom to allow drainage (model RLC4, Stuewe & Sons, Tangent OR, USA). They were planted in a soil mix of Fafard 4P (Conrad Fafard, Inc., Agawam, MA). The tubes were suspended in a grid that held 100 tubes. Plants were grown in a growth chamber (Environmental Growth Chambers, Chagrin Falls, OH) for 5 weeks at 13°C with a 12-h photoperiod at 380 µmol m^2/s. After 5 weeks, plants were transferred to a different growth chamber for cold-acclimation at 3°C with a 12-h photoperiod at 300 µmol m^2/s for 3 weeks. Plants were watered daily and three times weekly with a complete nutrient solution (Livingston 1991).

Freeze test

After cold-hardening, plants were removed from tubes. Soil was removed and rinsed from roots in ice-water. Excess water was eliminated by briefly blotting the washed plants on paper towels. Plants were trimmed to 2 cm of roots and 5 cm of leaves as measured from the base of the crown. Crowns were inserted into slits cut into 4 cm high × 7.5 cm diameter circular sponges so that the crown and the root portions of the plants were completely covered by the sponges. Sponges were pre-soaked in ice-water and squeezed to remove excess water. Sponges with crowns were placed in plastic bags sprinkled with ice shavings to promote nucleation and prevent super-cooling when frozen. The bags were loosely tied to prevent desiccation. Sponges were placed on iron flanges to promote thermal stabilization. Sponges and crowns were kept in freezers at -3°C for 6 h to allow complete freezing. The temperature was reduced at 1°C/h to -10°C and kept there for 3 h. The temperature was then raised to 3°C at 2°C/h. After the freeze-test roots were trimmed to the base of the crown. Crowns were transplanted into trays containing Fafard 4P soil mix and allowed to recover for 21 days in a growth chamber with similar conditions to the germinating step, at 13°C with a 12-h photoperiod at 380 µmol m^2/s.

Carbohydrate extraction

Water-soluble carbohydrates were extracted from whole crowns (harvested as described above) immediately after their removal from sponges (day 0) after the freeze test and at 1 day, 3 days and 7 days during recovery. Crowns were rinsed in ice water to remove soil particles. Side tillers or secondary and tertiary tillers (**Fig. 2**) were removed from crowns with a razor leaving the primary tillers (the central and largest tiller in diameter) (**Fig. 2F**). The outer leaves of the primary tiller were slowly peeled off exposing the shoot apex. Under a dissecting microscope, the shoot apex was cut off using a razor-knife and collected into a tared 1.5-ml Eppendorf® tube on ice. Three 1mm sections representing the transition zone, the crown core, and the lower crown (**Fig. 3**) were collected after isolation of the shoot apexes. Seven plant samples per zone were placed into the Eppendorf® tubes which also contained three

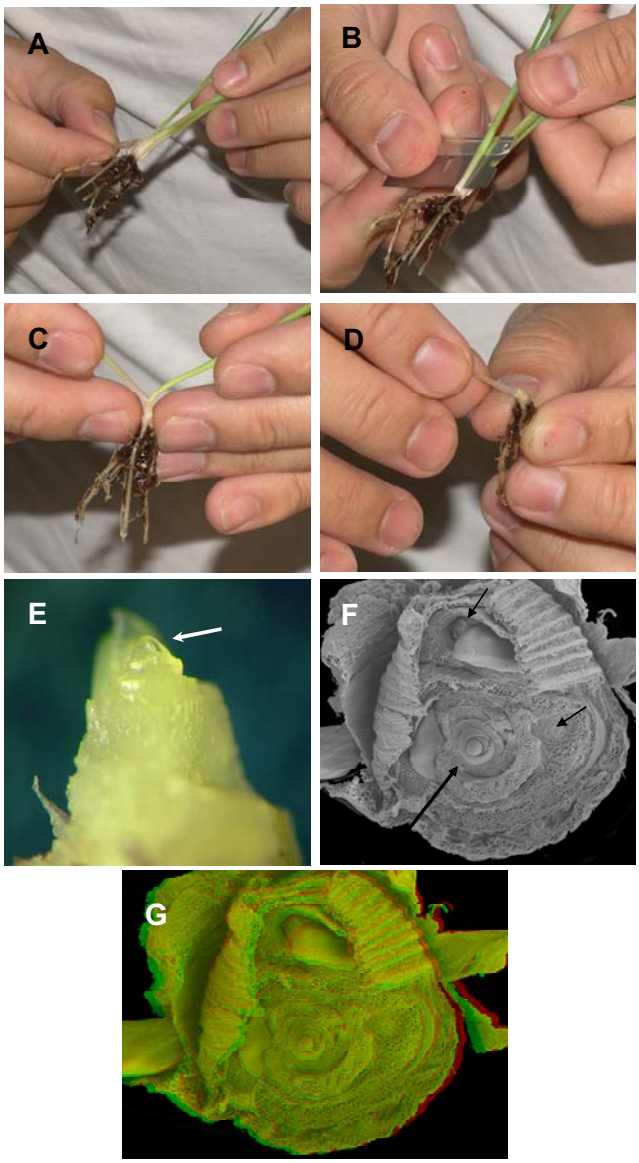

Fig. 2 Sequence used to expose the shoot apex in preparation for dissection of the 4 zones in Fig 1. (**A**) Peeling off the outer leaves of an intact plant. (**B**) Cutting off the secondary and tertiary tillers. (**C**) Peeling back the outer leaves of the primary tiller. (**D**) Exposing the shoot apex of the primary tiller. (**E**) The shoot apex (arrow) of the primary tiller. (**F**) Scanning electron micrograph of the top of an exposed shoot apex (single large arrow). Secondary and tertiary tillers (2 small arrows) remain in place. (**G**) The same photograph in **F** but in 3 dimensions. To see the image in 3-d the viewer must wear red-green glasses.

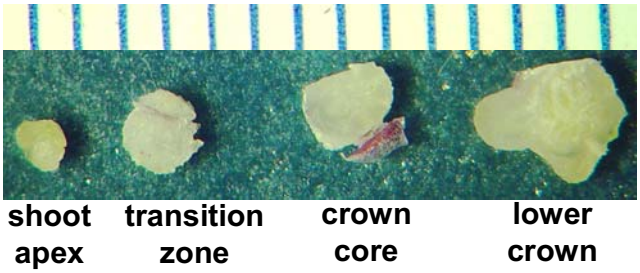

Fig. 3 The four zones that were removed from a single primary tiller of an oat plant. The segments in blue above the tissue are 1 mm apart.

glass beads (0.33 mm in diameter). The weight of the tube with beads was recorded before adding plant tissue. Fresh weight was determined by subtracting the weight of tube and beads from the total weight. Tubes were placed immediately in liquid nitrogen after weights were recorded. Samples were shaken for 30 s using a Silamat S5 amalgamator (Ivoclar Vivadent). Four hundred mL of HPLC-grade water was added to the powdered plant sample in each tube. Tubes were briefly vortexed, placed in 90ºC water bath for 15 min, and centrifuged at 500 × *g* for 5 min at 4°C using a Beckman GS-6R centrifuge (Fullerton, CA). Fifty µL of supernatant from each sample was used for carbohydrate analysis.

Carbohydrates from unfrozen plants were used as controls. Unfrozen controls were subjected to the same treatments as the frozen plants. However, instead of being frozen, they were kept in a 3°C chamber for the duration of the test.

Extracted samples were injected into a Bio-Rad (Richmond, CA) Aminex HPX-42A (silver-based) analytical HPLC column (7.8 × 300 mm). 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 0.6 mL/min. Carbohydrates were detected with a Waters (Millipore Corp, Milford, MA) 410 Refractive Index Detector and quantitated using external standards.

Data analysis

The experiment was conducted and analyzed in a completely randomized design. Three replications were done for each zone for all treatments. Each replication contained 7 randomly chosen recovering plants. Concentrations of glucose, fructose, sucrose and fructans with degree-of-polymerization (DP) greater than 5, 5, 4, and 3 were measured using Class-VP 7.x Software (Shimadzu Corp., Kyoto 604-8511, Japan). Analysis of variance (ANOVA) was performed using SAS V. 9.1.3 (SAS Institute, Cary, NC).

RESULTS

Regrowth following freezing

Immediately after thawing, leaves appeared water soaked as described by other researchers (Palta *et al.* 1978) but by the time plants were transplanted and photographs taken (approximately 1 h), any difference in leaf appearance between frozen and unfrozen controls was imperceptible (not shown). By day 1 (24 h after thawing) the extension of stems in plants without leaves was obvious. By day three, significant extension of stems was observed. Many stems which grew initially, stopped expanding after day 3 and by day 7 had begun to die. By day 7 new growth was visible from the center whorl of leaves of tillers which had survived the freeze test. Most of these tillers completely re-grew and produced a new plant.

Changes in total carbohydrates in the primary tiller

Significant differences between the 3 tillers were found in carbohydrates (not shown). Therefore, to reduce experimental variability caused by tiller differences when all 3 tillers are bulked, the primary tiller was selected in which to measure carbohydrates.

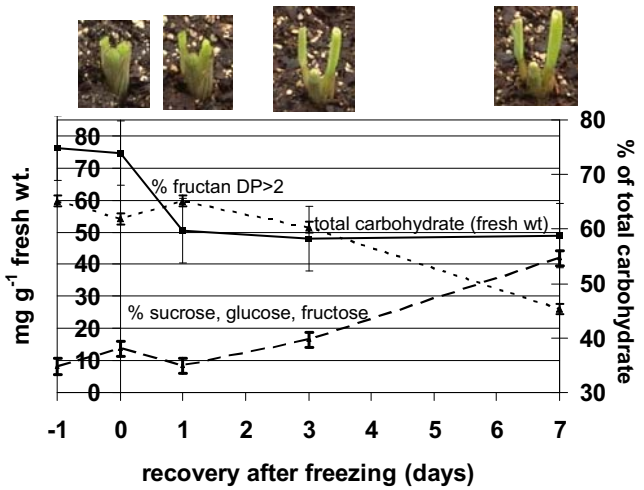

Fig. 4 Total carbohydrates in the entire crown (four zones combined) of the primary tiller of oats (left-hand Y-axis) and the percentage of fructan and simple sugars (right-hand Y-axis) showing how they change over time after being frozen. Day 0 is immediately after freezing and Day -1 is just prior to freezing (unfrozen, cold-acclimated plant). Photographs above the graph show how the plant re-grows after freezing at each time period. Error bars at each data point represent the least significant difference at a probability of 0.05.

Very little difference in total carbohydrate per mg fresh wt was observed between the primary tiller of unfrozen plants (77 mg/g) and that of plants that were frozen (75 mg/g) (**Fig. 4**) at Day 0. However, even though total carbohydrate remained nearly the same, a small but statistically significant percentage of fructan was reduced while an equivalent percentage of the sugars, sucrose, glucose and fructose increased.

During the first day of recovery from freezing total carbohydrate went down 25 mg/g fresh weight while the percentage of fructan went up with a concomitant decrease in simple sugars.

After day 1, changes in total carbohydrate were not statistically significant up to day 7. During this time period the percentage of fructan continually decreased from its high of 65% at day three down to 45% at day 7. Sugars on the other hand gradually increased during the same period from 35 to 55% at day 7.

Total carbohydrate changes in tissue dissected from primary tillers

The crown was divided into 4 sections on the basis of differences in damage observed histologically (Livingston *et al.* 2009). In the uppermost zone, the shoot apex, when frozen, complete deterioration was observed with no discernable cellular structure remained (Livingston *et al.* 2009). Just below the shoot apex, the transition zone contained considerable dark-staining regions that appeared to be barriers (Livingston *et al.* 2009). Below the transition zone was the crown core in which freeze-damage was characterized primarily by tissue disruption. The lowermost region of the crown called the lower crown was a region that had more of what appeared to be vessel plugging than any other region (Livingston *et al.* 2009).

The concentration of total carbohydrates was half as high in frozen tissues as it was in tissues dissected from unfrozen plants (**Fig. 5**). By day 1 total carbohydrates in the shoot apex (**Fig. 1**) were unchanged from day 0 while they were slightly reduced in the transition zone and in the crown core. Total carbohydrates in the lower crown were about 3.5 times lower in the frozen plants after 1d in recovery than they were in unfrozen plants (**Fig. 5**). This trend continued up to day 3 with carbohydrates in the lower

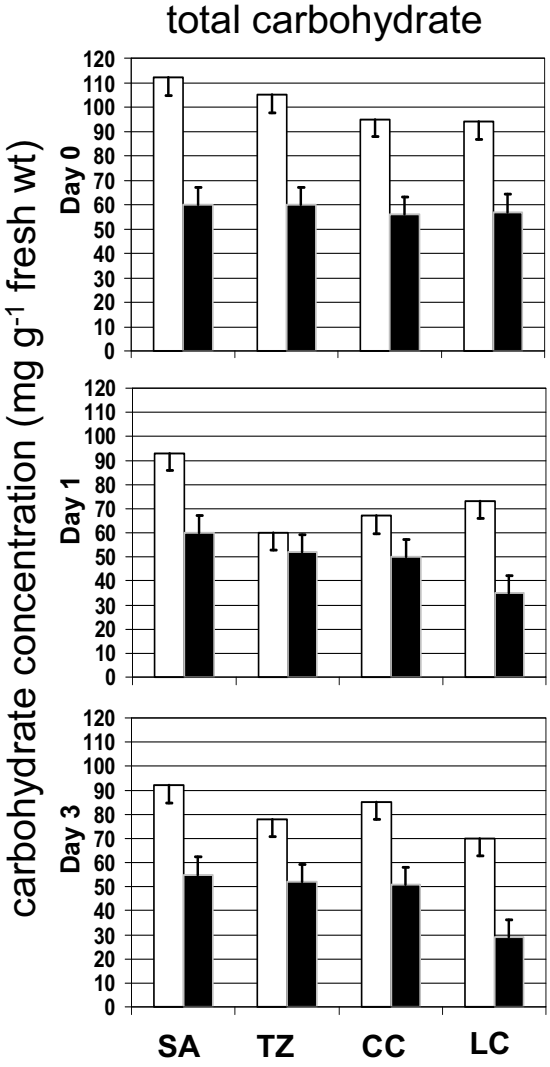

Fig. 5 Changes in total carbohydrate on a fresh weight basis in the first 3 days after freezing. Note the higher concentration in control plants (white bars) that were grown and extracted with the recovering plants (black bars) but had not been frozen. SA is the shoot apex (see **Fig. 1**), TZ is the transition zone, CC is the crown core, and LC is the lower crown. Error bars above graphs indicate the least significant difference at a probability of 0.05.

crown 5 times lower than they were in unfrozen plants (**Fig. 5**).

Change in percentages of carbohydrates in crown tissues over time

The largest concentration of carbohydrate (between 30 and 40%) was DP>5 fructan and the second largest, (between 20 and 30%) was sucrose (compare **Figs. 5-8**). The percentages of various carbohydrates were significantly different between tissues and between days after recovery but only in day 0 in the lower crown and in day 1 of the crown core and lower crown were percentages of DP>5 fructan significantly higher than unfrozen controls (**Fig. 6**). Significant differences from unfrozen controls in sucrose (**Fig. 6**), glucose and fructose percentages (**Fig. 8**) were observed in all 4 regions of the crown as well as in every day after freezing, except for day 1 in glucose.

Differences from unfrozen controls were very small in all tissue during recovery in DP5 Fructan (**Fig. 7**). Slight differences from controls were observed in DP4 and DP3 fructan (**Fig. 7**). Fructose and glucose in frozen plants were significantly higher in all 4 zones (except for glucose at day one (**Fig. 8**)).

DISCUSSION

Cold-acclimation is a biochemical adaptation to above freezing temperatures that allow plants to withstand a certain level of freezing stress. With out cold-acclimation the ability of plants to withstand freezing is restricted. Under controlled conditions the LT_{50} of non-acclimated oats was -4.9°C while the LT_{50} of cold acclimated oats was -10.7°C (Livingston *et al*. 2006). Other species exhibit similar effects from cold acclimation with wheat increasing in freezing tolerance during cold acclimation up to 4°C (Houde *et al*. 1995; Herman *et al*. 2006) and Arabidopsis up to 7°C (Gilmore *et al.* 1988).

Cold-acclimation is an important component of overall freezing tolerance, and is evaluated prior to a freezing treatment. However, except for measuring plant survival, how the plant adapts *after* being frozen, is generally overlooked. In contrast to freezing tolerance, studies involving biotic stress (such as plant disease), evaluate how plants react biochemically to a pathogen after exposure to stress. The ability of, or extent to which, the plant adapts to this stress helps determine the nature of its resistance. One common pathogenic response is the production of phenolic compounds which are thought to inhibit ingress of the pathogen by formation of lignin (see Nicholson and Hammerschmidt 1992 for a review of the role of phenols; Carver *et al*. 1996).

In most pathology studies where it is measured, total carbohydrate, including sucrose, increases after exposure to a pathogen (Hwang *et al.* 2008; Junqueira *et al.* 2004; Lehrer *et al.* 2007; Lobato *et al.* 2009). In our study, total carbohydrate, including sucrose, decreased after freezing and thawing. This is presumably because once the plant thaws the stress is removed and if it was not severely frozen, some growth initially occurs. Growth after thawing is presumably fueled by carbohydrates that accumulated during cold-acclimation. This would explain the most rapid decrease in carbohydrates just prior to when the most rapid growth was noted (**Fig. 4**). After day three, total carbohydrate remained the same (**Fig. 4**) while the percentage of simple sugars increased (sucrose **Fig. 6**; glucose and fructose **Fig. 8**). This is presumably because the plant had grown new leaves and was able to synthesize the carbohydrates it needed to grow further.

Response of separate zones within the crown

Total carbohydrate concentration at day 0 (immediately after thawing) could have been significantly lower than unfrozen controls in all 4 zones (**Fig. 5**) because of an increased water content in the tissues being sampled. The percent moisture of tissue in day 0 and day 1 was slightly higher than unfrozen controls and differed between zones (not shown). By day 3, percent moisture had returned to levels of that in unfrozen controls. Previous studies indicated a significant increase in apoplastic water content (Herman *et al.* 2006) in crowns of plants that had been frozen at -3°C. Because of this variation in water content between frozen and unfrozen plants as well as the 4 zones, percentage of total carbohydrate was calculated for individual carbohydrates (**Figs. 6-8**).

Despite the return to more normal percent water at day 3, total carbohydrates were significantly lower in the lower crown than they were in the other 3 zones. This could be because this was the region of the crown where damage was most apparent. In plants frozen at -12°C, complete deterioration of the lower crown was reported (Livingston *et al.* 2005). However, this could not be confirmed in this study because carbohydrate extraction is a destructive form of sampling, therefore it was not possible to perform a histological analysis on the same tissue that was used for carbohydrate determination. The survival of barley was highly correlated with tissue damage (as assessed by tetrazolium staining) in the central area of the base of the crown (Olien 1981; Pearce *et al.* 1998). However, the apical meristem of *Dactylis glomerata* was less freezing tolerant than that of

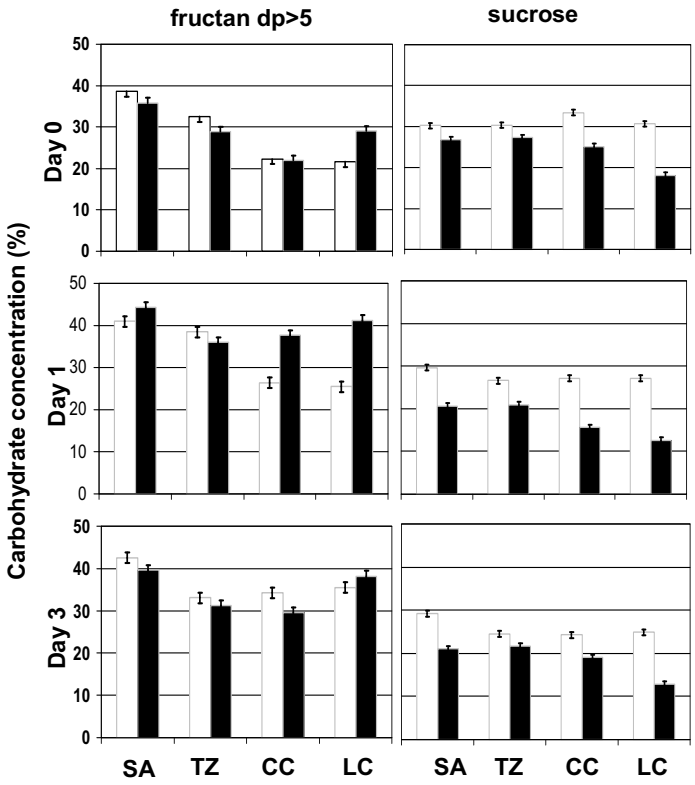

Fig. 6 Changes in percentage of large DP fructan and sucrose in the first 3 days after freezing. Control plants (white bars) were grown and extracted with the recovering plants (black bars) but had not been frozen. SA is the shoot apex (see **Fig. 1**), TZ is the transition zone, CC is the crown core, and LC is the lower crown. Error bars above graphs indicate the least significant difference at a probability of 0.05.

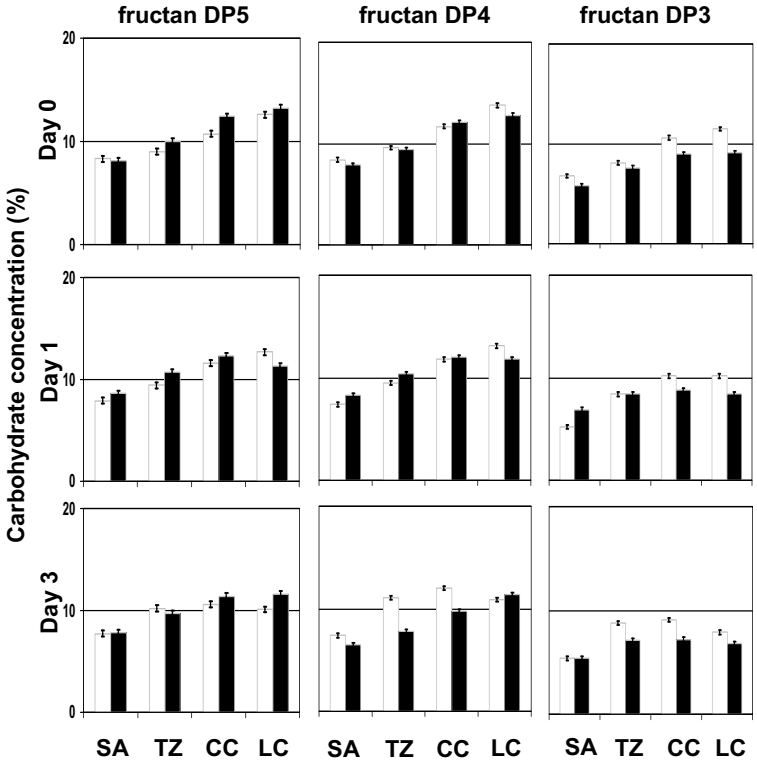

Fig. 7 Changes in percentage of fructan DP 5,4, and 3 in the first 3 days after freezing. Control plants (white bars) were grown and extracted with the recovering plants (black bars) but had not been frozen. SA is the shoot apex (see **Fig. 1**), TZ is the transition zone, CC is the crown core, and LC is the lower crown. Error bars above graphs indicate the least significant difference at a probability of 0.05.

the lower crown as evidenced by histological analysis (Shibata and Shimada 1986); the apical meristem in non-acclimated wheat was also less freezing tolerant than that of the lower crown (Tanino and McKersie 1985).

While carbohydrates in some regions of the crown on some days were not significantly affected by freezing (see for example fructan DP>5 in the shoot apex, transition zone and crown core in day 0 and the shoot apex and transition zone on day 1 and day 3 (**Fig. 6**)), other regions, as judged by their difference from unfrozen controls, were significantly affected by freezing. See for example fructan DP>5 in the lower crown on day zero, and the crown core and lower crown on day 1, as well as every region on all 3 days for sucrose (**Fig. 6**).

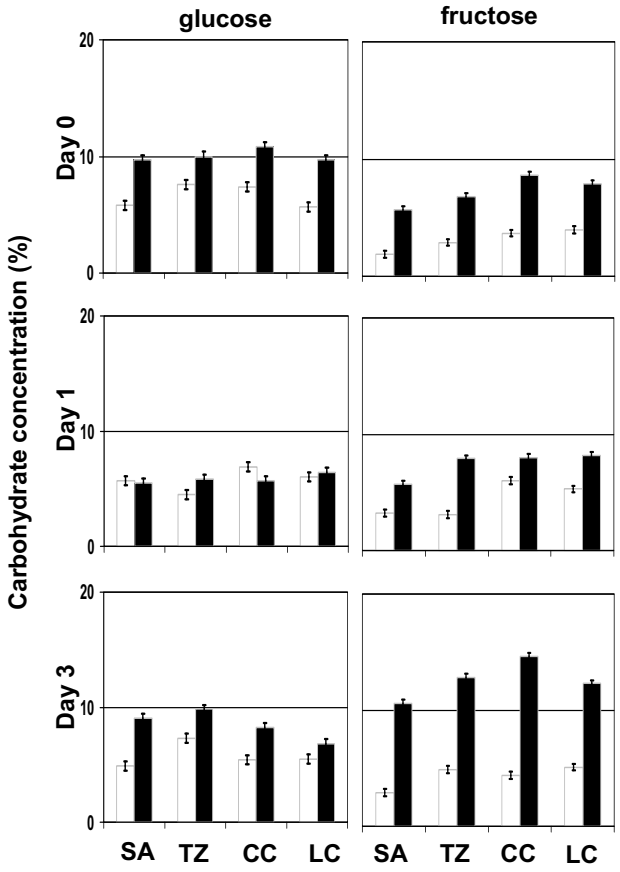

Fig. 8 Changes in percentage of glucose and fructose in the first 3 days after freezing. Control plants (white bars) were grown and extracted with the recovering plants (black bars) but had not been frozen. SA is the shoot apex (see **Fig. 1**), TZ is the transition zone, CC is the crown core, and LC is the lower crown. Error bars above graphs indicate the least significant difference at a probability of 0.05.

The higher percentages of glucose and fructose in plants that had been frozen suggests a higher invertase activity but this will need to be confirmed. Besides providing monomers for synthesis of other substrates involved in cell repair, it is not known how a higher invertase activity could be associated with recovery from freezing.

These differences between different zones underscore the need to analyze regions of the crown (as opposed to the whole crown) if one is to discover biochemical reactions of the plant that are causatively related to specific histological responses.

CONCLUSION

Using carbohydrates as a model, we have shown that statistical differences between regions of the crown can be identified from dissected tissue. It is tempting to speculate that carbohydrate differences in response to freezing are related to differences in the forms of freeze damage or how the plant responds that damage in the 4 zones. However, more information is needed to make this assumption. For example what is the composition of the barrier in the transition zone? Or, why is tissue within the crown core more susceptible to disruption? What is the material found in plugged vessels of the lower crown? Experiments are underway to investigate these and other questions in an attempt to determine how recovery from freezing tolerance might be affected by various biochemical responses.

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