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Understanding the response of winter cereals to freezing stress through freeze-fixation and 3D reconstruction of ice formation in crowns

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ABSTRACT

One of the more difficult aspects of discovering mechanisms involved in winterhardiness is detecting where ice is formed and how it interacts with tissues in the frozen state. Many tissues recover and change shape during thawing which prevents a clear picture of ice formation and how individual cells might have responded to this form of stress. Cryo-sectioning and related techniques, while providing valuable information, only allow a two-dimensional view of what is in fact, a three-dimensional phenomenon. In this study, an established freeze-fixation protocol was used in conjunction with histology to visualize empty spaces or voids created by ice within crowns of oat. Images of sections were aligned and background color was cleared to provide 3D visualization of voids that had formed within tissues as a result of freezing. Reconstruction in 3 dimensions revealed that ice had formed continuously in the roots but terminated at the root-shoot junction. This supports previous research that a barrier exists at the base of the crown in freezing tolerant cultivars of winter cereals. In addition, ice-induced voids within the crown were narrow and vertically inclined; they did not form large spherical shapes as had previously been suggested from two-dimensional analysis. Within apical regions of the crown, voids always formed just below the epidermis on what would eventually become the lower surface of the leaf. The 3D structure of these formations resembled a curtain with a termination point at the top of the transition zone and which extended continuously into the leaves. These results suggest that multiple mechanisms must be operative concurrently for the crown to survive. This underscores the need for a variety of approaches that includes clear and detailed observational data to fully comprehend winter survival of cereal crops.

1. Introduction

Hardiness in winter cereals can most simply be thought of in terms of four stages: (1) cold-acclimation, (2) freeze-acclimation, (3) freezing and thawing event(s), and (4) recovery from freezing. During each stage, gene expression within the crown of the plant interacts with numerous fluctuating environmental conditions producing nearly an infinite set of circumstances that determine if a plant will ultimately survive and produce a crop. From an environmental perspective, the duration and rate of change during each stage provides for a most challenging phenomenon to study.

While each of the 4 stages of winterhardiness has been researched, the stage with perhaps the most noticeable effect on winterhardiness is cold acclimation (CA). For that reason, this aspect of winterhardiness has received the greatest attention (Hughes and Dunn, 1996; Pearce, 2004; Thomashow, 1998; Cook et al., 2004). Numerous biochemical changes occurring during a cool but above-freezing period have been associated with the ability of plants to withstand freezing temperatures.

Freeze-acclimation has been less studied and while resulting in comparatively less of an increase in freezing tolerance than cold-acclimation, it is none the less essential if maximum hardiness is to be attained (Trunova, 1965; Olien, 1984; Livingston, 1996; Herman et al., 2006).

Arguably, the most difficult stage to study, for technical reasons, is the actual freezing event(s) to which the plant is exposed. Various attempts have been made to visualize ice formation in plants (McCully et al., 2009) beginning with a report by Prillieux in 1869 (Cited by McCully et al., 2004). The amount of water in and around the plant affects the dynamics of freezing (Olien, 1974) and the rate of temperature change determines if freezing will take place in an equilibrium or non-equilibrium manner (Olien, 1973; Olien and Livingston, 2006). These and other factors determine precise types of stresses within the plant and result in different kinds of injury to tissues. The interface between ice and plant tissues that results in adhesions (Olien and Smith, 1977) and in other interactions (Pearce,

Abbreviations: CA, cold acclimation; mFAA, formaldehyde/acetic acid fixative with methanol instead of ethanol.

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2001; Pollack, 2013) take the complexity of winterhardiness to levels that will afford topics of research for years to come.

Recovery from freezing has been studied from a histological as well as a metabolic point of view in several plant species (Eagles et al., 1993; Livingston et al., 2005, 2013; Olien and Marchetti, 1976; Tanino and McKersie, 1985). These studies demonstrate that the most freezing tolerant tissue within the crown is the transition zone subtending shoot meristems. However, if vessels that transport water and nutrients are destroyed, the meristems may grow initially but will soon die. Because of this, while most of the central portion of the crown can be killed by freezing, as long as root and shoot meristems survive as well as the vessels supplying water and nutrients, the plant will regrow after being frozen.

This study is an attempt to provide information related to mechanisms involved in responses of plants to the actual freezing event. More specifically, our purpose is to provide a basis for understanding the interaction of ice with specific cells and tissues within the crown of oat (*Avena sativa* L.). Furthermore, since formation of ice occurs spatially in 3 dimensions it would be most informative to visualize these tissues in 3D. Therefore, a recently developed histological reconstruction technique (Livingston et al., 2010) was used to identify ice formation within crowns in 3D.

2. Materials and methods

2.1. Plant growth

Wintok oat has been used as a cold hardy oat check cultivar in the Uniform Oat Winterhardiness Nursery since 1964. Under our growing and freezing conditions it has an LT50 of −12 °C. Individual plants were grown in cylindrical tubes (12.5 mm diameter) in a soil mix (Fafard 2P, Fafard, Inc., Anderson, SC). The tubes had an opening at the bottom to provide drainage. They were grown in a growth chamber for 5 weeks at 13 °C and 300 μmoles of light and then at 3 °C for 3 weeks (CA) under 300 μmoles of light as described (Livingston et al., 2005).

2.2. Freezing and fixing crowns

After CA, 5 plants to be used for histological analysis were washed free of soil mix, and excess water was removed by patting plants dry with a paper towel. They were then placed in a sponge and inserted into the opening of a 125 mL Erlenmeyer flask. The sponge held the lower part of the leaves and the crown was suspended in the air inside the flask. The roots at the bottom of the flask were submerged in ice shavings to ensure freezing was initiated when the freezer temperature went below zero. We did not monitor the precise tissue in which freezing initiated, so it is possible, despite roots being submerged in ice, that ice initiation began in leaves. Flasks were placed inside a plastic bag with the opening tied shut to help limit desiccation. Flasks with plants were placed in a freezer at −1 °C and held for 1 h before ramping at 1 °C per h to the test temperature of −12 °C. They were held at −12 °C for 3 h and then roots and leaves were quickly trimmed inside an insulated glove box. The resulting crown (bottom 2 cm of the stem with about 2 mm of roots) was plunged into −12 °C fixative inside the same chamber. This process was repeated 3 times with a total of 15 plants to be used for histological observations.

The fixative was based on FAA described by Johansen (1940) but with methanol substituted for ethanol (hereafter referred to as mFAA). This change reduced shattering during sectioning of crown tissue and resulted in significantly less disruption of cell contents (Livingston et al., 2009) typical of formaldehyde based fixatives.

To determine the length of time needed to fix crowns in mFAA fixative at −12 °C, crowns that had been frozen for 3 h and left in fixative for 24 h at −12 °C were compared to crowns that had been frozen 3 h and left in fixative for 7 days. No difference in void size/location or in tissue structure was observed (not shown) between the 2 fixing times. In addition, ice (1.5 g) at −12 °C placed in mFAA fixative, also at −12 °C, completely melted in 20 min. These preliminary experiments suggested that freeze-fixing crowns for 24 h at −12 °C would result in complete fixation.

After fixing, crowns were removed from the freezer and allow to warm to room temperature, whereupon they were processed using microwave dehydrating and paraffin-embedding as described previously (Livingston et al., 2009).

Embedded crowns were sectioned at 20 μ, stained with Safranin and fast green and photographed with a Sony DSC707 camera mounted on a Nikon 50i light microscope.

2.3. Image processing

For 3D reconstruction, 186 sequential JPEG images of freeze-fixed and stained sections were imported into After Effects (Adobe Systems Inc., San Jose, CA). Images were aligned and background color was removed as described (Livingston et al., 2010). Voids were considered to be a result of ice formation only if at least 3–5 sequential images showed the same tissue/cell separation at the same anatomical location in the section. In addition, sections from frozen plants were carefully compared to unfrozen controls and only voids that were not present in sections from unfrozen plants were considered to be caused by ice formation. Voids were identified and color coded using the Roto-Brush tool within After Effects and an animated video was created as described (Livingston and Tuong, 2014). While 5 crowns were selected for reconstruction, only one is presented here as a representative.

2.4. Tetrazolium

To identify live and dead tissue, 10 crowns that had been frozen and allowed to recover at 13 °C for 3 days, along with unfrozen controls, were cut longitudinally and incubated in 0.5% 2,3,5-tryphenyl tetrazolium chloride in a 50 mM HEPES solution (pH 7.3) for 24 h at 21 °C. The cut surface of the incubated crowns was photographed under a dissecting microscope at 20X (see AOSA, 2000; Briggs et al., 2009 for more procedural details).

2.5. Aniline blue

To determine the ability of conducting vessels to transport water and nutrients, 5 plants that had frozen and allow to recover for 3d, along with unfrozen controls, were placed in a 1% solution of aniline blue in a phosphate buffer at pH 4.0. The blue color of the solution was most visible at this pH and the color was not affected by its migration through the plant even up to the tips of leaves (not shown).

3. Results

3.1. Ice formation

The freeze-fixation process used here was developed to observe ice formation in rodent hearts (MacKenzie et al., 1975) and was adapted for use in plants by Ashworth (1990). Ashworth demonstrated that the tissue voids in frozen and fixed plant samples corresponded to ice crystals that had formed in plant tissues. Olien (1964) observed voids that were filled with ice in crowns of barley, in the same regions we describe here. We have determined that ice melts within 20 min at −12 °C in the fixative used here (see Section 2) and that crown tissue is indeed fixed by mFAA (Livingston et al.,

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