Increased Cell-Wall Mass and Resistance to Freezing and Snow Mold during Cold Acclimation of Winter Wheat under Field Conditions

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Abstract: Accumulation of soluble carbohydrates plays an important role in enhancement of resistance to freezing and snow mold of plants during cold acclimation. Nevertheless, few studies have examined whether changes in cell wall properties are involved in enhancement of resistance during cold acclimation. In this study, four winter wheat cultivars were sown in a field on six different dates during August–October, and their resistance to freezing and snow mold were compared in relation to soluble carbohydrate content and cell-wall mass in leaves. Resistance to freezing and snow mold was much higher in the plants sown on 23 September than in those sown on 9 September. The percentage of cell-wall mass in leaf to total dry mass (%CW) and water-soluble carbohydrate content also increased considerably during 9–23 September. Multiple regression analyses revealed that %CW contributed significantly to freezing resistance, whereas total water-soluble carbohydrate content contributed significantly to snow mold resistance. These results suggest that increased %CW enhances freezing resistance during cold acclimation.

Key words: Carbohydrate accumulation, Cell-wall mass, Cold acclimation, Freezing resistance, Snow mold resistance.

Resistance to freezing and resistance to snow mold are important traits for winter crops in cold regions that are exposed to low temperature and deep snow accumulation in winter. Although freezing injury and snow mold damage are derived from different physiological mechanisms (Hofgaard et al., 2003), cold acclimation enhances resistance to freezing and snow mold. Accumulation of soluble carbohydrates is an important physiological mechanism that enhances resistance to freezing and snow mold (Levitt, 1972; Yoshida et al., 1998; Gaudet et al., 1999; Wanner and Junttila, 1999). However, because the cold acclimation process involves altered expression of many genes and corresponding biochemical and physiological changes (Thomashow, 1998), many physiological changes, in addition to accumulation of soluble sugars, are involved in enhanced resistance to freezing and snow mold.

Mechanical properties of cell walls are reportedly associated with dehydration tolerance of cells and with resistance to infestation of pathogens. For example, rigid cells can resist physical disruption of the cell structure that is caused by freezing-induced dehydration (Rajashekar and Burke, 1996). Murai and Yoshida (1998) showed that the cell wall plays important roles in increasing freezing resistance during cold acclimation in Helianthus tuberosus. On the other hand, a thick cell wall retards penetration by pathogenic microorganisms (Sherwood and Vance, 1980). However, roles of the cell wall in enhancing resistance to freezing and snow mold during cold acclimation have not been well studied under field conditions. We focused on changes in nonstructural and structural carbohydrates during cold acclimation, and hypothesized that, in addition to nonstructural carbohydrates, changes in the amount of structural carbohydrates (cell-wall mass) are related to enhancement of the resistance to freezing and snow mold in winter wheat under field conditions.

In this study, four winter wheat cultivars that show different resistance to freezing and snow mold were sown in a field on six different dates from August to October. We examined: (1) whether cell-wall mass varies during cold acclimation; and (2) how changes in cell-wall mass are associated with changes in the resistance to freezing and snow mold.

Materials and Methods

1. Plant materials

In this study, we used four winter wheat cultivars differing in levels of resistance to freezing and snow mold: Norstar, Valuevskaya, Haunsberg, and PI 173438. Norstar and Valuevskaya, developed in Canada and Russia, respectively, have strong resistance to freezing and moderate resistance to snow mold. Haunsberg and PI 173438, developed in Austria...
and originating in Turkey, respectively, have strong resistance to snow mold and moderate resistance to freezing (Yoshida et al., 1998). Seeds of the four cultivars were sown on moistened filter paper in plastic trays at two-week intervals on August 12, August 26, September 9, September 23, October 7, and October 21, 2001, referred to hereafter as groups I, II, III, IV, V and VI, respectively (Table 1). Germinating seeds were transplanted to nursery flats that were compartmented into 253 (23 rows × 11 columns) square cells (2 cm × 2 cm × 3 cm in depth) filled with sterilized nursery soil containing 0.36 g N, 0.54 g P₂O₅, and 0.36 g K₂O per 1 kg soil (Ikubyou, CI Agrisystem, Tokyo, Japan). Each flat was separated into five blocks, each consisting of four rows; a single cultivar was sown in each row. Seedlings were grown in an unheated glasshouse and watered every day. After roots appeared from the bottom holes of the flats, the flats were moved to experimental fields of the Faculty of Agriculture and Life Science, Hirosaki University, where they were grown until harvest. Plants were harvested at 36 days after sowing. Table 1 shows the temperature, day length and solar radiation during cultivation.

2. Evaluation of freezing resistance

Freezing resistance was evaluated by measuring the amount of ions that leaked from leaf cells after exposure to several low temperatures (Kamata and Uemura, 2004). Young mature leaves of each cultivar were harvested from ten plants in one block of the flat. The leaves were cut into ca. 3 mm sections after being washed with deionized water. About 20 leaf sections were put into a test tube (ø 16 × 110 mm) with 100 µl of deionized water. To examine the effects of six low temperatures, we used 12 test tubes for each cultivar with two replications. Test tubes were placed in a freezer with a programmable temperature controller (LU-112; Tabai Espec. Corp., Tokyo, Japan). The freezer temperature was first set at 5°C; then it was cooled at a rate of 1°C h⁻¹. Small pieces of ice (2-mm cubes) were put into test tubes for ice nucleation when the freezer had reached −2°C. Temperatures in each experiment were selected to cover the range of freezing points of experimental plants, which were determined in preliminary studies. Samples were removed from the freezer at designated temperatures. They were then thawed overnight in an incubator at 4°C. Then, after adding 7 ml of deionized water, the test tubes were shaken at 25°C for 2.5 h. The ion concentration in each test tube was measured with a conductivity meter (Cyberscan 100; Iuchi Corp., Tokyo, Japan). After killing leaf cells by exposure to 80°C for 2 h, test tubes were shaken for 2.5 h and the ion concentration in each tube was measured again. The electrolyte leakage, EL, was calculated as follows:

\[ EL = \frac{\text{conductivity after freezing treatment}}{\text{conductivity after killing tissues}}. \]

The EL₅₀, representing the temperature for 50% leakage, was calculated using linear regression of EL against the treatment temperature.

3. Snow mold resistance

Snow mold resistance was tested by the method of Nakajima and Abe (1990). In this study, a speckled snow mold fungi, Typhula ishikariensis (M-1), was used for testing snow mold resistance. Plant survival after incubation with snow mold fungi was used as a measure of snow mold resistance. Spores of the fungi were cultured in a wheat-bran vermiculite medium (wheat bran: vermiculite: distilled water, 10 : 10 : 15 by volume) at 10°C in the dark until inoculation. At harvest, a single flat of plants grown in a field was moved to a laboratory and the medium, including cultured fungi, was scattered uniformly over the surface of the flat. The flat was covered with a 2-mm-thick moistened cotton sheet and enclosed in a black plastic bag to maintain high humidity and dark conditions; then incubated at 15°C for 5–36 days depending on the group. Five blocks of the flat were assigned to five incubation treatments. Plants were then grown for 30 days at 20°C in a heated greenhouse and survival was recorded referring to production of new leaves or roots in ten individuals of each cultivar for each incubation period. The number of lethal incubation days for 50% killing (LE₅₀) was calculated using logistic regression.

4. Soluble carbohydrate assay

Leaf tissues at each harvest were frozen in liquid nitrogen and stored at −30°C until measurement. Leaf tissues (50–100 mg) were cut into 3-mm sections and extracted in 1 ml of distilled water at 80°C for 20 min. The solution was centrifuged at 1500 g for 5 min and the supernatant was recovered. The extraction procedure was repeated five times. Supernatants were pooled and distilled water was added to give a final volume of 10 ml. The extract solution was passed through a 0.45-µm pore filter; 20 µl was injected into an HPLC with a column of 8 mm × 300 mm (Ionpak KS802; Shodex/Showa Denko K.K., Tokyo, Japan) kept at 50°C. Deionized water was used as the solvent at a flow rate of 0.5 ml min⁻¹. Peaks were detected according to their refractive index. Detected peaks were identified as fructose, glucose, sucrose, and polysaccharides (>3 degree of polymerization, DP) and were quantified using an internal commercial standard. Inulin was used as an internal standard of polysaccharides (Wako Pure Chemical Industries Ltd., Tokyo).

5. Cell-wall mass

The cell-wall mass of each leaf sample was analyzed according to the method of Osaki (1990). Dried leaf tissues (50–100 mg) were weighed and then incubated
in 100 ml of boiled neutral detergent solution (pH 6.9), containing 100 mM sodium dodecyl sulfate (SDS), 45 mM EDTA-2Na, 5 mM Na₂B₄O₇, 3 mM Na₂PO₄, 10 ml 2-ethoxyethanol, and 2 ml of C₁₀H₁₈ for 10 min. The solutions were warmed on a hot plate at 80ºC for 1 h and then filtered with a vacuum pump through glass filter paper (GA-200; Toyo Corp.). The residue was washed with boiled water and with acetone until lather had disappeared; it was then dried overnight at 80ºC and weighed. Percentage of cell-wall mass in leaf to total leaf dry mass (%CW) was calculated as follows:

\[
\%\text{CW} = \frac{\text{dry residue mass}}{\text{leaf dry mass before extraction}} \times 100\%
\]

### 6. Statistical analysis

Differences in plant weight, %CW, and water-soluble carbohydrate contents among the groups with different sowing dates and cultivars were assessed using the split plot design of analysis of variance (ANOVA) using groups as the main plot and cultivars as subplot. EL₅₀ was calculated from a linear regression of the electrolyte leakage to treatment temperature for each replication in each cultivar. EL₅₀ was tested by analysis of covariance (ANCOVA). LI₅₀ was calculated from a logistic regression for each cultivar because of the nonlinear relationship between the survival rate and incubation days. The differences in LI₅₀ between cultivars or sowing dates were tested by logistic regression analysis using the survival rate as a dependent variable, incubation days as a covariate and sowing dates and cultivars as independent variables. Chi Square test was applied to twice the negative log likelihood for each effect (SAS Institute, 2000). Standardized multiple linear regression analysis was conducted to examine the effects of water-soluble carbohydrate contents and %CW on EL₅₀ and LI₅₀.

All statistical tests were performed using JMP (SAS Institute Inc.).

### Results

Table 1 shows climatic variables (mean temperature, minimum temperature, accumulated temperature, mean solar radiation, and minimum day length) in each group with different sowing dates. The temperature and day length declined gradually with the delay of the sowing date, from group I to VI (Fig. 1). The mean temperature decreased from 22.0ºC in group I to 6.6ºC in group VI. The minimum day length decreased from...
12.5 h to 9.6 h. The mean solar radiation in group VI (5.51 MJ m$^{-2}$) was half that in group I (11.81 MJ m$^{-2}$).

$EL_{50}$ and $LI_{50}$ showed significant differences among groups with different sowing dates and among cultivars (Fig. 2 and Table 3). Fig. 2 shows the changes in $EL_{50}$ and $LI_{50}$ depending on the sowing date in each cultivar. The freezing resistance changed slightly with delayed sowing date and then much increased levels in all cultivars. The mean $EL_{50}$ decreased from $-7.05^\circ$C in group III to $-12.61^\circ$C in group IV. Valuevskaya had the highest freezing resistance among the four cultivars in all groups except for group VI. Snow mold resistance also increased in group III to IV: $LI_{50}$ increased from 19.16 days to 31.40 days. In contrast to freezing resistance, however, snow mold resistance declined in group V (29.83 days) and group VI (24.91 days). Haunsberg had the least resistance in group V and VI (Fig 2). The differences in snow mold resistance among four cultivars were smaller than those in freezing resistance.

Fig. 3 shows the differences in total leaf dry mass and %CW depending on the sowing date. The two traits showed significant differences among groups (sowing dates) and among cultivars (Table 2). The leaf dry mass increased from group I to II and then decreased until group VI. %CW showed no clear differences between group I and III, but increased with delay of sowing date from group IV. Fig. 4 shows the differences in contents of water-soluble carbohydrates (fructose, glucose, sucrose, and polysaccharide) among cultivars. Total water-soluble carbohydrate content (WSC), particularly polysaccharide content, was much higher in group IV than in group III. Fructose and glucose showed no significant differences among cultivars, although sucrose, polysaccharide, and total carbohydrate content showed significant differences among them (Table 2). These results suggest that the increases in freezing resistance and snow mold

| Table 2. Analysis of variance for shoot dry matter weight, cell-wall mass, and water-soluble carbohydrate content. |
|-------------------------------------------------------------|
| **Mean Square**                                              | **Sowing date (S)** | **Error(1)** | **Cultivar (C)** | **S×C** | **Error(2)** |
| Degree of freedom (df)                                       | 5                  | 20           | 3               | 15     | 72           |
| Shoot dry matter weight (g plant$^{-1}$)                    | 0.0451***          | 0.001        | 0.0098***       | 0.0021* | 0.001        |
| Cell-wall mass (%)                                          | 1301.22***         | 8.5          | 117.63***       | 92.13** | 7.76         |
| Glucose content (mg g$^{-1}$)                               | 159.98***          | 14.4         | 11.5**          | 34.81** | 15.1         |
| Fructose content (mg g$^{-1}$)                              | 380.23***          | 10.0         | 8.51            | 16.54   | 10.4         |
| Sucrose content (mg g$^{-1}$)                               | 710.6***           | 33.6         | 81.2            | 44.6    | 32.7         |
| Polysaccharide content (mg g$^{-1}$)                        | 2531.9***          | 65.6         | 567.4***        | 250.9** | 101.5        |
| Total carbohydrate content (mg g$^{-1}$)                    | 7331.3***          | 102.9        | 1101.5**        | 632.7** | 271.6        |

* and ** represent significance at 5%, 1% and 0.1% levels, respectively. Sowing date was tested against error(1), and those of cultivars and S x C were tested against error(2).
Table 3. Statistical test for freezing resistance ($EL_{50}$) and snow mold resistance ($LI_{50}$).

|                        | df | $EL_{50}$ Mean square | p | $LI_{50}$ Chi Square | p |
|------------------------|----|-----------------------|---|----------------------|---|
| Freezing temperatures or incubation days | 1  | 10.288***             |   | 672.2***              |   |
| Sowing group (G)       | 5  | 0.364***              |   | 248.3***              |   |
| Cultivars (C)          | 3  | 0.063**               |   | 12.2***               |   |
| $S \times G$           | 15 | 0.025**               |   | 28.4*                 |   |

*, ** and *** represent significance at 5%, 1% and 0.1% levels, respectively.

$EL_{50}$ was tested by analysis of covariance, and $LI_{50}$ was tested by logistic regression analysis.

Fig. 3. Shoot dry weight and cell-wall mass in each group. Error bars show one standard error.

Fig. 4. Content of water-soluble carbohydrates (glucose, fructose, sucrose and polysaccharide) in each cultivar in each group.
resistance by cold acclimation correspond to the change in %CW and SWC.

As shown in Fig. 5, freezing resistance (EL_{50}) negatively correlated with WSC (r = -0.76**) and %CW (r = -0.85**), while snow mold resistance (LI_{50}) positively correlated with WSC (r = 0.83**) and %CW (r = 0.74**). Since there was a close correlation between WSC and %CW (r = 0.86**), high correlation of EL_{50} with WSC and %CW does not necessarily represent independent effects of these traits on freezing resistance and snow mold resistance. Table 4 shows the results of multiple regression analyses of freezing resistance and snow mold resistance against cell-wall mass and total water-soluble carbohydrate content. Standardized regression coefficients are shown for each independent variable.

Table 4. Standardized multiple regression analysis of freezing resistance (EL_{50}) and snow mold resistance (LI_{50}) against cell-wall mass and total water-soluble carbohydrate content. Standardized regression coefficients are shown for each independent variable.

|                      | Cell-wall mass | Total soluble carbohydrate content |
|----------------------|----------------|-----------------------------------|
| Freezing resistance  | -0.7137**      | -0.0902                           |
| Snow mold resistance | 0.0580         | 0.7572**                          |

**: significant at 1% level.

Discussion

In this study, cold-acclimation under natural conditions in a field increased resistance to freezing and snow mold. However, the plants under natural conditions are exposed to complicated changes in environmental factors. Among various environmental factors, low temperature and short day length have been reported to be critical signals that act independently on induction of cold acclimation (Welling et al., 2002). Freezing resistance and snow mold resistance were markedly higher in the plants in group IV (sown on 23 Sept) than in group III (sown on 9 Sept). Polysaccharide and %CW were much higher in group IV than in group III. The minimum temperature in group III and IV was 5.8°C and 1.2°C, respectively, and the day length was 11.2 h and 10.6 h, respectively, which suggests that minimum temperature below 5°C and/or day length shorter than 11 h are important environmental factors for cold acclimation.

It is uncertain how cell wall components change...
during cold acclimation and how these changes are involved in the development of resistance. Under conditions of natural cold acclimation in the field, the %CW was much higher in group IV (about 65%) than in group III (about 50%). This great difference in %CW seemed to enhance resistance to freezing and snow mold. Multiple regression analysis revealed a significant contribution of %CW to freezing resistance, suggesting the important role of %CW on enhancement of freezing resistance during cold acclimation. Although %CW did not show any significant contribution to snow mold resistance in the multiple regression analysis, this is partly due to the fact that snow mold resistance decreased at the fifth and six sowing dates but not for %CW. The cell wall structure is associated closely with resistance to freeze-induced cell dehydration (Rajashekar and Burke, 1996). The stress acting on the cell surface increase in cell wall thickness (Nobel, 1991). Increased %CW in this study might have resulted from cell-wall thickening, which increased resistance to freeze-induced cell collapse. In addition, a rigid cell wall structure can contribute to avoidance of freeze-induced desiccation stress by creating negative pressure within cells (Rajashekar and Burke, 1996). Rajashekar and Lafta (1996) showed that cold-acclimated apple and grape plants had smaller cell-wall pores and higher cell-wall breaking pressure than did non-acclimated apple and grape plants. These studies revealed the physiological mechanisms of the relationship between freezing resistance and %CW.

Accumulation of soluble carbohydrate is well known to play an important role in the enhancement of freezing resistance and snow mold resistance during cold acclimation (Sakai and Yoshida, 1968; Levitt, 1972). Plant freezing injury mostly results from severe cell dehydration caused by extracellular ice formation (Ashworth and Pearce, 2002). Accumulation of carbohydrates decreases the osmotic potential of cell sap and decreases the cell dehydration rate (Kamata and Uemura, 2004). Furthermore, accumulation of simple sugars can stabilize the membrane structure against dehydration-induced damage resulting from interactions with the membrane surface or surrounding water (Thomashow, 1998; Uemura and Steponkus, 2003). However, multiple regression analyses showed no significant contribution of watersoluble carbohydrate content to freezing resistance, partly because freezing resistance were constantly higher, even in groups IV~VI, whereas total water-soluble carbohydrate content was slightly higher (Haunsberg and PI 173438) or lower (Norstar and Valuevskaya) in groups V and VI.

Although soluble carbohydrate accumulation contributed significantly to increased snow mold resistance, the mechanism of the increase in snow mold resistance differs from that of the increase in freezing resistance. The increase in all soluble carbohydrate contents was caused mostly by increased polysaccharide content (Fig. 4). The content of stored carbohydrates such as fructan provide a long-term supply of respiratory energy to plants under snow cover and increases the tolerance of plants to snow mold attack (Gaudet et al., 1999).

Strong freeze-resistant cultivars, Norstar and Valuevskaya, tended to have higher freezing resistance over the all sowing dates than a moderate resistant cultivar, PI 173438. However, a moderate freeze-resistant cultivar, Haunsberg, had the highest freezing resistance in group VI (Fig 2), which may result from high soluble carbohydrate accumulation in this group (Fig 4). On the other hand, snow mold resistance did not reveal consistent differences between strong resistant cultivars, PI 173438 and Haunsberg, and moderate resistance cultivars, Norstar and Valuevskaya, although significant differences between cultivars were found (Table 3). The cultivar differences between freezing resistance and snow mold resistance do not seem to be strongly related to the differences in nonstructural and structural carbohydrate contents.

Snow mold resistance decreased in groups V and VI. The decrease in snow mold resistance seems to be related to reduced plant size and carbohydrate accumulation, which is caused by low temperature and low radiation energy levels. On the other hand, freezing resistance was constantly higher until the last sowing date, suggesting that freezing resistance is less influenced by plant size.

This study hypothesized that change in %CW has an important role in enhancement of freezing resistance and snow mold resistance in winter wheat exposed to natural cold acclimation in fields. The cold acclimation period necessary to increase the resistance to freezing and snow mold corresponds to the period necessary to increase %CW and water-soluble carbohydrate content. Furthermore, multiple regression analysis revealed a significant contribution of %CW to freezing resistance, although no significant contribution to snow mold resistance was found. These results suggest important role of cell-wall mass in enhancement of freezing resistance during cold acclimation.

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