

Changes in Sugar Content during Cold Acclimation and Deacclimation of Cabbage Seedlings

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The relationship between freezing tolerance and sugar content in cabbage seedlings was investigated. Seedlings exposed to non-freezing low temperature (5 °C) acquired freezing tolerance down to –6 °C. The degree of freezing tolerance increased with duration of exposure to low temperature (up to 10 d). Sucrose, glucose, fructose and *myo*-inositol were detected as soluble sugars in cabbage leaves, and all soluble sugars, except for *myo*-inositol, and starch increased gradually during cold acclimation such that their levels were positively correlated with the degree of freezing tolerance. The induced freezing tolerance was attributed not to ontogenetic changes but to cold acclimation. However, the induced freezing tolerance was lost after only 1 d of deacclimation at control temperatures, and this change was associated with a large reduction in sugar content.

These results reveal that the sugar content of cabbage leaves is positively correlated with freezing tolerance.

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Key words: *Brassica oleracea* L., cabbage, cold acclimation, deacclimation, freezing tolerance, sugars.

INTRODUCTION

Cold and frost limit the growing seasons and geographic distributions of plants (Burke *et al.*, 1976; Alberdi and Corcuera, 1991) but most overwintering plants are able to increase their freezing tolerance when exposed to non-freezing low temperatures, a process known as cold acclimation. It has been reported that cold acclimation is accompanied by biochemical changes such as solute accumulations (Jung and Smith, 1961; Perras and Sarhan, 1984; Alberdi and Corcuera, 1991).

Sugar has been considered to be one of the most important factors in freezing tolerance. In woody plants, sugars accumulate from autumn to winter as freezing tolerance increases (Sakai and Yoshida, 1968). In some herbaceous plants, including wheat (Perras and Sarhan, 1984), cloud-berry (Kaurin, Junttila and Hansen, 1981), and spinach (Guy, Huber and Huber, 1992), changes in sugar content are correlated with those of freezing tolerance. In contrast, Pollock, Eagles and Sims (1988) reported that in *Lolium perenne* there was little correlation between LT₅₀ and soluble sugar content at the end of the hardening period. The physiological role of sugar in the mechanism of freezing tolerance in plants is, therefore, still unclear.

Cabbage, one of the most important vegetables, is commonly injured by low temperatures in spite of the existence of many cultivars for winter cultivation. Although Kohn and Levitt (1965) confirmed that cabbage seedlings could be acclimated by exposure to low temperatures, the biochemical mechanism of cold acclimation has not yet been studied. Elucidation of the biochemical mechanism of cold acclimation may therefore help in the development of cropping in cold regions and the stable production of cabbage and other crops.

With this aim in view this study was conducted to investigate the changes in sugar and starch contents which occur during cold acclimation and deacclimation in cabbage seedlings.

MATERIALS AND METHODS

Plant materials and low temperature treatment

Seeds of cabbage (*Brassica oleracea* L. cv. Banchurisou) were sown in plastic pots and grown in a growth chamber at the control temperature (20/15 °C, day/night) and 12 h photoperiod. Irradiance at plant height was about 300 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Three weeks after sowing, when two leaves had unfolded, the seedlings were acclimated in a growth chamber at 5 °C for 10 d under the same light conditions as above. In the experiment to determine the effect of deacclimation on freezing tolerance and sugar content, the seedlings were cold-acclimated for 7 d, then deacclimated by transferring them again to the growth chamber at control temperature.

Freezing tolerance test

The degree of freezing tolerance of seedlings was expressed in terms of the electrolyte leakage determined by the methods described by Sukumaran and Weiser (1972) and Pearce and Willison (1985), with modifications. Leaf discs (10 mm in diameter) were cut from the second leaf of 12 seedlings in each treatment. Two leaf discs were placed in a test tube and transferred to a chamber where the temperature was lowered from 15 to –4 or –6 °C at a rate of 0.25 °C min⁻¹. When the temperature in the chamber

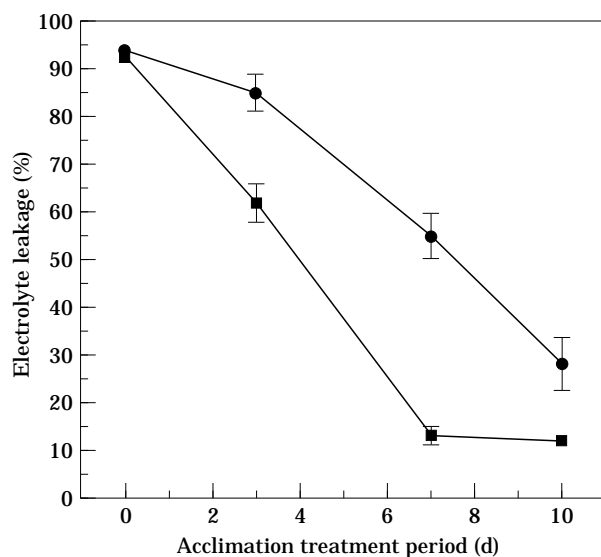


FIG. 1. Freezing tolerance of leaves of cabbage seedlings exposed to low temperature acclimation (5 °C). Freezing tolerance is expressed in terms of percentage electrolyte leakage from leaves after a freezing test. (■) Freezing at -4 °C; (●) freezing at -6 °C. Vertical bars indicate s.e. ($n = 3$).

reached -2 °C, the test tubes were maintained at this temperature for 1 h and the leaf discs were sprayed with deionized water to initiate extracellular freezing. The test tubes were maintained at -4 or -6 °C for 30 min and then allowed to thaw at room temperature for about 1 h. The warming rate was about 0.4 °C min⁻¹. Deionized water (15 ml) was added and the tubes were stored overnight at room temperature. Then conductivity of the solution was measured, and again after heating in boiling water for 20 min. The degree of electrolyte leakage was calculated as the conductivity of the solution before the heating as a percentage of that after heating.

Determination of soluble sugars and starch contents

The first and second leaves were used to examine the effect of low temperature treatment (cold acclimation), whereas only the second leaf was used to examine the effect of returning to the control temperature (deacclimation). After treatment, leaves were cut from the plants and their midribs removed. The remainder was extracted in 10 volumes of 80% (v/v) ethanol at approx. 70 °C for 30 min. After cooling at room temperature, xylose (0.5% of the sample weight) was added as an internal standard, and the sample was homogenized. The homogenate was centrifuged at 1500 g for 10 min. The pellet was re-extracted twice in 5 volumes of 80% (v/v) ethanol, the three supernatants were combined and dried using a centrifugal evaporator, and the dried residue was dissolved in 1 ml of distilled water. The sample was passed through an SEP-PAK C18 cartridge (Millipore Corporation, Massachusetts, USA) which had been equilibrated with water, after which 2 ml of distilled water was eluted. An aliquot (20 µl) of the eluate was

subjected to HPLC using an 830-RI refractive index detector (JASCO, Tokyo, Japan) and a SUGAR SP0810 column (Showa Denko Co., Tokyo, Japan). The column temperature was 80 °C, and the mobile phase was distilled water at a flow rate of 0.8 ml min⁻¹.

For determination of starch content, the ethanol-insoluble residue after extracting soluble sugars was lyophilized. One hundred milligrammes of the residue was heated in 5 ml DMSO (dimethyl sulphoxide) at 100 °C for 30 min. After cooling to room temperature, the samples were stirred and centrifuged at 1500 g for 10 min. The pellet was re-extracted twice in 5 ml DMSO, and the resulting supernatants combined. Aliquots (0.02 ml) of the sample were digested with 4 units of glucoamylase (*R. niveus*, Lyo., Seikagaku kogyo Co., Ltd. Tokyo, Japan) in 0.8 ml of 0.05 M acetate buffer (pH 4.8) at 37 °C for 3 h. To inactivate the glucoamylase activity, the samples were heated in boiling water for 5 min. The liberated glucose was measured enzymatically using the glucose B test (Wako Pure Chemical Industries, Ltd, Osaka, Japan).

RESULTS

Changes in freezing tolerance and sugar contents during low-temperature acclimation

The seedlings grew more slowly under cold acclimation (5 °C) than under control conditions (20/15 °C); the fresh weight of seedling shoots doubled during the 10 d of cold acclimation (data not shown) and the number of unfolded leaves increased from two to three.

High electrolyte leakage from leaves of control seedlings occurred in the freezing tolerance test at -4 °C (Fig. 1). The degree of freezing tolerance of leaves (expressed in terms of electrolyte leakage) increased progressively with increasing duration of cold acclimation, and after 10 d of cold acclimation, the electrolyte leakage value had fallen to 27.9% in the freezing tolerance test at -6 °C.

Soluble sugar contents increased markedly under cold acclimation for 3 d: sucrose, glucose and fructose increased by about two, six and three-fold, respectively (Fig. 2A). By day 10 of cold acclimation, the level of sucrose had begun to decrease, whereas glucose and fructose levels appeared to have reached a plateau after 7 d of treatment. Regarding the other sugars, *myo*-inositol was detected in the cabbage leaves, but did not increase under cold acclimation (data not shown). Total contents of sucrose, glucose and fructose showed a positive correlation ($r = 0.70$, $P < 0.02$) with freezing tolerance at -6 °C, suggesting that the increase in soluble sugar content during cold acclimation could be related to the acquisition of freezing tolerance.

Starch content increased about five-fold during cold acclimation for 7 d therefore remaining steady up to 10 d (Fig. 2B). As for the soluble sugar content, starch content was also correlated ($r = 0.67$, $P < 0.02$) with freezing tolerance at -6 °C. However, changes in sugar or starch contents did not always coincide with the change in the freezing tolerance (e.g. limited biochemical changes between 7 and 10 d of acclimation associated with a significant increase in freezing tolerance at -6 °C (Figs 1 and 2). This

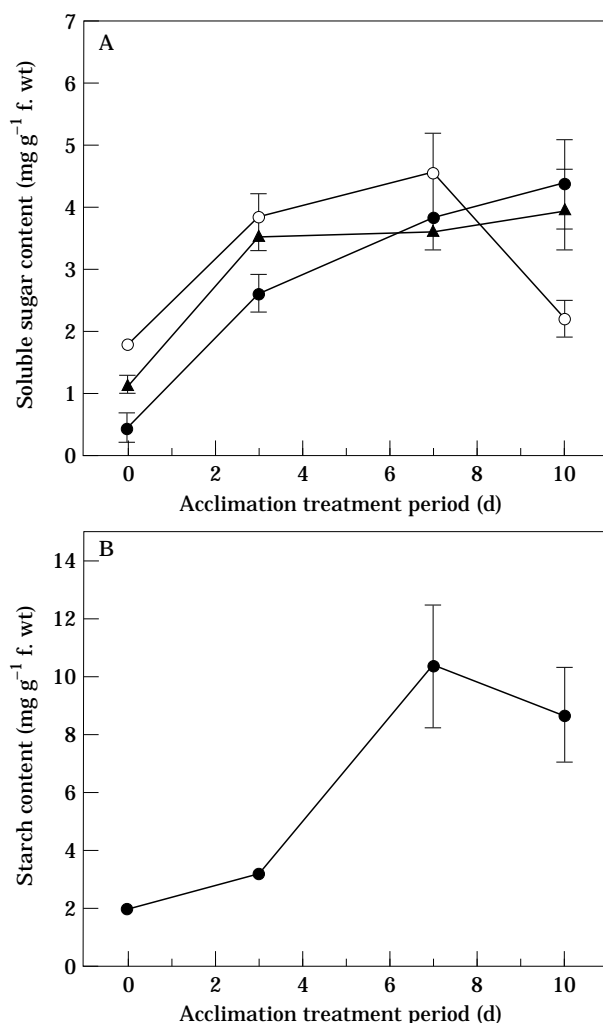


FIG. 2. Soluble sugar (A) and starch (B) contents of cabbage leaves during exposure to low temperature (5 °C). (○) Sucrose; (●) glucose; (▲) fructose in (A). Vertical bars indicate s.e. ($n = 3$).

indicates that freezing tolerance of cabbage seedlings is not explained solely by the accumulation of sugars.

Freezing tolerance and sugar contents at different growth stages

As described above, sugar contents of leaves were correlated with degree of freezing tolerance. To analyse the relationship, effects of growth stage and cold acclimation by low temperature had to be separated, since the plants developed more slowly at low temperature (e.g. for cabbage seedlings at two-leaf stage, 3 and 7 d were required for the unfolding of one new leaf under the control and acclimation conditions, respectively). Therefore, the tolerance of leaf 2 to -4 °C was measured at the same growth stage. The level of electrolyte leakage from the second leaf of control plants before imposing treatments was $88.2 \pm 9.2\%$ (mean of three replications \pm standard error). Cold acclimation for 7 d decreased electrolyte leakage to $23.2 \pm 7.2\%$, whereas the

TABLE 1. Soluble sugar contents (mg g⁻¹ f. wt) of leaves of different insertions at different stages of development. I-1, Leaf 1 before imposing treatments; I-2, leaf 2 before imposing treatments; N-1, leaf 1 grown under control conditions for 3 d; N-2, leaf 2 grown under control conditions for 3 d; L-1, leaf 1 exposed to cold acclimation for 7 d; L-2, leaf 2 exposed to cold acclimation for 7 d. The results are means of three determinations \pm s.e.

Leaf insertion and growth stage	Sucrose	Glucose	Fructose
I-1	1.58 \pm 0.67	0.91 \pm 0.14	0.85 \pm 0.26
I-2	2.14 \pm 0.89	1.31 \pm 0.37	1.33 \pm 0.23
N-1	1.87 \pm 0.69	0.81 \pm 0.59	0.57 \pm 0.16
N-2	2.84 \pm 0.36	0.69 \pm 0.07	0.90 \pm 0.09
L-1	3.93 \pm 0.56	6.67 \pm 1.20	4.84 \pm 0.99
L-2	4.29 \pm 0.13	5.81 \pm 2.60	5.58 \pm 0.84

TABLE 2. Soluble sugars and starch contents (mg g⁻¹ f. wt) of cabbage leaves exposed to low temperature acclimation (5 °C) and deacclimation at control temperatures (20/15 °C). Treatments were: C, control temperature; 7a, 7 d cold acclimation; 7a-1d, 7 d acclimation followed by 1 d deacclimation; 7a-2d, 7 d acclimation followed by 2 d deacclimation. The results are means of three determinations \pm s.e.

Treatment	Sucrose	Glucose	Fructose	Starch
C	1.39 \pm 0.25	0.51 \pm 0.10	0.75 \pm 0.23	0.77 \pm 0.15
7a	6.54 \pm 0.46	5.74 \pm 0.88	6.07 \pm 0.55	9.47 \pm 2.30
7a-1d	2.09 \pm 0.17	0.35 \pm 0.08	0.53 \pm 0.08	0.47 \pm 0.06
7a-2d	1.99 \pm 0.27	0.92 \pm 0.35	0.88 \pm 0.30	0.77 \pm 0.24

value for plants (of the same development stage) grown at a normal temperature for 3 d was $92.3 \pm 1.2\%$. This indicates that acquired freezing tolerance is not attributed to ontogenetic changes but to low temperature. Sucrose, glucose and fructose levels in leaves 1 and 2 increased markedly at low temperature, but did not increase at the control temperature. The increases in glucose and fructose content were larger than in sucrose content (Table 1).

Changes in soluble sugar and starch contents during cold acclimation and deacclimation

Seedlings that had acquired freezing tolerance by cold acclimation at low temperature for 7 d were deacclimated by returning them to the control temperature; their freezing tolerance at -4 °C was then examined as before by measuring electrolyte leakage. Cold acclimation for 7 d decreased electrolyte leakage values from $82.6 \pm 9.4\%$ to $31.4 \pm 4.8\%$, whereas deacclimation for 1 d caused an increase to $67.5 \pm 6.4\%$, indicating that the period required for deacclimation is much shorter than acclimation.

Sucrose, glucose and fructose contents raised by cold acclimation fell substantially after only 1 d of deacclimation (Table 2). This was consistent with the observed degree of

freezing tolerance. Starch content was similarly raised by cold acclimation and lowered by deacclimation.

DISCUSSION

The results of the present study show that cabbage seedlings acquired freezing tolerance by exposure to non-freezing low temperature, and that this change was associated with the accumulation of sugars. Freezing tolerance continued to increase with increasing sugar contents up to 10 d of low temperature treatment. These changes in freezing tolerance and sugar content were not due to ontogenetic trends but were caused by cold acclimation by low temperature. The freezing tolerance and sugar content of cold-acclimated plants were reduced by deacclimation at higher temperatures. These results are consistent with findings from various species (Kaurin *et al.*, 1981; Perras and Sarhan, 1984; Guy *et al.*, 1992). Thus, sugars are probably one of the important factors of freezing tolerance in cabbage seedlings.

Koster and Lynch (1992) reported that the soluble sugars of rye had reached their maximal levels before the LT_{50} reached its lowest temperature during cold acclimation. In this study, we observed that sugar contents were not always consistent with the degree of freezing tolerance. This result and the above findings indicate that factors other than sugar may also affect freezing tolerance.

Although sugar contents are raised by exposure to low temperature in several species, the types of sugar accumulated vary among plant species. Sucrose and fructose contents increased during cold stress in rye (Antikainen and Pihakski, 1994); sucrose content increased quickly following exposure to lower temperature in cloudberry (Kaurin *et al.*, 1981); and in spinach, the contents of sucrose, glucose and fructose increased on exposure to low temperature (Guy *et al.*, 1992). In wheat (Tognetti, Calderón and Pontis, 1989) and temperate grasses (Pollock *et al.*, 1983), low temperature led to fructan synthesis. The present study with cabbage plants has shown that sucrose, glucose and fructose contents also increased on exposure to low temperature. However, it is not clear to what extent the freezing tolerance of plants depends on the types of sugar accumulated at low temperature.

One of the effects of sugar is colligative; i.e. causing a lowering of osmotic potential (O'Neill, 1983). Although monosaccharides are more effective than oligosaccharides per unit mass in lowering the osmotic potential, sugars other than monosaccharides did accumulate as mentioned above. Besides such colligative effects, sugars have been shown to function as protectants of plasma membranes and proteins from the effects of freezing and dehydration (Sakai and Yoshida, 1968; Santarius, 1973; Steponkus, 1984) and they may act to suppress ice nucleation (Mackenzie, 1977). To clarify the physiological role of sugar in freezing tolerance, the localization of sugars in the intracellular compartments needs to be analysed.

Starch serves generally as the dominant storage carbohydrate in higher plants. In cabbage seedlings, starch accumulated during cold acclimation and decreased during deacclimation. Guy *et al.* (1992) showed that chloroplastic starch in the leaves of spinach increased under cold

acclimation but the increase was not related to freezing tolerance. Further investigation is needed to clarify the role of starch in freezing tolerance.

Freezing tolerance induced in cabbage seedlings by low temperature was reduced substantially after only 1 d at the control temperature. The period required for deacclimation was much shorter than that for cold acclimation, and was accompanied by a rapid reduction in sugar content. These results, which are consistent with the findings reported for spinach by Guy *et al.* (1992), indicate that sugars accumulated in leaves at low temperature were rapidly metabolized on deacclimation at normal growing temperatures.

In conclusion, low temperature induces freezing tolerance and accumulation of sugars and starch in the leaves of cabbage seedlings, but these changes can be reversed rapidly by returning the plant to normal temperatures. The relationship between induced freezing tolerance and induced sugar accumulation remains to be explained.

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