PERGAMON

Phytochemistry 50 (1999) 755–761

PHYTOCHEMISTRY

Effect of apoplastic proteins on freezing tolerance in leaves

Ökkeş Atıcı[a], Barbaros Nalbantoğlu[b,c,*]

[a]Department of Biology, Faculty of Science and Arts, Atatürk University, 25240 Erzurum, Turkey
[b]Department of Biochemistry, Faculty of Science and Arts, Atatürk University, 25240 Erzurum, Turkey
[c]Biotechnology Application and Research Center, Atatürk University, 25240 Erzurum, Turkey

Received 11 December 1997; revised 14 July 1998

Abstract

Freezing tolerance was determined in cabbage (*Brassica oleracea* cv Acephala) and winter wheat (*Triticum aestivum* cv Doğu-88) leaves growing under control and cold conditions. Freezing injury was less in cold-acclimated leaves than in control leaves. In cold-acclimated leaves, the freezing injury increased when apoplastic solution is extracted. In addition, ice nucleation activity was lower with apoplastic proteins extracted from cold-acclimated leaves than from control leaves. These results suggest that the proteins present in the apoplast during cold acclimation are an important component of the mechanism by which cabbage and winter wheat leaves inhibit extracellular ice formation. Winter wheat has greater freezing tolerance than cabbage because winter wheat leaves have lower freezing injury and apoplastic proteins in winter wheat leaves have lower ice nucleation activity.

Keywords: *Brassica oleracea*; Brassicaceae; Cabbage; *Triticum aestivum*; Poaceae; Winter wheat; Cold-acclimated leaves; Apoplastic proteins; Freezing tolerance; Freezing injury; Ice nucleation activity

1. Introduction

Freezing is lethal to most cellular organisms. Dehydration of the intracellular environment and physical damage by ice crystals are the major causes for freezing injury and death. One of the more challenging problems in modern plant physiology is the elucidation of the mechanism of plant-cell freezing tolerance and the genetic elements that govern the acquisition of this tolerance. The understanding of this process is not only of fundamental scientific importance but could also be of immense economic value (Singh & Laroche, 1988).

It has been clearly demonstrated in freezing tolerant organisms such as insects, reptiles, and mollusks that the process of ice formation is controlled by specific proteins (Storey & Storey, 1988; Duman, Xu, Neven, Tursman, & Wu, 1991; Griffith, Ala, Yang, Hon, & Moffatt, 1992). Overwintering plants that are frost-tolerant survive freezing temperature by forming ice in extracellular spaces within their tissues (Burke, Gusta, Quamme, Weiser, & Li, 1976). In plants, for the first time, winter rye was chosen to investigate proteins in apoplastic spaces. Some specific proteins have been purified from the apoplastic region of winter rye leaves after cold acclimation. These proteins have been shown to exhibit antifreeze activity (Griffith et al., 1992; Marentes, Griffith, Mlynarz, & Brush, 1993; Hon, Griffith, Chong, & Yang, 1994).

In this study, for only the second time in plants, cabbage and winter wheat were chosen to investigate the effect of apoplastic proteins on freezing tolerance of leaves during cold acclimation. Freezing injuries and ice nucleation activities were determined in cabbage and winter wheat grown in control and cold acclimating conditions.

2. Results

2.1. Effect of apoplastic extract on freezing injury

Cabbage and winter wheat plants were grown in control and cold acclimation, made in imitation of natural conditions (Table 1). Freezing injuries were

* Corresponding author. Fax: +90-442-233-1062.

0031-9422/99/$ - see front matter
PII: S0031-9422(98)00533-0

Table 1
Growth conditions for cold acclimation

Time (day)	Day/night temperature (°C)	Day/night (h)
1–7	20/18	16/8
8–11	15/12	16/8
12–14	12/9	15/9
15–18	11/7	15/9
19–21	8/4	15/9
22–28	5/2	14/10
29–35	5/2	12/12
36–42	5/2	10/14
43–60	5/2	8/16

investigated in cabbage and winter wheat leaves growing in both control and cold acclimation. In order to express the freezing injury, the LT_{50} (temperature at which 50% of leaves are injured) value was used for simple quantitative comparisons.

Freezing injury was determined in control leaves of the both plants (Fig. 1). In cabbage, LT_{50} values were −4.5°C for 15 day, 30 day and 45 day leaves. In winter wheat, LT_{50} values were −5.5°C for 15 day and 30 day, and at −7°C for 45 day leaves. Freezing injuries of both plants reached 85% at −9°C and became constant between 85 and 100% when the temperature was lowered from −10 to −20°C. Freezing injury was also determined in CA (cold-acclimated) leaves of both plants (Fig. 2). In cabbage, LT_{50} values were −4.5°C for 15 day, −4°C for 30 day, −9.5°C for 45 day and 60 day leaves. In winter wheat, LT_{50} values were −8.5°C for 15 day, −13°C for 30 day and 45 day, and −16°C for 60 day leaves.

In order to investigate the effect of cold acclimation on freezing injury, after solutions of the apoplastic regions had been extracted from leaves of both 60 day plants, freezing injuries in extracted leaves were determined (Fig. 3). In cabbage, while the LT_{50} of unextracted leaves was −9.5°C, the LT_{50} of extracted leaves was −7.5°C. In winter wheat, the LT_{50} in unextracted leaves was −16°C, whereas the LT_{50} in extracted leaves was −11°C. The LT_{50} values of cabbage and winter wheat leaves were compared in order to see effect of apoplastic extracts on freezing injury (Fig. 4).

2.2. Effect of apoplastic proteins on ice nucleation activity

In order to investigate the function of apoplastic components produced during cold acclimation, precipitated proteins from apoplastic extracts were used to determine ice nucleation activity (Fig. 5). In cabbage, the threshold ice nucleation temperatures of apoplastic proteins were −4°C for 45 day control and 15 day CA leaves, −5°C for 30 day and 45 day CA leaves, and −6°C for 60 day CA leaves. In winter wheat, the threshold ice nucleation temperatures of apoplastic proteins were −6°C for 45 day control and 15 day CA leaves, −7°C for 30 day CA leaves, −9°C for 45 day CA leaves, and −11°C for 60 day CA leaves.

3. Discussion

In this study, for only the second time in plants, cabbage and winter wheat were chosen to investigate the effect of apoplastic proteins in leaves on freezing tolerance during cold acclimation. Freezing injuries and ice nucleation activities were determined during control and cold acclimation.

The temperatures of freezing injury are summarized for both plants (Figs. 1–4). When the temperature was lowered to −9°C, freezing injuries are 85% in both plant leaves growing at control condition while freezing injuries are 45% and 18%, respectively, in cabbage and winter wheat leaves growing at CA condition (Fig. 3). It is seen that freezing injuries were lower in CA leaves than control leaves, even at −20°C. When LT_{50} values of 45 day control and 60 day CA leaves are compared, LT_{50} values decreased from −4.5 to −9.5°C in cabbage and from −7 to −16°C in winter wheat (Fig. 4). After the soln of the apolastic region from 60 day CA leaves was extracted, LT_{50} values are increased from −9.5 to −7.5°C in cabbage and from −16 to −11°C in winter wheat (Fig. 4). From these results it is seen that the freezing injury is greater in extracted leaves than in unextracted leaves.

In plants, for the first time, the effect of apoplastic extraction on freezing tolerance was investigated in winter rye leaves (Marentes et al., 1993). At that study, it was shown that apoplastic extract has roles in controlling extracellular ice formation. Proteins that directly affect ice formation are classified as ice nucleators and antifreezes (Griffith et al., 1992; Hon et al., 1994; DeVries, 1983, 1986; Feeney & Burcham, 1986; Yang, Sax, Chakrabarty, & Hew, 1988; Raymond, Wilson, & DeVries, 1989; Hightower, Baden, Penzes, Lund, & Dunsmuir, 1991). Therefore, in our study, apoplastic proteins were precipitated from apoplastic extract and then these proteins were used to investigate ice nucleation activity. When ice nucleation activities of apoplastic proteins in 45 day control and 60 day CA leaves are compared, ice nucleation activities were found to decrease from −4 to −6°C in cabbage and from −6 to −11°C in winter wheat (Fig. 5). These results show that ice nucleation activity is lowered more with apoplastic proteins extracted from CA leaves than from control leaves. It is known that ice nucleator proteins initiate freezing at a temperature very close to 0°C, which are produced by certain bacteria to help them invade plants by inducing frost damage (Feeney & Burcham, 1986). However, here

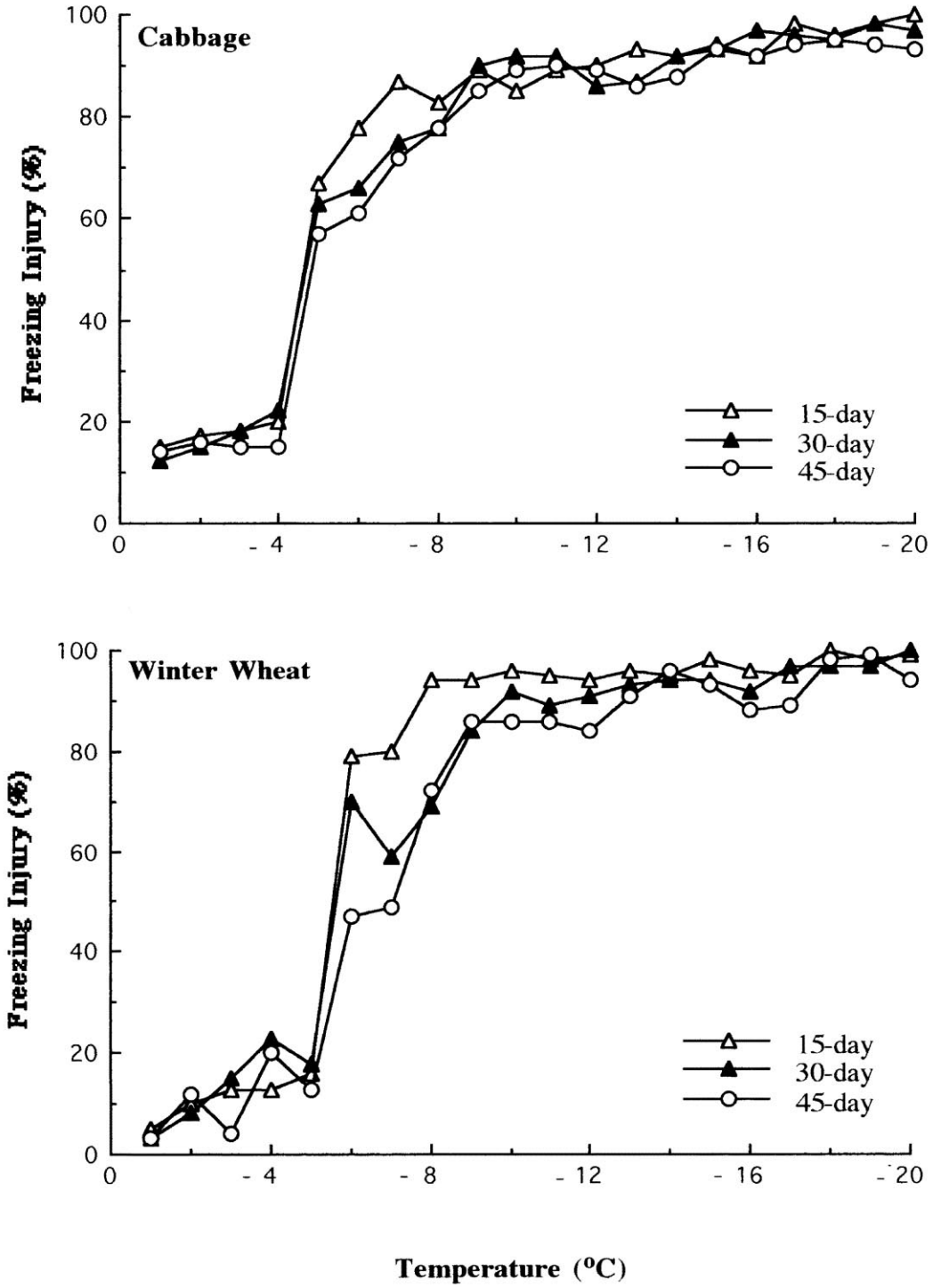

Fig. 1. Freezing injury % values in cabbage and winter wheat leaves growing at control condition.

can be seen the opposite effect. In addition to ice nucleator proteins, it is known that antifreeze proteins inhibit ice formation (Feeney & Burcham, 1986). Since ice nucleation activity is lowered in the present study, these results suggest that the presence of antifreeze proteins may be essential in controlling the growth of ice within cooled plant tissues (Marentes et al., 1993).

When the freezing tolerances of the both plants are compared with each other, winter wheat has more freezing tolerance than cabbage because winter wheat leaves have lower freezing injury and apoplastic proteins have lower ice nucleation activity than cabbage. In the present paper, we present evidence that apoplastic proteins play important roles on freezing

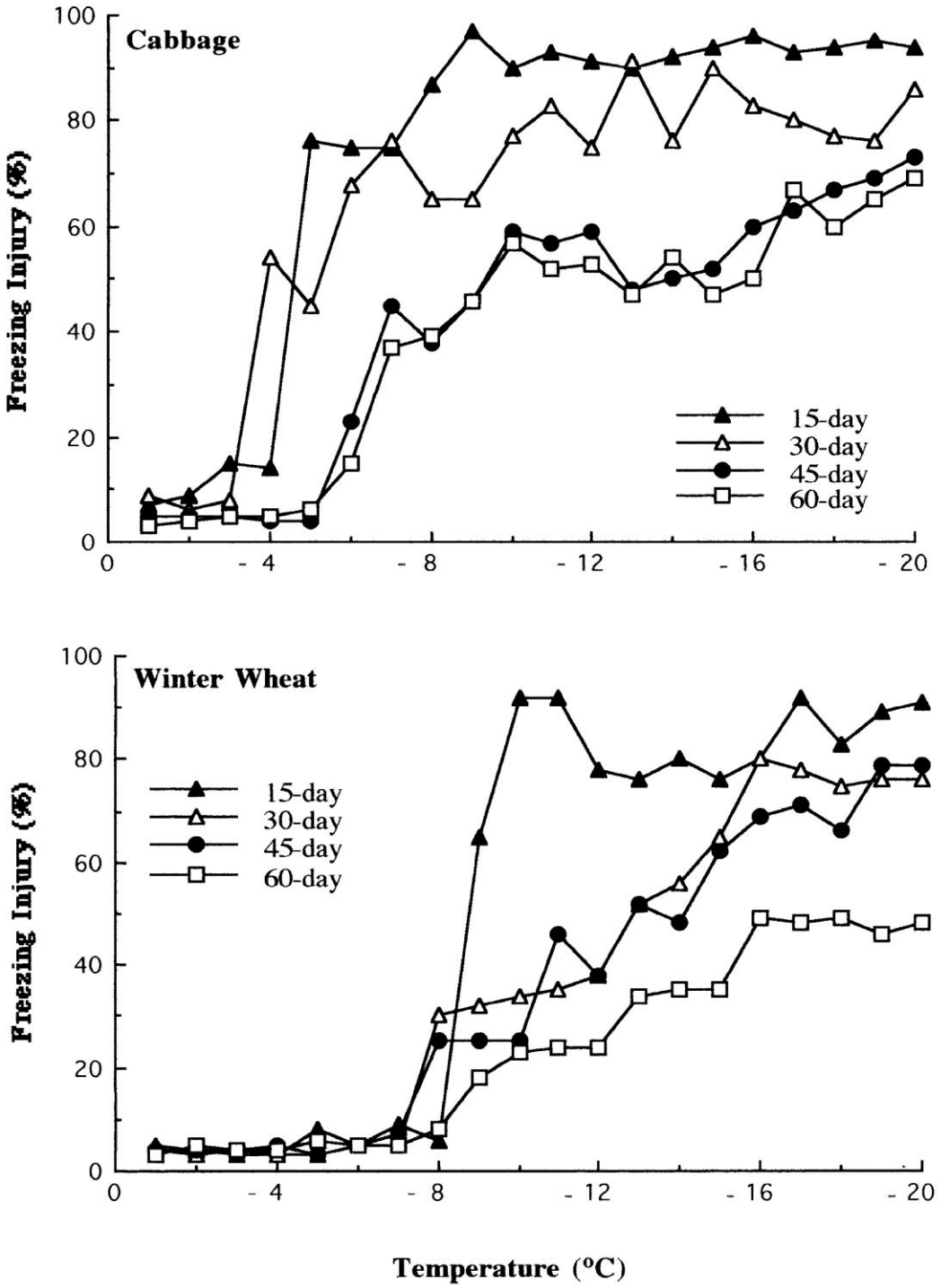

Fig. 2. Freezing injury % values in cabbage and winter wheat leaves growing at cold acclimation conditions.

tolerance in cabbage and winter wheat leaves. We can conclude, therefore, that the leaves of both plants accumulate antifreeze proteins in the apoplasts and that these proteins directly influence the process of extracellular ice formation. Some specific proteins have been purified from apoplastic region of winter rye leaves after cold acclimation. All these proteins have been shown to exhibit antifreeze activity (Griffith et al., 1992; Marentes et al., 1993; Hon et al., 1994).

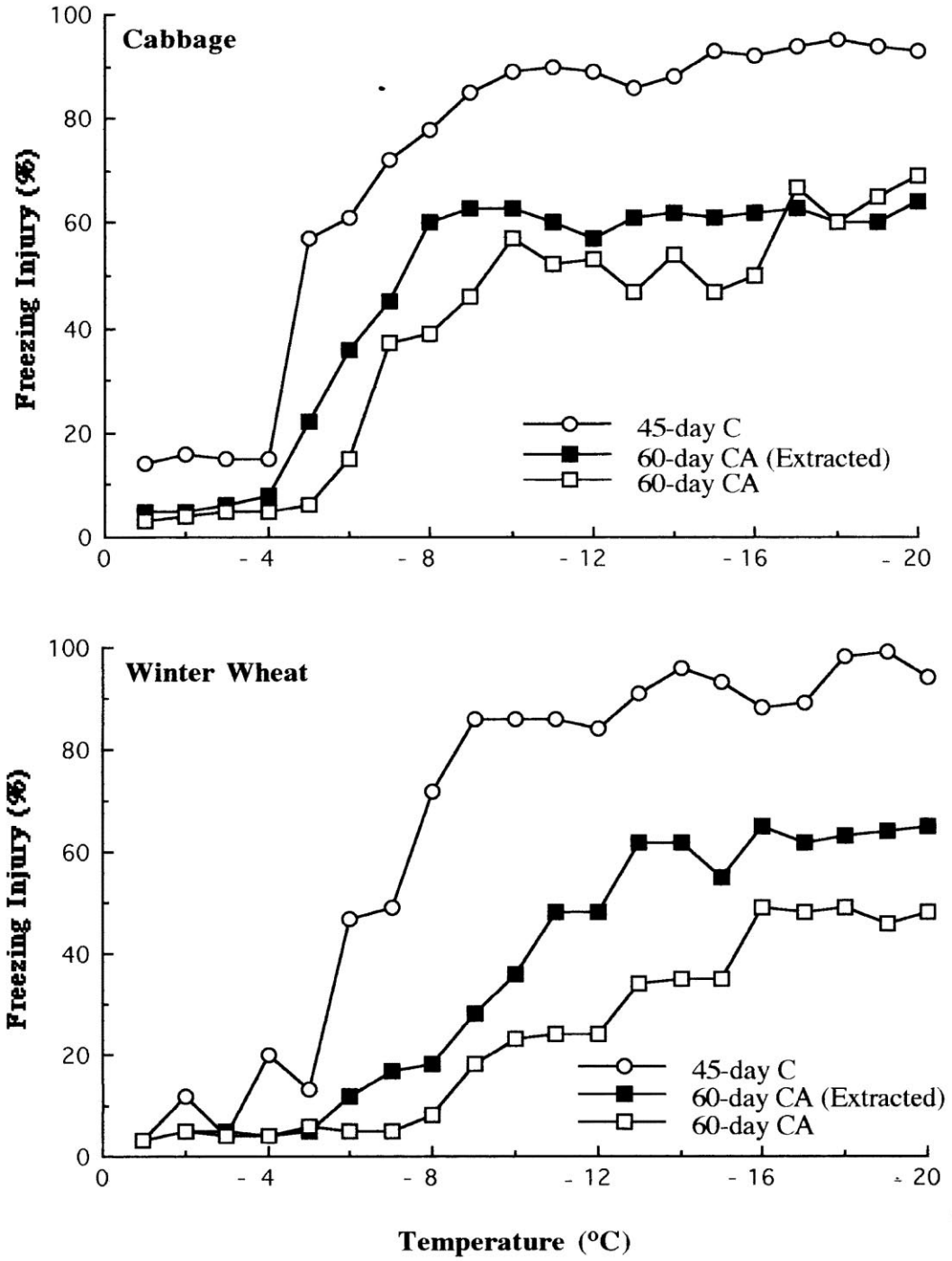

Fig. 3. Freezing injury % values in cabbage and winter wheat leaves growing at different conditions. C: control, CA: cold-acclimated, CA (extracted): solution of apoplastic region was extracted from cold-acclimated.

4. Experimental

4.1. Plant material

Cabbage (*Brassica oleracea* cv Acephala) and winter wheat (*Triticum aestivum* cv Doğu-88) seeds were planted in coarse vermiculite in 15 cm pots. They were maintained in a 20/18°C (day/night) growth chamber with a 16 h day length for 7 days to initiate germination. After 7 days, for control plants, some of the plants were maintained at 20/18°C for another 38 days. The other plants were transferred for cold acclimation for another 53 days at which growth conditions were slowly changed from 20/18°C 16 h daylength to 5/2°C 8 h daylength as shown in Table 1. The plants were watered routinely with modified

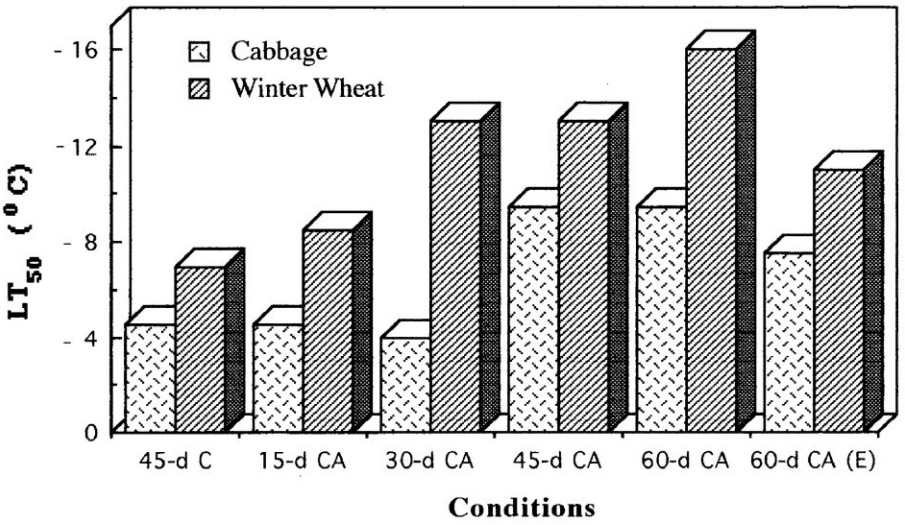

Fig. 4. LT_{50} values in cabbage and winter wheat leaves growing at different conditions. d: day, C: Control, CA: cold-acclimated, CA (E): solution of apoplastic region was extracted from cold-acclimated.

Hoagland's soln (Huner & Macdowall, 1976). Cabbage and winter wheat leaves growing in both control and cold acclimating conditions were cut at 15 day intervals to determine freezing injury and ice nucleation activity. When both freezing injury and ice nucleation activity between control and cold-acclimated leaves were compared, the same growth stage of plant leaves were considered in the results (Marentes et al., 1993).

4.2. Extraction of apoplastic proteins

Apoplastic proteins were extracted from cabbage and winter wheat leaves using a modification of the method of Rohringer, Ebrahim-Nesbat, and Wolf (1983). Fresh leaves (7 g) were cut into 2 cm lengths, and rinsed in 6 changes of H_2O to remove cellular proteins from the cut ends. At the end of each rinsing, the removing of cellular proteins was controlled by determining of $A_{280\ nm}$. The leaves were then vacuum-infiltrated for 30 min in 20 mM ascorbic acid. The leaves were blotted dry and placed vertically in a 20 ml syringe. The syringes were placed in centrifuge tubes. The apoplastic extract was collected from the bottom of the tubes after the leaves were centrifuged at $1500 \times g$ for 20 min.

4.3. Determination of freezing injury

Freezing injuries were determined in cabbage and winter wheat leaves allowed to freeze spontaneously

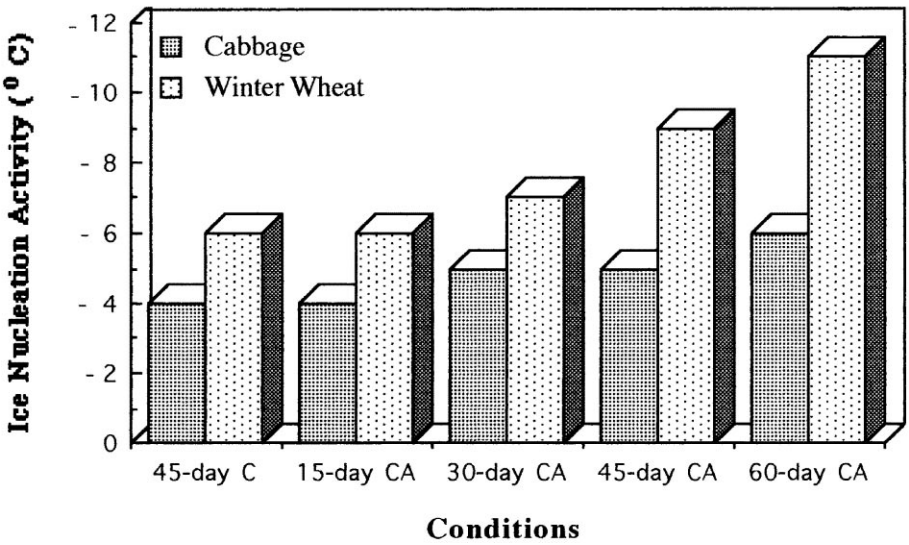

Fig. 5. Ice nucleation activity for apoplastic proteins extracted from cabbage and winter wheat leaves growing at different conditions. C: control, CA: cold-acclimated.

using a modification of the method of Marentes et al. (1993). Fresh leaves were cut into 2 cm lengths and rinsed in 6 changes of H_2O to remove cellular proteins from the cut ends. At the end of each rinsing, the removing of cellular proteins was controlled by determining of $A_{280\ nm}$. Then, leaves (0.1 g) were placed in each of 20 tubes and the tubes were positioned in a freezing bath (low temperature circulator LTD 6 G, Grant Instruments, Cambridge, England). After equilibration at −1°C for 30 min, the temp. was lowered stepwise by 1°C intervals from −1 to −20°C. The tubes were allowed to equilibrate at each temp. for 15 min. The tubes were then removed from the freezing bath one by one at each temperature and 4 ml cold HPLC-grade H_2O was added in each tube containing the frozen leaves. These tubes were stored at 4°C for 24 h. The degree of freezing injury was assessed by ion leakage (Dexter, Tottingham, & Graber, 1932). The conductivity of the solution in each tube was measured at room temp. with an electrical conductivity bridge. Ion leakage was calculated as the conductivity of the frozen sample divided by the conductivity of the same sample after it was boiled for 3 min. 60 day CA leaves were also assayed for freezing injury as described above after the soln of apoplastic region had been extracted. All experiments were performed 3 times and the average of the values was used.

4.4. Determination of ice nucleation activity

Apoplastic proteins obtained from the leaves (7 g) were used to determine ice nucleation activity using a modification of the method of Lindow (1990). Proteins were precipitated from apoplastic extracts by adding 1.5 vol. of ice-cold MeOH containing 1% HOAc and incubating the samples overnight at −20°C. After centrifugation for 20 min at 3500 *g*, the protein pellets were washed with 100% ice-cold EtOH and 70% ice-cold EtOH. The dried protein pellets in Eppendorf tubes were dissolved in 1 ml HPLC-grade H_2O and the tubes were then positioned in a freezing bath (low temp. circulator LTD 6 G, Grant Instrument, Cambridge, England). After equilibration at −1°C for 30 min, the temp. was lowered stepwise by 1°C intervals. The tubes were allowed to equilibrate at each temperature for 15 min. The tubes were then removed from the freezing bath after the apolastic protein soln in each tube had been frozen. The freezing temp. was used as a threshold for ice nucleation activity. All experiments were performed 3 times and the average of the values was used.

References

Burke, M. J., Gusta, L. V., Quamme, H. A., Weiser, C. J., & Li, P. H. (1976). *Annual Review of Plant Physiology, 27,* 507.

DeVries, A. L. (1983). *Annual Review Physiology, 45,* 245.

DeVries, A. L. (1986). *Methods in Enzymology, 127,* 293.

Duman, J. G., Xu, L., Neven, L. G., Tursman, D., & Wu, D. W. (1991). In: D. E. Lee, Jr. & D. L. Denlinger, *Insects at low temperature* (p. 94). New York: Chapman and Hall.

Dexter, S. T., Tottingham, L. F., & Graber, L. F. (1932). *Plant Physiology, 7,* 63.

Feeney, R. E., & Burcham, T. S. (1986). *Annual Review of Biophysics and Biophysical Chemistry, 15,* 59.

Griffith, M., Ala, P., Yang, D. S. C., Hon, W.-C., & Moffatt, B. A. (1992). *Plant Physiology, 100,* 593.

Hightower, R., Baden, C., Penzes, E., Lund, P., & Dunsmuir, P. (1991). *Plant Molecular Biology, 17,* 1013.

Hon, W.-C., Griffith, M., Chong, P., & Yang, D. S. C. (1994). *Plant Physiology, 104,* 971.

Huner, N. P. A., & Macdowall, F. D. H. (1976). *Canadian Journal of Biochemistry, 54,* 848.

Lindow, S. E. (1990). In: D. C. Sands, Z. Klement, & Y. Rudolph, *Methods in phytobacteriology* (p. 428). Budapest: Akademia Kiado.

Marentes, E., Griffith, M., Mlynarz, A., & Brush, R. A. (1993). *Physiologia Plantarum, 87,* 499.

Raymond, J. A., Wilson, P., & DeVries, A. L. (1989). *Proceeding of the National Academy of Science USA, 86,* 881.

Rohringer, R., Ebrahim-Nesbat, F., & Wolf, G. (1983). *Journal of Experimental Botany, 34,* 1589.

Singh, J., & Laroche, A. (1988). *Biochemistry and Cell Biology, 66,* 650.

Storey, K. B., & Storey, J. M. (1988). *Physiological Reviews, 68,* 27.

Yang, D. S. C., Sax, M., Chakrabarty, A., & Hew, C. L. (1988). *Nature, 333,* 232.