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Immunological Assays of Freezing Tolerance in Barley using Antifreeze Proteins Antisera

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ABSTRACT : In order to measure an antifreezing tolerance, antifreeze proteins accumulated upon cold acclimation in apoplast were analyzed. As Dongboriiho were cold-acclimated for 3 to 74 days there was an abrupt increase in apoplastic proteins up to 30 days and then decrease to the similar levels. Among the known antifreeze proteins, CLP produced in *E. coli.* and TLP purified from apoplast were used to generate antisera that allow to measure and localize the proteins in leaves of barley. The CLP of 27.7 kDa and TLPs of 6, 26, 27, 30, and 31 kDa were increased in their amounts in apoplast as cold treatment being longer. There was a correlation among the amounts of those proteins accumulated in apoplast and freezing tolerance as shown in field and ion leakage tests for five cultivars. The deposit of CLP was localized in the marginal area and the area adjacent to leaf vescular bundle cells in an increasing manner according to duration of cold acclimation but no variation was observed in terms of it's distribution. Based on the close correlation between levels of antifreeze proteins and degrees of freezing tolerance, the immunological methods was to develop to estimate a freezing tolerance in barley.

Keywords **:** antifreeze protein, apoplast, CLP(chitinase like protein), TLP(thaumatin like protein), CA(cold-acclimation)

A freezing injury in plant results from an ice nucleation and it's growth within apoplast. The resulting ice of bigger size breaks the adjacent cells in irreversible manner so that the cells in plant tissue become injured permanently (Griffith *et al.*, 1992). In order for plant to protect against freezing stress antifreezing proteins are accumulated in apoplastic space during cold acclimation since a gradual decrease in temperature as occurring in nature, induces freezing in the intercellular space earlier than in intracellular space (Pearce & Ashwortu, 1992; Hwang, 1995). The cold-acclimated rye could survive at temperatures lower than -30°C otherwise injured at -5°C (Guy *et al.*, 1987; Thomashow, *et al.*, 1998). Once cold-acclimated for seven weeks, rye accumulates proteins in apoplast at a concentration higher than 0.3 mg per gram of fresh weight and decreased abruptly once returning to 20°C (Griffith *et al.*, 1993). Dongboriiho was also shown to accumulate proteins of 70, 21, 16, and 14 kDas in apoplast during cold treatment and it turned out that they were belonged to antifreeze proteins such as chitinase like protein (CLP) and thaumatin like protein (TLP) as shown in rye (Hwang, 1995; Griffith *et al.*, 1995). The formation of oligomeric complexes with CLP, TLP, and GLP was known to bind to surface of ice and to inhibit further addition of water molecules to the growing ice so that the amounts of the antifreeze proteins determine the degrees of tolerance against freezing (Chun & Griffith, 1998; Griffith & Yu, 1999). Besides an inhibitor of GA biosynthesis, trinexapacethyl was shown to induce TLP in apoplast and to enhance freezing tolerance (Hwang, 1999) and transformation of a CLP gene into tobacco also showed an increase in freezing tolerance in plant (Hwang *et al.*, 2000). With aims on development of an assay for freezing tolerance in plants, antisera against the antifreeze proteins were produced and used for quantitative estimation of the proteins in five cultivars of barley with different degrees of tolerance.

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MATERIALS AND METHODS

Plant Materials and Cold Acclimation

Five cultivars of barley, ranged from high to low in freezing tolerance, were provided by National Crop Experiment Station, Suwon. Based on the field test from 1976 to 1977 by Cho, *et al.* (1979), Dongboriiho(D) is the highest and Olbori(O), Saechalssalbori(S) are the next to Dongboriiho

Table 1. Degree of freezing tolerance measured from field test among different cultivars of barley (NCES, 1999~2000).

Degree of Freezing Tolerance	Cultivars of barley	Survival (%)
1	Dongboriiho(D)	*
2	Olbori(O)	96.7
	Saechalsalbiri(S)	95.1
3	Albori(A)	82.3
4	Doowonchapsalbori(DO)	0

* : data is not available but expected as 100% based on cho *et al.* (1979).

and Albori (A) and Doowonchapssalbori (DO) are shown to be lower in freezing tolerance (Table 1).

Barley seeds were soaked in water for 8 hours before germinating at 30°C for 16 hours and seeding onto pot of 30×40 cm. The barley plantlets were grown at 18/20°C (day/night for 8/16 hours). Once reaching at 2-3 leaves stage the plants were subjected to 6/4°C (day/night for 8/16 hours) for cold acclimation of 3, 10, 20, or 40 days. As controls, plants of the equal stage without the cold acclimation were used.

Extraction of Apoplast Proteins

Leaves with removal of both ends of 1 cm were sliced into 0.5 cm and 5 g of the sliced tissues were vacuum-infiltrated with apoplast solution (20 mM ascorbic acid, 20 mM $CaCl_2$) at room temperature for 30 minutes. The excess solution on the surface of the tissues was blotted out with kitchen towel and tissues were loaded into a syringe hold by a tube for a centrifugation at 760 g for 30 minutes. Four times volume of ice-cold acetone were added to the apoplastic solution collected on the bottom of the tube, and the solution was stored at -20°C for 16 hours. The precipitated apoplastic proteins were dried and resolved in PBS (Phosphate-Buffered Saline) at a volume of 10 μl per 1 g of fresh tissue and stored at -20°C before used (Hon *et al.*, 1994).

Preparation of Polyclonal Antiserum Against CLP and TLP

The cDNA for CLP was isolated from cold-acclimated Dongboril ho and constructed in pET32 vector(Novagen) and introduced to BL21(DE3). The BL21(DE3) with pET32 containing a CLP gene was cultured in LB medium until the cell density reached at 0.6 of OD_{600}. Four hours after adding IPTG to make 1 mM, the cells were harvested for the CLP synthesized. Following the manufacture's manual, total cell proteins were extracted and separated in 15% SDS-PAGE and purified by gel-elution using a diffusion buffer (50 mM Tris pH8.0, 0.1% SDS). The TLP of 16 KDa was purified from total apoplastic proteins of cold acclimated Dongboril ho using a 15% SDS-PAGE and gel-elution methods.

The purified proteins were quantitated by a BCA (Bicinchoninic Acid Protein Assay Kit, Sigma) method. 100 μl of the proteins was mixed with a 500 μl of Freund's adjuvant (Sigma) in two syringes connected with a 22G needle connected before injecting into a rabbit for four times at a weeks' interval.

Western Analysis and *In situ* Immunolocalization

10 μl of the apoplast protein equivalent to 1 g of fresh tissue was separated in 15% PAGE and then the gel was electro-blotted onto a nitrocellulose membrane (Protran, S&S) in Towbin buffer (18.3 mM Tris-base, 150 mM Glycine 20% Methanol) at 70 V for 2.5 hours. The membrane was blocked in TBST (100 mM Tris HCl pH 8.0, 150 mM NaCl, 0.05% Tween 20) with 5% of nonfat dry milk. The primary antiserum of either TLP or CLP at a dilution of 1:10,000 and secondary anti-rabbit IgG AP conjugate (Promega) at a dilution of 1:7,500 were used. Visualization with NBT/BCIP (nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate, Promega) was performed at room temperature.

For immunolocalization, the tissue of leaf barley were separated and fixed at room temperature by infiltration under a vacuum for 30 min with a FAA fixing solution [formalin 10 ml, glacial acetic acid 50 ml, ethyl alcohol (95%) 50 ml, DDW 35 ml] incubated for 4 hours in the same solution and then dehydrated using 50-100% alcohol series. The fixed and dehydrated samples were cleared in alcohol:xylene (1:1) for 60 min and then incubated in absolute xylene for another 60 min at room temperature. The samples were embedded with paraffin embedding medium (Paraplast Plus, Sigma). Transverse sections (10 μm in thickness) were made from the embedded samples with a microtome. The sections were placed on slide warmer at 45°C overnight to "bake" the ribbon piece. The paraffin was removed from the sections by immersing the slides twice for 10 min in fresh absolute xylene and then rehydrating the tissue by passing through a graded series of ethanol (95%, 85%, 75%, 60%) for 5 minutes each. After washings with TBS containing 0.05%(v/v) of Tween20 for 5 minutes the tissue was incubated for 1 hour at room temperature in the same buffer containing 5% nonfat milk powder. The sections were then incubated in a 1:500 dilutions of preimmunization serum as negative controls or anti-CLP serum for 1 hour at room temperature. After washing, secondary goat anti-rabbit IgG AP conjugate (Promega) at a dilutions of 1:1000 was added and CLP was detected by reacting with a solution of NBT/BCIP (Promega) at room temperature for 2 minutes by light microscope (Maria *et al.*, 1998; Gurpreet *et al.*, 2000).

Ion Leakage Test

Leaves with 1 cm of tip removed were sliced into 0.5 cm and 0.2 g of the sliced tissues in glass tube were incubated in a low temperature water bath. Temperature was controlled by lowering from room temperature to 2°C for 2 hours and then to -2°C for 10 minutes before adding ice chips as an ice nucleator. The decreasing rate of 4°C per 2 hours was applied down to -14°C and the samples were collected at -2, -6, -10, and -14°C. The amounts of ions leaked from the tissues frozen at respective temperatures were measured at 265

nm after releasing by shaking at 220 rpm for 3 hours (Sulc & Kenneth, 1991). Based on the total amounts of ions released from the tissues broken by autoclaving, the relative levels of freezing-induced leakage per each tissue were estimated.

RESULTS AND DISCUSSION

Ion Leakage Test For Freezing Tolerance

In an attempt to quantify a freezing tolerance, the levels of ion leaked from leaves upon freezing were measured in five cultivars of barley. It was shown that a UV of 265 nm was used to detect level of leakage including amino acid, peptides, protein, organic acids, and sugars (Sulc & Kenneth 1991). Without a cold pre-treatment, all cultivars except Saechasalbori showed the similar level of tolerance to freezing. As the temperature for freezing was lowered, the amounts of the leakage was negatively correlated to the freezing tolerance known as the order of Dongbori1ho, Olbori, Saechasalbori, Albori and Doowonchapsalbori.

As the longer cold acclimation is treated, the less amounts of ion leakage were observed. It indicated that a cold pre-treatment increased the freezing tolerance in all of the five cultivars tested (Fig. 1). However there were variations in degree of the tolerance among cultivars and the differences were shown to be correlated with what the field test had shown in terms of survival under freezing conditions (Table 1). Even among non-acclimated samples, some variations in freezing tolerance were also observed but only Albori showed a significant difference in freezing tolerance (Fig. 1).

Antifreezing Test Using CLP Antiserum

In order for antifreezing-related CLP to be synthesized, pET-32 vector was used for construction and expression. Upon a treatment of IPTG, the CLP of 42.5 kDa was increased and reached peak 3 hours after the treatment (Fig. 2).

A primary antiserum produced against the CLP was confirmed to be specific by western analysis of apoplastic proteins from Dongbori1ho in which a CLP of 27.7 kDa was increased as cold acclimation progressed. This is corresponding to what Hwang *et al.* (2000) and Chun & Griffith (1998) had observed in barley and rye showing an abrupt increase in the amount of apoplastic proteins in between 3 days and 15 days after cold acclimation. The gene used for

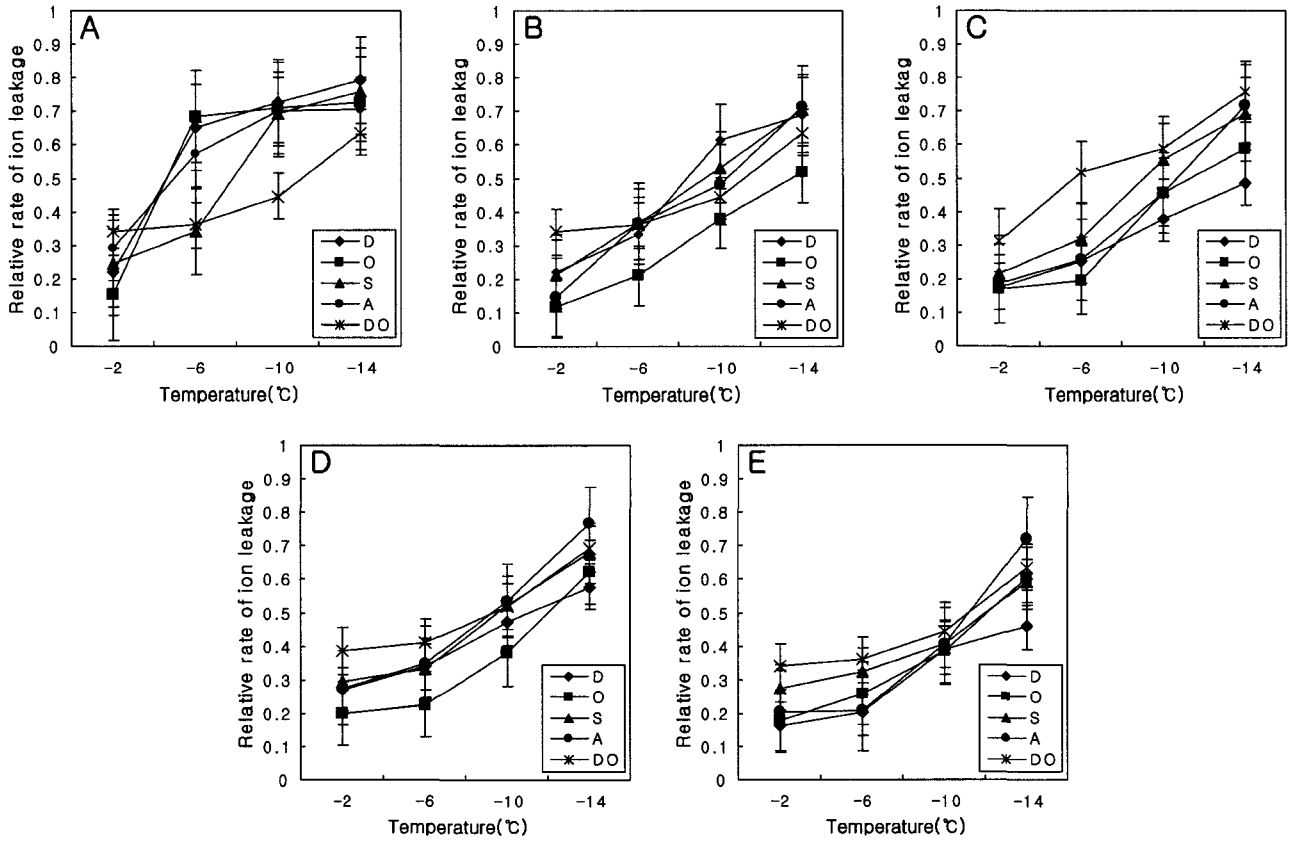

Fig. 1. Ion leakage test at non cold acclimation (A), CA 3 days (B), CA 10 days (C), CA 20 days (D), CA 40 days (E) after cold acclimation.

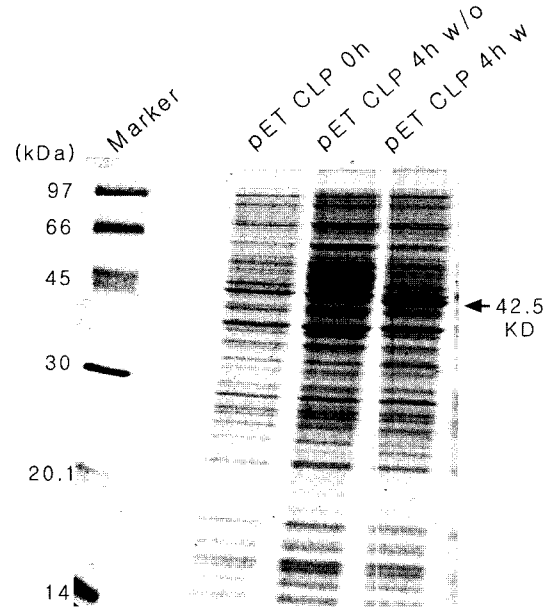

Fig. 2. Expression of CLP fused with thioredoxin protein using a pET-32 expression system in *E. coli*.

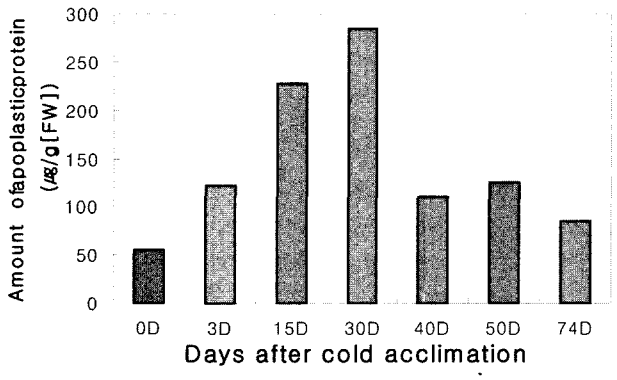

Fig. 3. Concentration of extracellular proteins accumulated in apoplast of Dongbori1ho leaves as the time increased for cold acclimation.

this study is a chitinase typeII lacking of chitin binding domain but containing a signal peptide toward apoplast via ER (Walther-Larsen *et al.*, 1993). A close correlation observed between amounts of the CLP and the duration of cold acclimation (Fig. 3, Fig. 4). In facts, an introduction of a CLP gene in tobacco had been shown to increase freezing tolerance (Hwang *et al.*, 2000).

Based on previous studies showing a role of CLP in freezing tolerance, the amounts of CLP accumulated among five different cultivars of barley having shown various degrees of the tolerance were measured.

Overall total proteins, in parallel to the CLP of 27.7 kDa, were found to be increased in apoplast as being cold-acclimated longer. It appears that a peak of CLP accumulation in apoplast was appeared at 25 days after cold acclimation

Fig. 4. Western analysis of the apoplastic proteins with CLP antiserum from leaves of Dongbiri1ho as the time for cold acclimation increased.

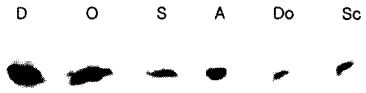

Fig. 5. Western analysis of apoplastic proteins accumulated during 40 days' cold acclimation. Using CLP antibody among 5 cultivars of barley; D(Dongbori1ho), O(Olbori), S(Saechalsalbori), A(Albori), DO(Doowonchapsalbori), Sc(non-acclimated of Saechalsalbori as an internal control to compare between each of membranes tested).

(Fig. 4). This may correspond to the change of total proteins in apoplast as shown in Fig. 3. Dongbori1ho and Albori showed higher levels of CLP even in the absence of cold acclimation.

On day 40 after cold acclimation, five cultivars showed different amounts of CLP in apoplast in the same order of Dongbori1ho, Olbori, Saechalsalbori, Albori and Doowonchapsalbori as having reported from field test of freezing tolerance (Fig. 5 and Table 1).

This may suggest a possibility of CLP as a molecular marker for freezing tolerance as long as plant undergoes cold acclimation for 40 days.

Antifreezing Test Using TLP Antiserum

TLP antiserum was prepared by using a TLP of 16 kDa separated in SDS-PAGE and eluted from the gel in Dongbori1ho cold acclimated for 53 days. The time course analysis of western hybridization showed a gradual increase of apoplast proteins immunoreacted with TLP antiserum but there were various induction patterns of individual TLP of different sizes though they commonly reacted with the TLP antiserum. In facts Griffith *et al.* (1994) had also reported TLPs of 16, 25, 28, 32, and 35 kDa found in apoplast of rye after cold acclimation. Overall it appears that the TLPs were accumulated drastically at 5 days of cold acclimation and then decreased more or less with change in composition of members of TLP family. With an emphasis on a TLPs of 16 and 30 kDa that was used as antigen, at day 10 their accumulation reaches at a maximum (Fig. 6).

In this Western analysis Dongbori1ho was used since it was expected to express the TLP to the highest level due to the highest freezing tolerance observed (Cho *et al.*, 1979). Five cultivars of barley showed the amount of total TLPs

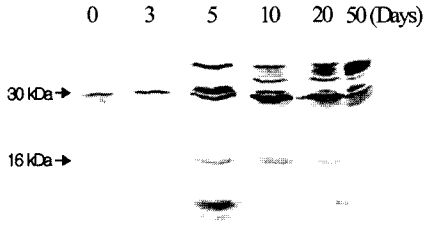

Fig. 6. Western analysis using TLP antibody of the apoplastic proteins from leaves of Dongboril ho cold acclimated in different time periods.

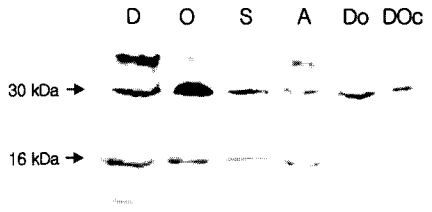

Fig. 7. Western analysis of apoplastic proteins accumulated during 40 days' cold acclimation using CLP antibody among 5 cultivars of barley; D(Dongboril ho), O(Olbori), S(Saechalsalbori), A(Albori), DO(Doowonchapsalbori), DOc (non-acclimated of Doowonchapsalbori as an internal control to compare between each of membranes tested).

accumulated in apoplast in an order of Dongboril ho, Olbori, Saechalsalbori, Albori and Doowonchapsalbori as reported from a field test of freezing tolerance. The lower band (16 kDa) of TLP showed more close correlation to freezing tolerance but the higher band (30 kDa) of TLP showed to be the highest in Olbori (Fig. 7).

It appears that the amounts of both CLP and TLP quite well correlate to the degrees of freezing tolerance measured from field test.

In Situ Immunoassay of CLP

There were many clues showing that a CLP is involved in both cold acclimation and freezing tolerance. In addition to such quantitative analyses, an *in situ* immunolocalization was performed to see any qualitative difference such as distribution of CLP in leaf tissue.

To subtract the background coming from nonspecific reaction from the signal, a pre-immune serum was used as a control. As shown in Fig. 8, there were much more signals for CLP found to be accumulated in margin of the bottom side of leaf acclimated for 40 days comparing to the non-acclimated. In the light of the fact that the empty space in bottom side make it more vulnerable to freezing than the upper side piled with palisade layer, the presence of highly concentrated CLP in the lower side of leaf may provide protection from freezing. In facts, the similar observation was reported

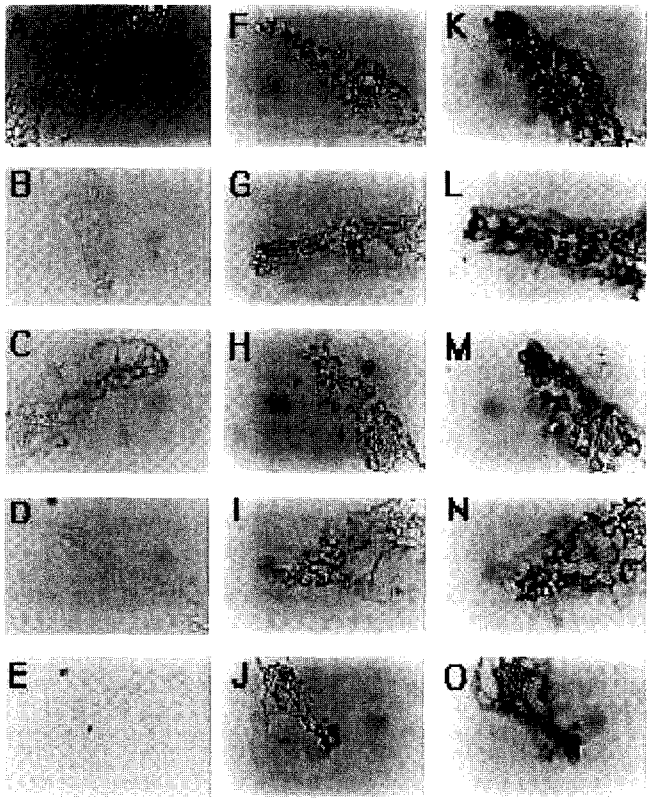

Fig. 8. *In situ* immunohistochmical localization of CLP in transverse sections of leaf margin at 0 day and 40 days after cold acclimation.
(A)~(E) CA 0 day with CLP antiserum, (F)~(J) CA 40 days with pre-immune serum, (K)~(O) CA 40 days with CLP antiserum.
(A), (F), (K): Dongboril ho ; (B), (G), (L): Olbori ; (C), (H), (M): Saechalsalbori ; (D), (I), (N): Albori ; (E), (J), (O): Doowonchapsalbori

also in rye by Mervi *et al.* (1996). When comparing both the amount and distribution of CLP, there was no clear difference observed in distribution of CLP among five cultivars of barley acclimated for 40 days. However it appeared that there was a correlation to some extents of the amounts of freezing tolerances such that Dongboril ho showed the highest but Doowonchapsalbori showed the least amounts of CLP accumulated (Fig. 8, 9).

In order to find how to measure a freezing tolerance in barley, Western analysis and *in situ* immunological methods had been applied to see both qualitative and quantitative differences of antifreeze proteins, CLP and TLP. The results showed that the levels of the proteins accumulated in leaf apoplast were correlated to those of antifreezing tolerance as observed with field and ion leakage tests. The CLP was found to increase in areas of margin and around vein as the plants were cold-acclimated but no difference was observed among cultivars by *in situ* localization (Fig. 8, 9).

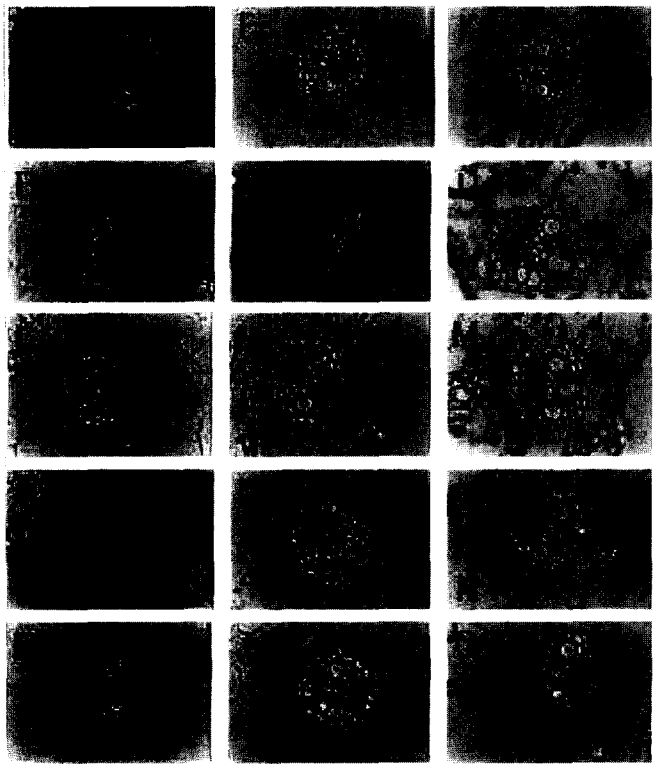

Fig. 9. *In situ* immunohistochmical localization of CLP in transverse sections of leaf vein at 0 day and 40 days after cold acclimation.
(A)~(E) CA 0 day with CLP antiserum, (F)~(J) CA 40 days with pre-immune serum, (K)~(O) CA 40 days with CLP antiserum
(A), (F), (K): Dongbori1ho ; (B), (G), (L): Olbori ; (C), (H), (M): Saechalsalbori ; (D), (I), (N): Albori ; (E), (J), (O): Doowonchapsalbori

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