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Class II chitinase accumulated in the bark tissue involves with the cold hardiness of shoot stems in highbush blueberry (*Vaccinium corymbosum* L.)

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ABSTRACT

To understand the development of cold hardiness in highbush blueberry plants (*Vaccinium corymbosum* L.), we investigated seasonal changes in the protein compositions of current-year and overwintered shoots. Electrophoresis using SDS-polyacrylamide gels indicated that the amounts of a few proteins increased in autumn, in association with an enhancement of cold hardiness. Of these proteins, 65- and 60-kDa proteins were confirmed to be dehydrins by western blotting. While the levels of most of the accumulated proteins decreased in April, a 27-kDa protein maintained its level in the overwintered stem during spring. The amino acid sequence deduced from a cDNA for this protein showed significant similarities with known chitinases. The stem segments from overwintered shoots sampled in July showed higher cold hardiness against sub-zero temperature than those from current-year shoots, and the bark tissue of overwintered shoots had higher cold resistance than that of current-year shoots. The 27-kDa protein (BC27) was mostly found in the bark tissue of stems. Appreciable accumulation of this protein in floral buds was not detected. The purified BC27 protein exerted a cryoprotective effect on protoplasts subjected to a freeze–thaw treatment. These results suggest that the stems of highbush blueberry develop a system of cold hardiness different from that of the buds and apices, and that the accumulation of chitinase is involved in tolerance to low temperature in winter or unseasonably low temperature in spring.

1. Introduction

Cold acclimation is a seasonal process in many overwintering temperate species. It is characterized by increased cold hardiness in fall, reaching a maximum in winter (acclimation), followed by a decrease in cold hardiness during spring (deacclimation) (Weiser, 1970; Guy, 1990; Ashworth and Wisniewski, 1991; Arora et al., 1992). While recent breakthroughs have increased our knowledge of the molecular basis of cold acclimation in herbaceous species, the mechanism underlying cold acclimation and tolerance of cold stress in woody plants, including fruit trees, is poorly understood.

In deciduous woody plants, proteins accumulate in considerable amounts during autumn. These proteins are translocated from leaves to storage sites in the bark during autumnal leaf senescence and degraded at the onset of spring growth (O'Kennedy and Titus, 1979; Wetzel et al., 1989; Wetzel and Greenwood, 1991). The accumulation of these bark storage proteins is induced by low-temperature treatment, by a short-day photoperiod and by nitrogen feeding under continuous long-day conditions (Coleman et al., 1991; Langheinrich and Tischner, 1991; van Cleve and Apel, 1993). Wisniewski et al. (2004) suggested that storage proteins have functional roles in addition to nitrogen storage. For example, dehydrin was reported to become abundant in bark tissue of peach (*Prunus persica* [L.] Batsch) during cold acclimation and exhibits antifreeze activity (Wisniewski et al., 1999, 2004).

Blueberries (*Vaccinium* sp.) have become one of the top berry crops of the world, and increased production is expected in the future (Strik, 2005). In order to improve the field performance of highbush blueberry under severe conditions, attempts have been made to identify proteins that play a role in cold acclimation in floral buds. And it is known that dehydrins accumulate in floral buds during autumn or in response to cold treatment and are known to contribute to increase in cold hardiness (Muthalif and Rowland, 1994; Arora et al., 1997; Levi et al., 1999; Dhanaraj et al., 2005). The synthesis of dehydrins is also induced in organs such as stems, leaves, and roots (Panta et al., 2001).

In northern areas, current-year shoots of highbush blueberry frequently suffer damage due to freezing during the winter or early spring. Therefore, increased cold hardiness in shoot stems is expected to improve productivity and the quality of crops.

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However, the role of shoot-stem proteins and their changing expression in the cold hardiness and cold acclimation of highbush blueberry plants are not yet fully understood.

In this study, we investigated seasonal patterns of cold hardiness and the protein composition of blueberry stems, and identified several proteins that show large changes from autumn to spring. The physiological role of the identified proteins in current-year and overwintered shoots was also examined.

2. Materials and methods

2.1. Plant material

Current-year and overwintered (1-year-old) shoots 15–20 cm long were collected from highbush blueberry plants (*Vaccinium corymbosum* L.) cv. Rancocas grown in field conditions on the campus of Hokkaido University, Sapporo, Japan. Collected shoots were placed in a box with ice and brought to the laboratory.

2.2. Protein extraction

Stems and floral buds were ground in a mortar and pestle in liquid nitrogen and homogenized with 10 vol of extraction buffer [8 M urea, 50 mM Tris–HCl, pH 7.6, 2% (w/v) Triton X-100, 5 mM EDTA, 10 mM dithiothreitol (DTT)] containing a protease inhibitor mixture (Complete; Roche Diagnostics, Basel, Switzerland). The homogenate was centrifuged at 16,000 × *g* for 10 min at 4 °C, and proteins were precipitated by the addition of 4 vol of acetone to the supernatant. After incubation at–40 °C for 2 h, the suspension was centrifuged at 2300 × *g* for 5 min. The pellet was washed with 75% acetone and air-dried. Bark, xylem, and pith tissues in January were cut from the stems with a razor, and proteins were extracted using the same method.

2.3. Polyacrylamide gel electrophoresis (PAGE)

Proteins were dissolved in a solution containing 1.2% (w/v) sodium dodecyl sulfate (SDS), 50 mM Tris–HCl, pH 6.8, 1% (v/v) β-mercaptoethanol, 20% (v/v) glycerin, and 0.001% (w/v) bromophenol blue and boiled in water for 5 min. SDS-PAGE was performed by using NuPAGE 4-12% Bis-Tris Gel (Invitrogen Co., Carlsbad, CA). For two-dimensional gel electrophoresis (2D-PAGE), proteins were dissolved in a solution containing 8.5 M urea, 2% (w/v) Nonidet P-40 (Nacalai Tesque, Kyoto, Japan), 2% (v/v) Servalyt 3-10 (Serva Feinbiochemica, Heidelberg, Germany) and 5% (v/v) β-mercaptoethanol. Insoluble protein was removed by centrifugation. Separation of proteins in the first dimension was achieved by nonequilibrium pH-gradient electrophoresis (NEpHGE), as described by O'Farrell et al. (1977), with 1.0% Servalyte 3–10 plus 1.0% Servalyt 3–7 as a carrier ampholyte. Separation in the second dimension was achieved by SDS-PAGE as described by Laemmli (1970), on a 12.5% polyacrylamide gel. Proteins in gels were stained with Coomassie brilliant blue R-250 and destained by diffusion.

2.4. Detection of dehydrins

To detect dehydrins, an immunoblot analysis was performed. Proteins extracted from stems and floral buds during winter were separated by SDS-PAGE and transferred to a polyvinylidene fluoride (PVDF) membrane by electrophoresis. The PVDF membrane was blocked by phosphate-buffered saline (PBS: 9.6 mM phosphate buffer, pH 7.4, supplemented with 137 mM NaCl and 2.68 mM KCl) containing 2% (w/v) bovine serum albumin. Blots were incubated in a 1:1000 dilution of rabbit anti-dehydrin polyclonal antibody (Stress Gen Biotechnologies, Victoria, BC, Canada) as the primary antibody and a 1:1000 dilution of alkaline phosphatase-conjugated donkey anti-rabbit IgG antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) as the secondary antibody, followed by staining with 0.3% (w/v) fast red TR salt and 0.05% (w/v) naphthol AS-MX phosphate (Sigma Chemical Co., St. Louis, MO) in 200 mM Tris–HCl, pH 8.2.

2.5. Protein microsequencing

After 2D-PAGE, protein spots excised from gels were partially digested in the gel with endoproteinase Glu-C or Lys-C and then separated according to the method described by Cleveland et al. (1977). The separated polypeptides were transferred to a PVDF membrane. The amino acid sequences were determined with a gas-phase microsequencer (model 491 Procise; Applied Biosystems, Foster City, CA).

2.6. Cloning and sequencing of cDNA

Sampled stems were quickly frozen in liquid nitrogen, ground to powder in a mortar and pestle, and homogenized with 10 vol of extraction buffer containing 2% (w/v) cetyltrimethylammonium bromide, 0.1 M Tris–HCl, pH 7.5, 20 mM EDTA, 1.4 M NaCl, and 1% (w/v) β-mercaptoethanol. After incubation at 65 °C for 15 min, the homogenate was extracted with chloroform/isoamyl alcohol (24:1), and the RNA in aqueous phase was precipitated by addition of an equal volume of isopropanol. The precipitates were dissolved in water, and LiCl was added to a final concentration of 2 M. Precipitated RNA was collected by centrifugation, dissolved in water, and extracted with phenol and chloroform. The RNA in the aqueous phase was precipitated again by adding LiCl to a final concentration of 2 M. The precipitates were washed with 75% ethanol twice.

To amplify cDNA for the 27-kDa protein, 3′-RACE was performed using a Smart Race cDNA Amplification Kit (Clontech, Palo Alto, CA), using the degenerate primer 5′-GA(AG)AC(CT)A-C(ATG)GG(ATC)GG(AG)TGGGC-3′, and the nested primer 5′-TGGGC(AG)AC(ATG)GC(AG)CC(ACG)GA(CT)GG-3′ as sense primers, which were designed based on the partial amino acid sequence of the original protein. 5′-RACE was performed using 5′-CATAGTACTTCTTCCCAGGAGCA-3′ as an antisense primer based on the cDNA sequence determined by 3′-RACE.

DNA sequencing was carried out using a Thermo Sequenase Cy5 Dye Terminator Cycle Sequencing Kit (Amersham Biosciences, Piscataway, NJ) on an AFLexpress DNA sequencer (Amersham Biosciences).

2.7. Computer analysis

The theoretical isoelectric point (p*I*) and molecular weight of amino acid sequence was analyzed with ExPASY Proteomics Tools (Wilkins et al., 1998). The amino acid sequence comparisons with the GenBank database were performed using BLASTP, and the multiple sequence alignment of selected amino acid sequences was performed by the BCM Search Launcher (Smith et al., 1996).

2.8. Cold-hardiness determination

The cold hardiness of the stem tissues was evaluated by use of the electrolyte leakage method of Arora et al. (1992), with a few modifications. Twig pieces about 10 cm long sampled from current and overwintered shoots in July were wrapped in aluminum foil along with moistened paper and transferred to a programmable freezer (MC-71; TABAI Co., Osaka, Japan). After 2 h at −2 °C, the temperature of the chamber was lowered to a given temperature at the rate of −0.17 °C/min. Samples kept for 30 min at a given

temperature were removed from the freezer and thawed at 4 °C overnight. Nonfrozen twigs were maintained at 4 °C overnight. "Freezing death" twigs were frozen with liquid nitrogen and thawed at 4 °C overnight. At each treatment temperature, twigs were cut into 3–4 mm thick slices, which were each placed in a 1.5 ml reaction tube containing 1 ml of deionized water and maintained at room temperature for 3 h. Subsequently, the conductivity of the effusion was recorded with a conductivity meter (B-173; Horiba, Kyoto, Japan). Samples were then heat-treated in the same water and conductivity was once again recorded at room temperature. Ion leakage was calculated as a percentage of the total (after heat treatment). Percentage injury was then calculated according to the method of Zhang and Willison (1987) using the modified expression:

$$\left[\%\text{injury} = \frac{\%\text{L(t)} - \%\text{L(c)}}{\%\text{L(d)} - \%\text{L(c)}}\right] \times 100$$

where %L(t), %L(c), and %L(d) are the measurements of percentage of ion leakage for the respective freeze-treatment temperature, nonfrozen control, and freezing death control, respectively.

2.9. Visualization of esterase activity by use of fluorescein diacetate (FDA)

After freezing and thawing according to the same method used in determining cold hardiness, twigs were sectioned and incubated in PBS containing 0.002% (w/v) FDA for 5 min at room temperature. The sections were washed with PBS and observed using a BX50 microscope equipped with epifluorescence optics (OLYMPUS, Tokyo, Japan).

2.10. Protein purification

The BC27 protein was purified by conventional liquid chromatography. In all steps, eluates were routinely monitored by measuring the absorbance at 280 nm. Bark tissues sampled in January were ground in a mortar and pestle with liquid nitrogen and homogenized with 10 vol of extraction buffer [50 mM Tris–HCl, pH 7.6, 2% Triton X-100, 5 mM EDTA, 10 mM DTT, 0.2 mM Pefabloc SC (Roche Diagnostics)]. The homogenate was centrifuged at 20,000 × *g* for 30 min at 4 °C, and the protein in the supernatant was precipitated with 60% saturation of ammonium sulfate. The precipitate was collected by centrifugation at 20,000 × *g* for 30 min at 4 °C and resuspended in 10 mM MES [2-(*N*-morpholino)ethanesulfonic acid]-KOH, pH 6.5, containing 0.1% (w/w) Triton X-100 and 1 mM DTT. The resulting suspension was dialyzed against deionized water and adjusted to 10 mM MES-KOH, pH 6.5, containing 0.1% (w/w) Triton X-100 and passed through the DEAE-Sepharose CL-6B (Amersham Biosciences) column equilibrated with the same buffer. Flow through was collected and applied to the CM-Sepharose CL-6B (Amersham Biosciences) column equilibrated with the same buffer. Bound proteins were eluted with a linear gradient of 0–300 mM NaCl in the same buffer at a flow rate of 0.5 ml/min. The fractions containing BC27 were dialyzed against deionized water, adjusted to 10 mM sodium phosphate, pH 6.8, and 1.8 M ammonium sulfate, and loaded on a *t*-Butyl HIC cartridge (Bio-Rad) that was equilibrated with 10 mM sodium phosphate, pH 6.8, and 1.8 M ammonium sulfate. Proteins were eluted from the column with a gradient of 1.8–0 M ammonium sulfate. Fractions containing BC27 were dialyzed against deionized water, concentrated by ultrafiltration (Molcut LGC; Millipore, MA), and stored at −40 °C.

2.11. Cryoprotective effect of BC27 on tobacco protoplasts

Tobacco (*Nicotiana tabacum* L.) BY-2 cell suspension cultures were maintained by subculturing aliquots every week in Nagata et al. (1981) modified version of Linsmaier and Skoog medium. The cells were harvested 4 days after subculture and filtered through a stainless steel sieve with an opening size of 250 μm. Fine cell clusters in the filtrate were pelleted by centrifugation at 500 × *g* for 2 min and suspended in a wash buffer (10 mM MES-KOH, pH 5.8, and 0.4 M sorbitol) for 15 min. Cells then were treated with an enzyme solution containing 1% (w/v) Meicelase (Meiji Seika Kaisha Ltd., Tokyo, Japan) and 0.1% (w/v) Pectolyase Y-23 (Seishin Pharmaceutical Co., Ltd., Tokyo, Japan) in wash buffer for about 1 h with gentle shaking. The resulting protoplasts were pelleted at 150 × *g* for 2 min, washed three times with a wash buffer, and suspended at the density of 1×10^4–10^5 protoplasts/ml in wash buffer.

The freezing experiments were conducted following Pihakaski-Maunsbach et al. (2003). Protoplast suspensions were mixed with BC27 or BSA in a 0.5 ml reaction tube at the concentration shown in the results and discussion section and kept at 4 °C for 30 min. After equilibration of protoplast suspensions at −3 °C for 30 min, the suspensions were ice-nucleated by dropping a small ice crystal into the tubes and maintaining the contents at −3 °C for an additional 10 min. The tubes containing frozen materials then were cooled at a rate of approximately −1 °C/min to the desired temperatures, where they were kept for 20 min. The samples were thawed at 25 °C, kept on ice, and subjected to the determination of viability. Viability of protoplasts was determined by staining with

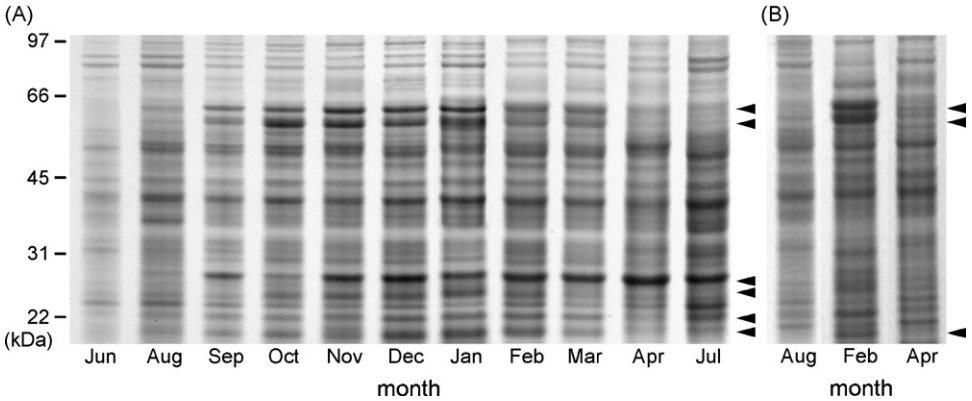

Fig. 1. SDS-PAGE protein profiles of highbush blueberry stems and floral buds. (A) Monthly SDS-PAGE profiles of proteins extracted from current-year shoots (left panel, months indicated at the bottom of electrophoretograms) and a typical SDS-PAGE profile of protein extracted from 1-year-old shoots in summer (rightmost lane, sampled in July). Apparent molecular masses are shown on the left side. The arrowheads indicate the position of 65-, 60-, 27-, 25-, 22-, and 20-kDa proteins. (B) SDS-PAGE protein profiles of floral buds in August, February, and April. The arrowheads indicate the position of 65-, 60- and 20-kDa proteins.

FDA (Pihakaski-Maunsbach et al., 2003). Protoplasts with fluorescence were counted using a BX50 microscope equipped with epifluorescence optics. All viability assays were conducted in triplicate. Differences in viability were analyzed using Student's *t*-test.

3. Results and discussion

3.1. Seasonal changes in highbush blueberry stem proteins

Seasonal changes in stem proteins of highbush blueberry were examined using SDS-PAGE (Fig. 1A). Three major proteins of 65, 60, and 27 kDa began to appear in September, and 22- and 20-kDa proteins in August. The 65-, 60-, 22- and 20-kDa proteins remained until March, then their levels diminished and they disappeared in April. The level of a 25-kDa protein started to increase in October and fell in March. The LT_{50} of the stem segments, determined by the TTC reduction test, began to decrease in September, showing the minimum value below −40 °C in February, and increased thereafter (data not shown). The accumulation of these proteins was likely related to the increase in cold hardiness.

O'Kennedy and Titus (1979) found that storage proteins in bark tissue from dormant apple shoots (*Malus pumila* Rehd.) completely disappeared as growth was resumed. Similarly, Wetzel et al. (1989) found that storage proteins were greatly reduced in the bark of temperate hardwoods, though they were not completely absent during spring, and supposed that these proteins act as a nitrogen reserve. The accumulated 65-, 60-, 25-, 22-, and 20-kDa proteins were degraded in spring and presumably utilized for nutrition. However, the 27-kDa protein maintained its level until at least July (Fig. 1A), suggesting that it could not be a storage protein. The 27-kDa protein was not detected in extracts from floral buds (Fig. 1B).

3.2. Identification of dehydrin proteins

Dehydrins of 65-, 60-, and 14-kDa accumulate in cold-acclimated floral buds, stems, leaves, and roots of blueberry, and have been suggested to be related to cold hardiness (Muthalif and Rowland, 1994; Arora et al., 1997; Levi et al., 1999; Dhanaraj et al., 2005). We detected 65- and 60-kDa dehydrins by immunoblot analysis of stems and floral buds proteins from cold-acclimated blueberry plants, while the 27-, 25-, 22-, and 20-kDa proteins of stems and the 20-kDa protein of floral buds did not react with anti-dehydrin antibody (Fig. 2A). Though the 77- and 45-kDa proteins from stems, and the 86-, 77-, and 45-kDa proteins from floral buds reacted with anti-dehydrin antibody, they showed little seasonal change (Figs. 1A & B and 2A). Dehydrin is one of late-embryogenesis abundant proteins and intrinsically known as protein related with protecting against or avoiding from dehydration stress (Close, 1996). It is possible that the 77- and 45-kDa proteins from stems, and the 86-, 77-, and 45-kDa proteins from floral buds relate to dehydration stress but not to cold acclimation.

3.3. Identification of a cDNA encoding the 27-kDa protein

The 27-kDa protein showed a distinctive change in banding patterns from other major proteins (Fig. 1A and B). Hence, identification of a cDNA for this protein was attempted. To acquire a partial amino acid sequence of the 27-kDa protein, we fractionated proteins from current-year stems in winter and from overwintered stems by 2D-PAGE. The 27-kDa protein ran in the basic p*I* range (p*I* 7.5) (Fig. 2B), and from it we derived the internal sequences TTGGWATAPDGPYA-GY-FL, QGNPPNY-VANQQWPCA-G, and YYGRGPIQISYN. This enabled us to identify a cDNA encoding the 27-kDa protein (*BC27*). The open reading frame encoded a protein of 264 amino acid residues with a calculated molecular

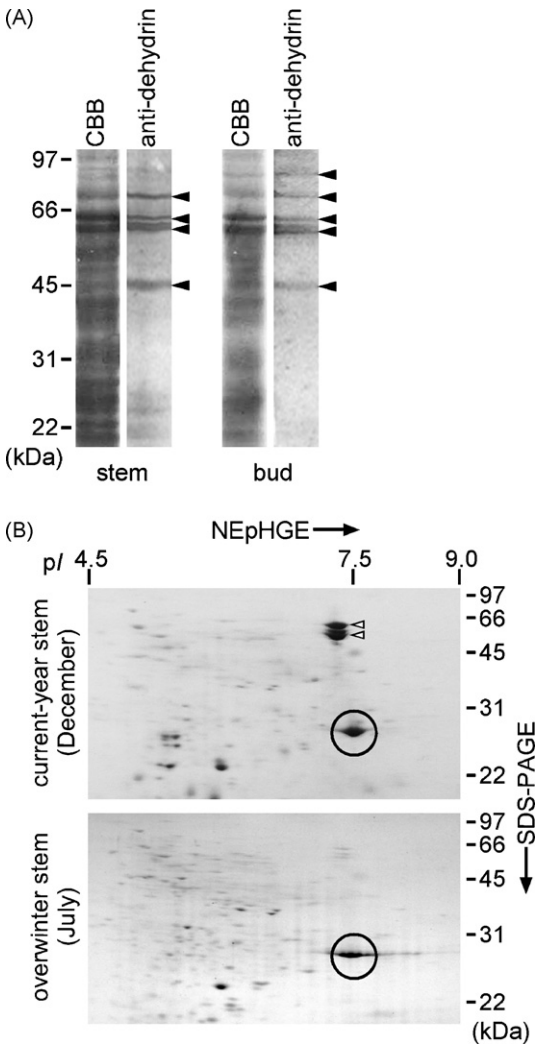

Fig. 2. An immunoblot analysis and two-dimensional gel electrophoresis (2D-PAGE) analysis of highbush blueberry. (A) The immunostaining of dehydrin in stem and floral bud of highbush blueberry in winter by western blotting. The primary antibody was rabbit anti-dehydrin polyclonal antibody and the secondary antibody was alkaline phosphatase-conjugated donkey anti-rabbit IgG antibody. The arrowheads indicate the position of 77-, 65-, 60- and 45-kDa proteins in stems and 86-, 77-, 65-, 60- and 45-kDa proteins in floral buds. The protein on the PVDF membrane was visualized by staining with Coomassie brilliant blue (CBB) R-250. (B) Fractionation by 2D-PAGE analysis of proteins extracted from stems of highbush blueberry. Proteins were fractionated first by nonequilibrium pH-gradient gel electrophoresis, then by SDS-PAGE, and were visualized by staining with CBB R-250. The open circle marks the position of the 27-kDa protein (p*I* 7.5). The arrowheads indicate the position of the 65- and 60-kDa (p*I* 7.3) dehydrin proteins.

mass of 28.4 kDa and a p*I* of 8.8. Alignment of the predicted amino acid sequence with those of other proteins in the databases (Fig. 3) suggested that BC27 protein must be a chitinase (EC 3.2.1.14). The homologous region extended throughout the glyco-hydro-19 (catalytic) domain. The catalytic domain was highly conserved across class I and II chitinases. The average similarity of BC27 protein to known class I and class II chitinases was 78.5% and 73.6%, respectively. As class II chitinases lack both an N-terminal chitin-binding domain and a glycine-rich hinge region, we assigned BC27 protein to the class II chitinases.

It is known that pathogenesis-related proteins, including chitinase, β-1,3-glucanase, and thaumatin, accumulate in the apoplasts of cold-acclimated monocot species. These proteins bind to the surface of potential ice nuclei, thereby inhibiting ice-crystal growth in vitro (Antikainen and Griffith, 1997; Yeh et al., 2000).

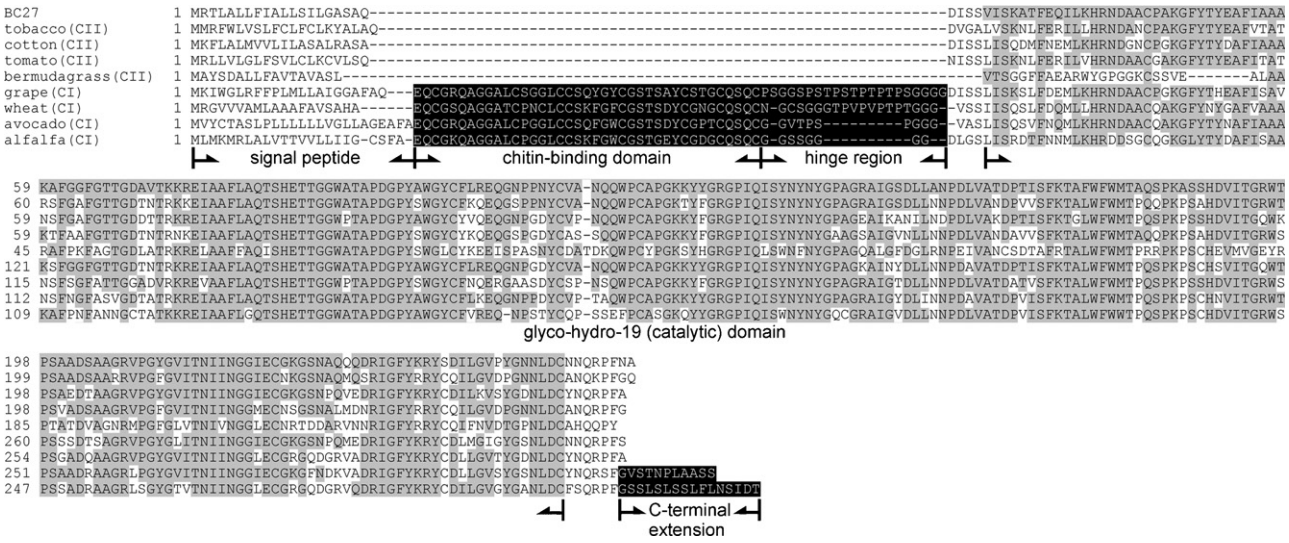

Fig. 3. Alignment of the 27-kDa highbush blueberry stem protein with the sequences of class I and II chitinases from other plant species. Class II accession numbers: tobacco, AB008892; cotton, Z68152; tomato, U30465; Bermuda grass, AF105425. Class I accession numbers: grape, AJ291506; wheat, X76041; avocado, Z78202; alfalfa, U83592. Identical amino acids in the catalytic domain are indicates by gray boxes, and the class I structural domains are highlighted on a black background.

Apoplastic proteins with endochitinase-like proteins extracted from *Phellinus weirii*-infected winter Douglas-fir (*Pseudotsuga menziesii* var. *menziesii*) needles also showed antifreeze activity (Zamani et al., 2003), suggesting that the expression of a chitinase protein may enhance cold hardiness.

3.4. Overwintered stems retain some level of cold hardiness

We compared the cold hardiness of current-year stems with that of overwintered stems to determine whether the accumulation of BC27 protein is related to cold hardiness. The current-year and overwintered stems were sampled in July and tested to estimate their cold hardiness. The proportion of freezing injury in current-year stems was 82% at −10 °C; in overwintered stems it was 50% at −10 °C and 88% at −25 °C (Fig. 4). Thus, the cold hardiness of overwintered stems was greater than that of current-year stems.

Because BC27 protein was present exclusively in the bark tissues of stems (Fig. 5, Lane 4), we examined the difference in cold hardiness among stem tissues by staining for esterase activity (Fig. 6). No tissue in the current-year stems frozen at −10 °C exhibited esterase activity, and only bark tissue from overwintered stems frozen at −20 °C showed esterase activity. The bark tissue displayed higher cold hardiness than the xylem and pith tissues. Similar results were obtained in a study of stem samples obtained from peach plants in winter (Arora et al., 1992).

We also examined the cryoprotective activity of exogenously applied BC27 protein to tobacco protoplasts. The activity is thought to represent protection effect on the plasma membrane. The BC27 protein was highly purified with ion-exchange chromatography and hydrophobic interaction chromatography (Fig. 5, Lanes 5 and 6). The viability of protoplasts after freezing and thawing was improved by the addition of 10 and 50 μg/ml of BC27 protein. The improvement was greater in protoplasts treated at −5 °C than those treated at −3 °C. The optimum concentration of BC27 addition was 50 μg/ml (Table 1). Addition of BSA to the protoplast suspensions had little promotive effect on the viability of protoplasts (Table 1), and addition of BC27 and BSA to cell suspensions had little effect on viability after the freeze–thaw treatment (data not shown). These results imply that BC27 protein released through the plasma membrane exhibits a cryoprotective

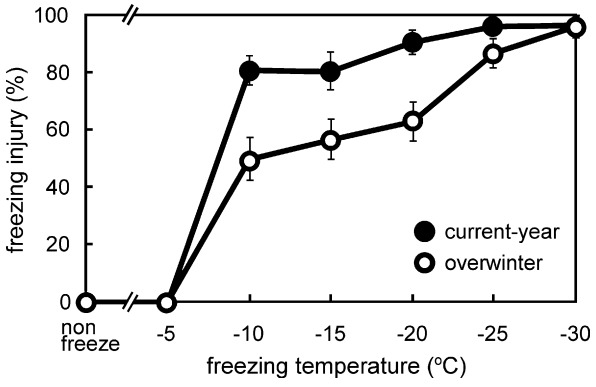

Fig. 4. Cold hardiness determination of current-year and overwintered highbush blueberry stems in July. Following freezing and thawing, the estimation of the degree of freezing injury to the stems was evaluated by using the electrolyte leakage method. The bars represent ±S.E. of three experiments.

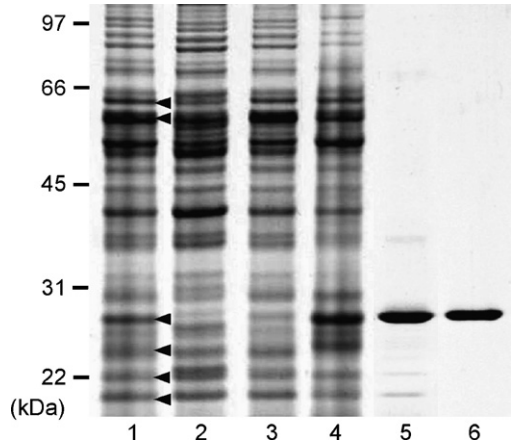

Fig. 5. SDS-PAGE of extracted stem protein and the purified BC27 protein in January. Apparent molecular masses are shown on the left side. The arrowheads indicate the positions of the 65-, 60-, 27-, 25-, 22-, and 20-kDa proteins. Lanes 1–4: proteins extracted from entire stem, pith, xylem and bark tissue, respectively (10 μg protein applied to each lanes). Lane 5: elution fraction of cation exchange chromatography (CM-Sepharose). Lane 6: a fraction of hydrophobic interaction chromatography (lanes 5 and 6, 3 μg protein applied).

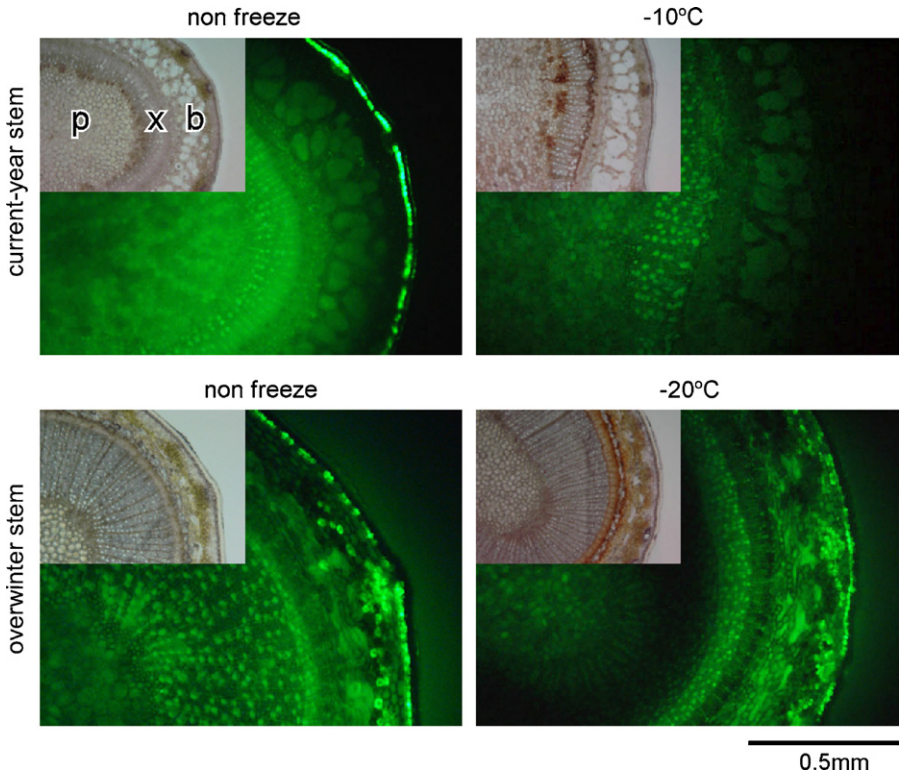

Fig. 6. Difference in the cold hardiness of highbush blueberry stems tissue. Stems sampled in July were frozen, thawed, and thin-sectioned. They were stained with FDA and examined under a fluorescence microscope. Inserts are light field images of the same fields; b = bark, x = xylem, and p = pith.

activity in the apoplast. This speculation agrees with the evidence that chitinases accumulated in apoplast of winter rye during cold acclimation exhibit antifreeze activity (Antikainen and Griffith, 1997; Yeh et al., 2000). However, the extent was limited at a higher concentration. The process of cold acclimation is very complex and involves many biochemical and physiological changes (Thomashow, 1999), and therefore it is required to identify other molecules that participate the development of cold acclimation.

Spring frosts often damage fruit trees (Rodrigo, 2000). Therefore, BC27 may help highbush blueberry plants avoid freezing injury during periods of unseasonably low temperatures in spring and increase cold hardiness in winter. The accumulation of some classes of chitinases in bark tissue, rather than xylem and pith tissues, should help to protect the outer tissue that is exposed to cold temperature in overwintered plants. Understanding the relationship between chitinases and cold hardiness in highbush blueberry plants will require further research.

Table 1
Effect of exogenous BC27 and BSA on the viability of BY2 protoplasts in freeze–thaw experiments.

Additives/concentration	Viability (%)[a]/effect of additive on the viability of protoplast at		
	0 °C	−3 °C	−5 °C
BC27/0 μg/ml	84.2 ± 2.8/1.00	25.0 ± 2.9/1.00	12.7 ± 3.1/1.00
BC27/10 μg/ml	76.2 ± 3.7/0.90	37.0 ± 5.7/1.48	21.6 ± 2.9/1.70
BC27/50 μg/ml	78.8 ± 8.3/0.94	36.7 ± 2.2/1.47	25.0 ± 6.1/1.97
BC27/100 μg/ml	83.7 ± 4.8/0.99	32.6 ± 5.1/1.30	14.7 ± 4.9/1.16
BSA/10 μg/ml	74.0 ± 2.6/0.88	27.1 ± 5.7/1.08	13.8 ± 2.9/1.09
BSA/50 μg/ml	76.4 ± 8.4/0.91	25.4 ± 8.1/1.01	12.0 ± 1.0/0.94
BSA/100 μg/ml	57.8 ± 0.3/0.69	12.0 ± 1.3/0.48	7.4 ± 1.4/0.58

[a] Relative values of viability of protoplasts treated in media containing the indicated concentrations of BC27 or BSA compared to the viability at the same temperature without the addition of proteins.

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