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Identification of Dehydrin-Like Proteins Responsive to Chilling in Floral Buds of Blueberry (*Vaccinium*, section *Cyanococcus*)[1]

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The level of three major polypeptides of 65, 60, and 14 kD increased in response to chilling unit accumulation in floral buds of a woody perennial, blueberry (*Vaccinium*, section *Cyanococcus*). The level of the polypeptides increased most dramatically within 300 h of chilling and decreased to the prechilling level with the initiation of budbreak. Cold-hardiness levels were assessed for dormant buds of *Vaccinium corymbosum* and *Vaccinium ashei* after different chilling treatments until the resumption of growth. These levels coincided with the level of the chilling-responsive polypeptides. Like some other previously described cold-induced proteins in annual plants, the level of the chilling-induced polypeptides also increased in leaves in response to cold treatment; the chilling-induced polypeptides were heat stable, resisting aggregation after incubation at 95°C for 15 min. By fractionating bud proteins first by isoelectric point (pI) and then by molecular mass, the pI values of the 65- and 60-kD polypeptides were found to be 7.5 to 8.0 and the pI value of the 14-kD polypeptide was judged to be 8.5. Purification of the 65- and 60-kD polypeptides, followed by digestion with endoproteinase Lys-C and sequencing of selected fragments, revealed similarities in amino acid composition between the 65- and 60-kD polypeptides and dehydrins. Indeed, antiserum to the lysine-rich consensus sequence EKKGIMDKIKEKLPG of dehydrin proteins cross-reacted to all three of the major chilling-responsive polypeptides of blueberry, identifying these as dehydrins or dehydrin-like proteins.

Throughout their life cycle, plants are subjected to many adverse environmental conditions such as drought, extreme temperatures, flooding, etc. that dramatically affect plant survival and limit productivity. To cope with such stresses, plants adjust metabolically and structurally. Stress-induced proteins play a definite role in protecting plants from possible damage by these conditions (Ho and Sachs, 1989).

Woody perennial plants of the temperate zone are exposed to freezing temperatures each winter. Their ability to survive is dependent on an evolved mechanism by which plants enter a state of dormancy and develop freezing tolerance (Powell, 1987). Leaf and flower bud meristems of most temperate woody perennials are formed in the summer and autumn (Saure, 1985). Following terminal bud formation, there is generally a period of time in which axillary buds can be forced to grow by removing the terminal bud that exerts apical dominance. In some plants, during late summer or early autumn, whole-tree defoliation will also stimulate bud growth (Powell, 1987). By mid to late autumn, as days become increasingly shorter and night temperatures become lower, inhibitory control of bud growth shifts to reside within the bud itself; buds are then referred to as endodormant (Lang, 1987). Plants are incapable of emerging from this type of dormancy by removal of terminal buds or by defoliation. Resumption of growth and budbreak require sufficient exposure to low temperatures, i.e. satisfaction of the CR (Scalabrelli and Couvillon, 1986). This requirement is determined genetically (Samish, 1954). CR is calculated slightly differently depending on the model used; one example is the number of hours between 0 and 7°C necessary for >50% budbreak upon exposure to higher temperatures. This requirement can range from 200 to 2000 h, depending on species and genotype. After the CR is satisfied, buds are capable of resuming growth upon exposure to 2 to 3 weeks of temperatures near 20°C. Buds with this capability that lack sufficient exposure to higher temperatures are termed ecodormant, the dormancy being imposed by unfavorable environmental conditions (Lang, 1987).

There is evidence from studies with numerous herbaceous plant species, *Arabidopsis thaliana* (Kurkela and Franck, 1990; Nordin et al., 1991; Gilmour et al., 1992), alfalfa (Mohapatra et al., 1989), spinach (Guy and Haskell, 1987, 1988), *Brassica* (Meza-Basso et al., 1986; Weretilnyk et al., 1993), and wheat (Perras and Sarhan, 1989), among others, that low, nonfreezing temperatures induce the accumulation of specific proteins in leaves and stems. Several cold-regulated proteins have been shown to have similar properties and amino acid similarity to proteins responsive to water stress and ABA treatments, identified as LEAs, Rabs, and dehydrins (dehydration-induced proteins) (Close et al., 1989; Dure et al., 1989; Jacobsen and Shaw, 1989; Danyluk and Sarhan, 1990; Lin et al., 1990; Gilmour and Thomashow, 1991; Houde et al., 1992;

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Abbreviations: BSP, bark storage protein; CR, chilling requirement; CU, chilling units; LEA, late embryogenesis abundant protein; pI, isoelectric point; Rab, ABA-responsive protein; LT_{50}, temperature at which 50% of flowers were killed.

Neven et al., 1993). These proteins are heat-soluble, hydrophilic, and they share a Lys-rich motif.

The question of how low-temperature exposure or CU accumulation is involved both in overcoming endodormancy and in the development of cold hardiness in woody perennials is poorly understood. A group of proteins called BSPs has been reported in apple (O'Kennedy and Titus, 1979), elder, locust (Nsimba-Lubaki and Peumans, 1986), maple, willow, poplar (Wetzel et al., 1989; Coleman et al., 1991), and peach (Arora et al., 1992). These have been shown to accumulate in protein storage vacuoles of inner bark parenchyma cells during autumn and winter (Greenwood et al., 1986, 1990; Sauter et al., 1988; Wetzel et al., 1989). The function of BSPs is unknown but it has been suggested that they might play a role in dormancy development, cold acclimation, and/or N_2 storage (Guy, 1990; Coleman et al., 1991; Arora et al., 1992).

In the study reported here, blueberry plants (*Vaccinium*, section *Cyanococcus*) were used to investigate changes in gene expression in floral buds of a woody perennial in response to CU accumulation and development of cold hardiness. Blueberry is a small-statured fruit crop amenable to greenhouse and growth chamber experimentation. Furthermore, blueberry plants have separate floral and vegetative buds, unlike many other fruit crops. Blueberry genotypes with high and low CR were used to study changes in protein profiles associated with CU accumulation. Three polypeptides were found to accumulate to a high level with chilling and to have similar properties to some cold-, ABA-, and dehydration-induced proteins. Using antiserum to the consensus sequence EKKGIMDKIKEKLPG found repeated one to many times within dehydrins, all three of the major chilling-responsive polypeptides of blueberry were identified as dehydrins or dehydrin-like proteins.

MATERIALS AND METHODS

Plant Material

Blueberry (*Vaccinium*, section *Cyanococcus*) genotypes with high (Bluecrop, approximately 1200 CU, and Berkeley, approximately 900 CU) and low (Tifblue, approximately 600 CU) CR were used. Bluecrop and Berkeley are highbush *Vaccinium corymbosum* cultivars; Tifblue is a rabbiteye *Vaccinium ashei* cultivar.

Beginning in October of each year, floral buds were collected periodically (every 300 CU) from field-grown plants of Bluecrop and Berkeley at the Blueberry/Cranberry Research Station (Chatsworth, NJ) and from field-grown plants of Tifblue at the Agricultural Research Center (Beltsville, MD) until the resumption of growth in the spring. Buds were collected during the winters of 1991, 1992, and 1993. After collection, buds were frozen in liquid N_2 and stored at −70°C until analyzed. CU, calculated using a biophenometer (Omnidata, Logan, UT) and weather data, were defined as the number of hours that plants were exposed to temperatures from 0 to 7°C.

To determine when the CR of each cultivar was met, approximately five shoots were cut at each sampling time and placed in jars of water at room temperature (about 20°C). Percent budbreak was determined after 3 weeks. If >50% of the buds broke, the CR was judged to be satisfied. In this way, the CRs of Bluecrop, Berkeley, and Tifblue were judged to be about 1200, 900, and 600 CU, respectively.

Shoots, 3 to 4 inches in length and having four to six floral buds, were also collected at the time of bud collection. Shoots were cooled at 4°C overnight and then used for cold-hardiness determinations (described below).

Leaf tissue was collected from greenhouse plants of Bluecrop in August, 1993. Plants were moved to an unlit cold room maintained at 4°C and leaf tissue was again collected after 1 and 2 weeks of cold treatment. Leaf tissue was frozen in liquid N_2 and stored at −70°C until analyzed.

Protein Extractions

Soluble proteins were extracted using a phenol-based procedure that lacked SDS in the extraction buffer (Shao-bing et al., 1989). Protein pellets were vacuum dried and resuspended in 10 mM Tris-HCl, pH 6.8. Proteins were quantified using a protein assay kit (Bio-Rad).

Gel Electrophoresis and Densitometry

One-dimensional SDS-PAGE (Laemmli, 1970) was carried out using 12.5% polyacrylamide gels. Polypeptides were visualized by staining gels with Coomassie brilliant blue using a modified procedure (Neuhoff et al., 1988) with improved sensitivity. According to this procedure, gels were fixed in 12% TCA for 1 h. TCA was removed with a distilled water wash. Gels were stained with 0.1% (w/v) Coomassie brilliant blue R-250 (dissolved first in water) in 2% (v/v) phosphoric acid and 10% (w/v) ammonium sulfate for at least 5 h. The stained gels were washed in 20% methanol until backgrounds were clear and stored in 20% ammonium sulfate in sealed containers at 4°C. Densitometric scans of photographs of gels were performed using the Personal Densitometer Image Quant (Molecular Dynamics). With this densitometer, protein abundance was calculated from peak volumes (area of protein bands × intensity of bands).

Cold-Hardiness Determinations

Cold hardiness of floral buds was determined using a method described by Takeda et al. (1993) with a few modifications. Shoots collected at different times during the winter were placed in 15-mL polypropylene tubes and the tubes were transferred to a manually controlled low-temperature bath (Polyscience model 910, Niles, IL) containing 95% ethanol. The bath was kept in a cold room at 4°C. Samples were cooled at a rate of 1.5°C/h until −4°C was reached and at a rate of 5°C/h thereafter to a minimum temperature of −30°C. Small pieces of ice were added to the bottom of the tubes at about −1.5°C to aid in ice nucleation. Samples were maintained at various temperatures from 4°C to −30°C for 30 min each. A fraction of the tubes was removed at each of these temperatures and shoots were thawed slowly at 4°C overnight. The following day shoots were placed in distilled water at room temperature and held for 48 h. Control samples were kept at 4°C. For each temperature, floral buds from

three to six shoots were removed and cross-sectioned by hand. Buds were examined using a dissecting microscope and the percentage of flowers that were killed at each temperature was determined. Flowers were judged to be dead if they turned black. From these data, LT_{50} values were calculated.

Heat-Stability Experiments

To investigate the heat stability of the chilling-induced polypeptides, tissue was homogenized in the first extraction buffer (as described above for extraction of soluble proteins) and divided in half. One-half of the slurry containing the soluble proteins was not heated, whereas the other half was heated to 95°C for 15 min. Heated samples were cooled on ice for 10 min and centrifuged to remove debris and coagulated proteins. The extraction procedure (Shao-bing et al., 1989) was then continued through to the end with the unheated samples and the supernatants of the heated samples.

Protein Purification, Cleavage, and Sequencing

Soluble proteins were extracted as described above from floral buds of Berkeley plants that had received 0 and 950 CU. Nine milligrams of protein from each time point were fractionated by preparative, free-solution IEF using the Rotofor (Bio-Rad). Fifty milliliters of protein solubilization buffer (1 mM Tris-HCl, pH 8.0, 8 M urea, 8 mM 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid, 5% [v/v] glycerol) with 2% (v/v) ampholytes (0.8% pH 5–7, 0.8% pH 4–6, 0.4% pH 3–10) (Bio-Rad) was prefocused in the Rotofor cell for 1 h at 12 W constant power. After prefocusing, the proteins, resuspended in 5 mL of protein solubilization buffer containing 2% ampholytes, were loaded through the port corresponding to the pH of the protein solution. Proteins were focused for 4.5 h at a constant 12 W. Cold ethanol was circulated to maintain the temperature at 10°C. The fractions were collected and the pH of the fractions was determined. Fractions were concentrated and aliquots of each were separated by SDS-PAGE.

Rotofor fractions containing the chilling-responsive 65- and 60-kD polypeptides were pooled, separated by SDS-PAGE, and electroblotted onto nitrocellulose membranes. After transfer, the polypeptides were reversibly stained with Ponceau S using the method described by Aebersold et al. (1987). Excess stain was removed by gentle agitation in 1% aqueous acetic acid for 1 to 2 min. Protein bands corresponding to the 65- and 60-kD polypeptides were cut out and shipped to the Harvard Microchemistry Department for sequencing. There the polypeptides were first subjected to endoproteinase Lys-C digestion and the resulting peptides were separated on a narrow-bore reverse-phase HPLC system (Aebersold et al., 1987). Peptides were detected by monitoring simultaneously at 210, 277, and 292 nm with a Waters 490 detector. Peptide fractions were collected based on A_{210} and a few well-separated peaks from each of the 65- and 60-kD polypeptides were selected for molecular mass determinations by matrix-assisted laser desorption mass spectrophotometry (Chait and Kent, 1992). Peptides were sequenced in a gas-phase sequenator.

Immunoblots

Polypeptides were electroblotted onto nitrocellulose membranes and immunostained with polyclonal antibodies (kindly provided by T.J. Close, University of California, Riverside) to a synthetic peptide of the consensus amino acid sequence EKKGIMDKIKEKLPG present in dehydrins (Close et al., 1993a, 1993b; Close and Lammers, 1993). Blots were immunostained using a 1:400 dilution of antiserum and a streptavidin-alkaline phosphatase immunoblotting kit for detecting rabbit antibodies (Gibco BRL, Gaithersburg, MD) following the manufacturer's instructions.

RESULTS

Identification of Chilling-Responsive Polypeptides in Floral Buds of Blueberry

To identify changes in gene expression in blueberry floral buds in response to CU accumulation, profiles of soluble proteins were examined from buds of field plants of the relatively high CR cultivar Bluecrop (1200 CU) and the low CR cultivar Tifblue (600 CU). Buds were collected about every 300 CU until the CR of each cultivar was satisfied, and again after the resumption of growth (Fig. 1). Buds had begun to swell in Bluecrop and Tifblue after 1800 and 1750 CU, respectively, and had broken by 2000 and 1900 CU, respectively. An increase in the level of three polypeptides of 65, 60, and 14 kD was observed with CU accumulation in both Bluecrop and Tifblue. An additional 10-kD polypeptide was observed in Tifblue to increase in concentration with chilling. The magnitude of the changes detected in the low-CR cultivar, however, was not as dramatic as the changes observed in the high-CR cultivar. The level of the three polypeptides appeared to decrease to about the 0 CU level when growth resumed.

Because of the likelihood that other proteins that do not respond to chilling comigrate with the chilling-responsive proteins, observable changes in the level of chilling-responsive proteins are expected to be minimized on one-dimensional gels. Nevertheless, densitometric scans of the gels presented in Figure 1 were performed for a relative comparison of the magnitude of the changes observed in Bluecrop to those in Tifblue. The results were corrected for slight differences in loading between lanes by scanning a protein band that did not appear to change with chilling (Fig. 2). In Bluecrop the level of the 65-, 60-, and 14-kD polypeptides increased most significantly during the initial 300 CU to 90, 71, and 91%, respectively, of the maximum levels observed. Although a 300-CU time point was not included on the gel of Tifblue, a similar trend was observed the previous year with Tifblue as well; the polypeptides increased to 60 to 80% of their maximum level during the initial 200 CU (data not shown). Of the time points analyzed, the maximum level of the polypeptides was reached in Tifblue by about 650 CU and in Bluecrop by about 850 CU. From these one-dimensional gels, the levels of the 65-, 60-, and 14-kD polypeptides appeared to increase in Bluecrop to a maximum of 2.1, 3.3, and 4.5 times the 0-CU level, whereas in Tifblue the levels increased by 1.6, 1.8, and 1.8, respectively, times the control level. The levels of the 60- and 14-kD polypeptides declined

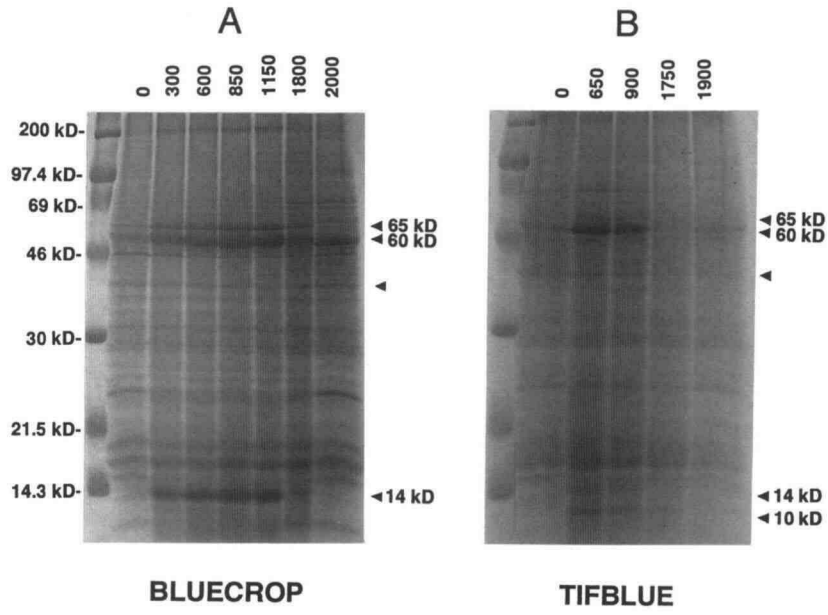

Figure 1. Comparison of profiles of soluble proteins from high-CR *V. corymbosum* cv Bluecrop (1200 CU) and low-CR *V. ashei* cv Tifblue (600 CU). Forty micrograms of soluble proteins, extracted from floral buds of field plants of Bluecrop (A) and Tifblue (B) collected at various times during CU accumulation until the resumption of growth, were fractionated on 12.5% gels by SDS-PAGE. CUs are indicated by the number above each lane. For Bluecrop buds, collection dates corresponding to CU and average CU accumulated per day for the week preceding collection were: October 7 (0 CU); November 10, 9 CU/day (300 CU); December 8, 19 CU/day (600 CU); January 4, 7 CU/day (850 CU); January 27, 11 CU/day (1150 CU); March 31, 7 CU/day (1800 CU); and April 26, 3 CU/day (2000 CU). For Tifblue buds, collection dates and average CU per day were: October 5 (0 CU); December 16, 19 CU/day (650 CU); January 5, 7 CU/day (900 CU); March 31, 7 CU/day (1750 CU); and April 26, 3 CU/day (1900 CU). In the far left lane are molecular mass markers. Arrows to the right mark the 65-, 60-, and 14-kD polypeptides that accumulate with chilling in both cultivars as well as the 10-kD polypeptide that accumulates with chilling in Tifblue. The additional arrow marks a protein band that did not appear to change with chilling. Data derived from densitometric scans (shown in Fig. 2) were corrected for slight differences in amount of protein loaded between lanes by scanning this protein band.

slightly by about 900 CU in Tifblue and the levels of all three polypeptides declined by about 1150 CU in Bluecrop and continued to decline with exposure to higher temperatures and resumption of growth, reaching a level nearly equal to or below the 0-CU level by the time of budbreak.

A control experiment was conducted to determine whether the 65-, 60-, and 14-kD polypeptides accumulate over time due to some developmental mechanism rather than due to CU accumulation. Protein profiles were examined from buds collected at monthly intervals in November, December, and January from Bluecrop plants kept in a heated greenhouse under natural lighting conditions (Fig. 3). Thus, plants received no chilling but were exposed to progressively shorter photoperiods through the winter solstice. Plants were dormant by November as judged by our inability to force budbreak after keeping shoots for 3 weeks at 20°C. In the absence of chilling, there was no detectable change in the level of any polypeptide. In a separate experiment, plants were kept in a heated greenhouse until after the first of January and then moved to a cold room. In this case, induction of the three polypeptides was observed with cold treatment (data not shown).

Association of Chilling-Responsive Polypeptides with Cold Acclimation

To determine if the level of chilling-responsive polypeptides was associated with the level of cold hardiness, changes in cold hardiness of floral buds of Bluecrop and Tifblue with CU accumulation were investigated (Fig. 4). Cold hardiness of floral buds of Bluecrop increased by 2.9 times from an LT_{50} value of −10°C for dormant, unchilled buds to a maximum LT_{50} value of −29°C after 850 CU. After the CR was satisfied and buds began to swell, cold hardiness decreased drastically to an LT_{50} of −4°C by the time buds opened. Cold hardiness of Bluecrop buds was found to increase most significantly, to 2.5 times the hardiness of unchilled buds, during the initial 300 CU. In the case of Tifblue, changes in cold hardiness followed a similar trend but the changes were not as dramatic as those of Bluecrop. Cold hardiness increased by 1.7 times during the first 300 CU and by 2.2 times overall to a maximum LT_{50} value of −22°C.

Induction of Chilling-Responsive Polypeptides in Leaves

Profiles of proteins extracted from leaves of Bluecrop plants after 0, 170, and 340 h in cold storage were examined also to

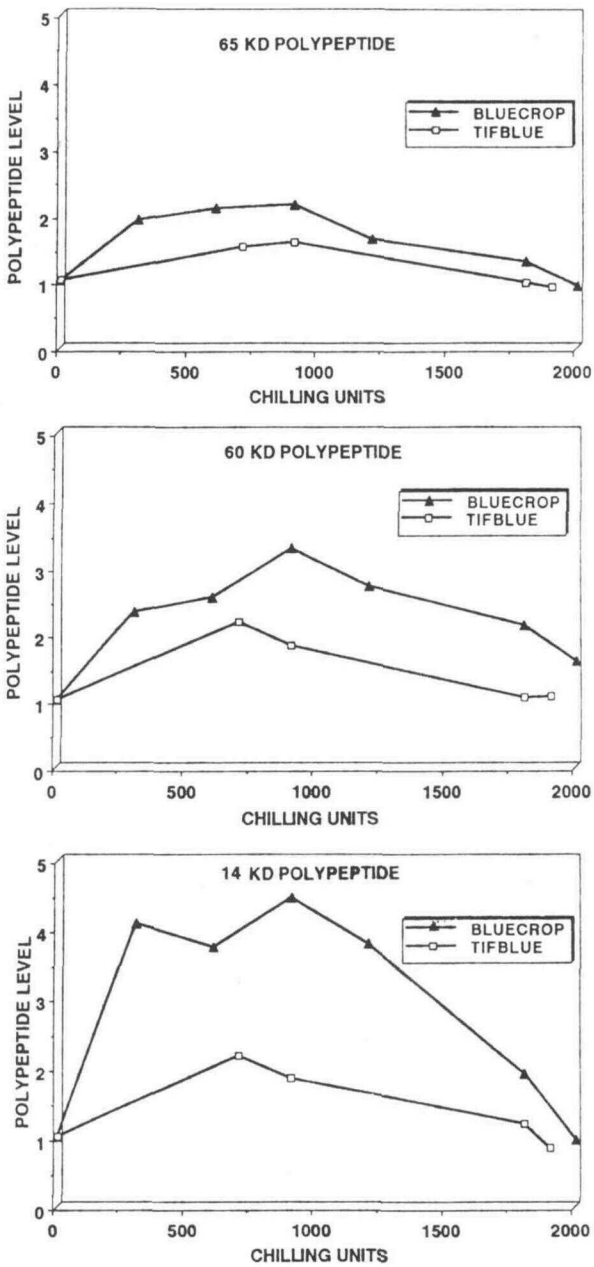

Figure 2. Comparison of relative changes in levels of 65-, 60-, and 14-kD polypeptides in floral buds of Bluecrop and Tifblue with CU accumulation. Data were derived from densitometric scans of gels presented in Figure 1 and corrected for slight differences in amount of protein loaded between lanes by scanning a protein band that did not appear to change with chilling (see Fig. 1). The gels shown in Figure 1 are representative of results obtained consistently over three separate years.

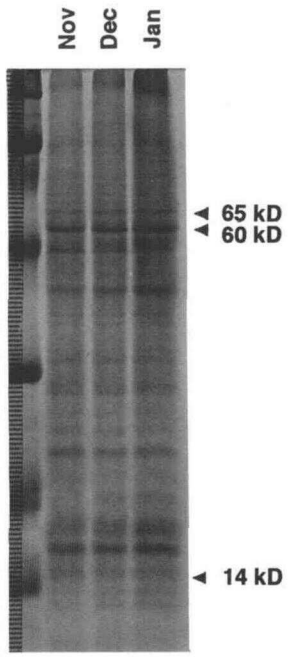

Figure 3. Profiles of soluble proteins from floral buds collected at monthly intervals from Bluecrop plants kept in a heated greenhouse under natural lighting. Equal amounts of proteins from each time point were fractionated on a 12.5% SDS-polyacrylamide gel. Shown from left to right are molecular mass markers followed by polypeptides from buds collected in November, December, and January. Arrows to the right mark the 65-, 60-, and the 14-kD polypeptides.

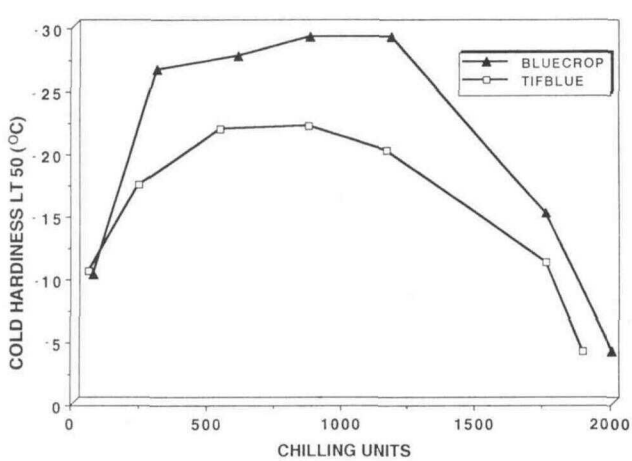

Figure 4. Cold hardiness of floral buds of Bluecrop and Tifblue as a function of CU accumulated. Shoots were collected from field plants after different lengths of chilling and subjected to increasingly lower temperatures to determine LT_{50}.

investigate whether the 65-, 60-, and 14-kD polypeptides were inducible in another organ and/or at another developmental stage (Fig. 5). Three polypeptides of 65, 60, and 14 kD did increase significantly in concentration after 340 h at 4°C, as did several other polypeptides.

Heat Stability of Chilling-Responsive Polypeptides

Because many ABA-, dehydration-, and cold-induced proteins have been reported to resist denaturation after heat treatments of 95 to 100°C (Close et al., 1989; Jacobsen and Shaw, 1989; Lin et al., 1990; Houde et al., 1992), the heat stability of the chilling-induced polypeptides of blueberry was investigated. Soluble proteins extracted from Bluecrop floral buds after 1150 CU were examined for their ability to remain in solution after a heat treatment of 95°C (as described in "Materials and Methods"). A comparison of profiles from the unheated and heated samples (Fig. 6) revealed that the 65-, 60-, and 14-kD polypeptides, as well as a few other polypeptides, remained in solution after heat treatment.

Purification and Sequencing of 65- and 60-kD Polypeptides

To determine the pI values of the 65-, 60-, and 14-kD chilling-responsive polypeptides and to purify the 65- and 60-kD polypeptides, bud proteins were fractionated by IEF in solution using the Rotofor followed by one-dimensional SDS-PAGE. Proteins were extracted from floral buds of a different high-chilling highbush cultivar (*Vaccinium corymbosum* cv Berkeley) because more bud tissue was available to us from Berkeley than from Bluecrop or Tifblue and because

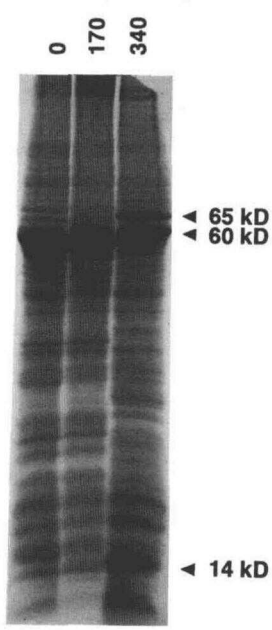

Figure 5. Profiles of soluble proteins from leaves of Bluecrop collected after 0, 170, and 340 h at 4°C. Equal amounts of proteins from each time point were fractionated on a 12.5% SDS-polyacrylamide gel. Arrows to the right mark the 65-, 60-, and 14-kD polypeptides.

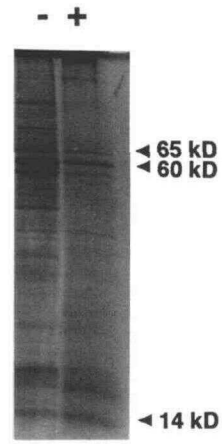

Figure 6. Profiles of soluble proteins from floral buds of Bluecrop field plants (1150 CU) before and after a 95°C heat treatment. Floral buds were homogenized in the extraction buffer described by Shao-bing et al. (1989). The slurry containing the proteins was divided in half and one-half was left untreated (−) while the other half was heated to 95°C for 15 min (+). Samples were fractionated on a 12.5% SDS-polyacrylamide gel. Arrows to the right mark the 65-, 60-, and 14-kD polypeptides.

the 65- and 60-kD polypeptides appeared to be induced to comparable levels in Berkeley and Bluecrop from one-dimensional SDS-PAGE. The 14-kD polypeptide was induced in Berkeley, too, but not as dramatically as in Bluecrop (data not shown). Rotofor profiles of proteins extracted after 0 and 950 CU were compared (Fig. 7). From a comparison of fractions 1 to 12 (from the acidic end of the Rotofor cell) of the 0- and 950-CU samples, no significant qualitative or quantitative differences in polypeptides were noted (data not shown). From a comparison of polypeptides present in fractions 13 to 20 of the 0- and 950-CU samples, several differences were observed. First, it should be noted that Rotofor fractionation itself varied slightly between the 0- and 950-CU samples. Proteins were shifted by one fraction toward the basic end in the 0-CU sample compared with the 950-CU sample. Thus, fractions 14 to 20 of the 0-CU sample were comparable to fractions 13 to 19 of the 950-CU sample. Higher levels of several polypeptides were observed in the 950-CU sample compared with the 0-CU sample; however, no polypeptides increased as dramatically in amount as did the 65-, 60-, and 14-kD polypeptides. Based on the pH of the fractions containing the chilling-responsive polypeptides, the pI values of the 65- and 60-kD polypeptides were found to be 7.5 to 8.0 and the pI of the 14-kD polypeptide was found to be 8.5.

The 65- and 60-kD polypeptides were purified and subjected to Lys-C digestion, and selected fragments were sequenced. From the amino acid sequences (Table I), the 65- and 60-D polypeptides appeared very similar. One fragment from each had an identical sequence (QDQLHGGYK) and another had a strikingly similar sequence (EGGGLVDK from the 65-kD polypeptide and EGGGLIYK from the 60-kD polypeptide). The peptide fragments were rich in Gly, Gln, and Lys.

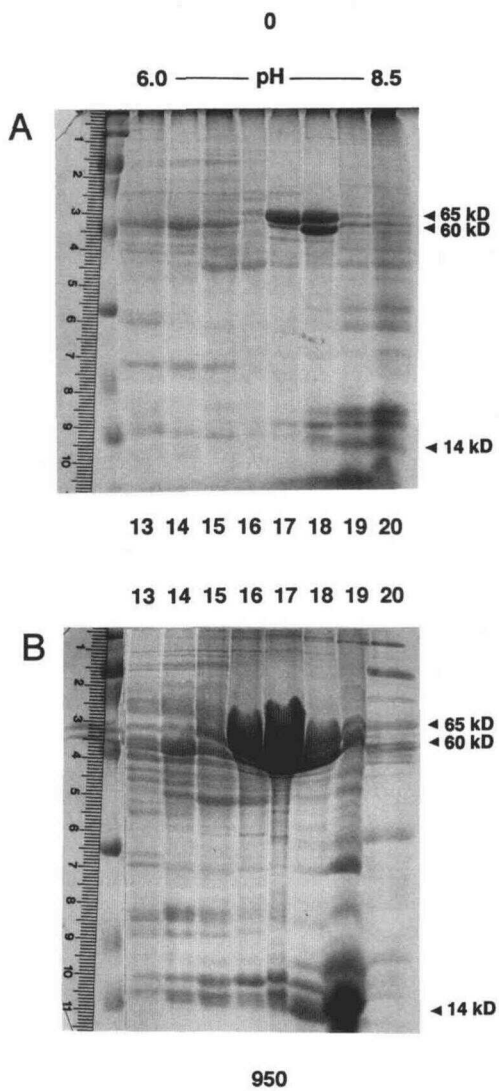

Figure 7. Comparison of profiles of soluble proteins, fractionated first by IEF (using the Rotofor) and second by one-dimensional SDS-PAGE, from floral buds of Berkeley (900 CU) collected after 0 and 950 CU. Rotofor fractionation was performed using 9 mg of proteins from each time point. Fractions 13 to 20 (from the basic end of the Rotofor cell) from the 0- (A) and 950-CU (B) samples are shown. pH of the fractions ranged from 6.0 to 8.5. In the far left lane of each gel are molecular mass markers. Arrows to the right mark the 65-, 60-, and 14-kD polypeptides.

Table I. *Amino acid sequences of peptide fragments from the 65- and 60-kD polypeptides*

The 65- and 60-kD chilling-responsive polypeptides were purified and digested with endoproteinase Lys-C, and selected fragments were sequenced.

Polypeptide	Peptide Sequences
65 kD	QDQLHGGYK
	EGGGLVDK
	QDQYRVG
60 kD	QDQLHGGYK
	EGGGLIYK

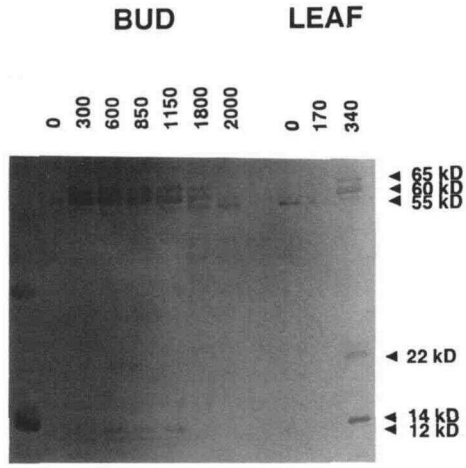

Figure 8. Blot of floral bud and leaf proteins from Bluecrop after different chilling treatments probed with dehydrin antiserum. Six micrograms of soluble proteins extracted from buds after 0, 300, 600, 850, 1150, 1800, and 2000 CU and from leaves after 0, 170, and 340 h at 4°C were fractionated on a 12.5% SDS-polyacrylamide gel and blotted to nitrocellulose. The blot was probed with antiserum to the Lys-rich consensus sequence EKKGIMDKIKEKLPG of dehydrins. Molecular mass markers are shown in the far left lane. Arrows to the right mark the 65-, 60-, and 14-kD polypeptides as well as the 55-, 22-, and 12-kD polypeptides, which reacted strongly to the antiserum. Preimmune serum also reacted to the 55- and 12-kD polypeptides (data not shown).

Immunological Similarity of Chilling-Responsive Polypeptides to Dehydrins

A blot of floral bud and leaf proteins, extracted from Bluecrop after different chilling treatments, was probed with antiserum to the Lys-rich consensus sequence EKKGIMDKIKEKLPG of dehydrin proteins (Fig. 8). The antiserum reacted strongly with the three chilling-responsive polypeptides of 65, 60, and 14 kD, identified by their molecular masses and induction profiles, and with three other polypeptides of about 55, 22, and 12 kD. A similar blot was probed with preimmune serum (data not shown). Preimmune serum did not react with the 65-, 60-, 14-, or 22-kD polypeptides but did react with the 55- and 12-kD polypeptides.

DISCUSSION

It has been shown that dormancy development, cold acclimation, and/or N_2 storage in woody plants involve changes in gene expression (Coleman et al., 1991; Arora et al., 1992). Our work with blueberry floral buds demonstrates that accumulation of CU is associated with an increase in the level of several polypeptides. The levels of three polypeptides of 65, 60, and 14 kD increase most dramatically with CU accumulation. The largest increase in the levels of these polypeptides is observed within the initial 300 h of chilling. The concentration of the three polypeptides returns to the 0-CU level after plants are exposed to high temperatures long enough for the initiation of budbreak.

For the two cultivars tested, Bluecrop and Tifblue, accumulation of the chilling-responsive polypeptides coincides

with cold acclimation. The largest increase in the level of the polypeptides (during the initial 300 CU) coincides with the largest increase in the level of cold hardiness. Also, the maximum level of cold hardiness is higher in Bluecrop than in Tifblue, as is the maximum level of the chilling-responsive polypeptides (relative to the 0-CU level). However, level of the chilling-responsive polypeptides declines slightly in Tifblue by about 900 CU and in Bluecrop by about 1150 CU. No decline in cold hardiness is observed until about 1200 CU for Tifblue and 1200 to 1800 CU for Bluecrop. Thus, if these polypeptides play a role in the development of cold hardiness, the decline in their levels by 900 CU in Tifblue and by 1150 CU in Bluecrop may not be significant enough to bring about a detectable change in levels of cold hardiness. Since satisfaction of the CR and release from dormancy occur during this same time frame, it is impossible at this time to discern if the chilling-responsive polypeptides might be related to development of dormancy rather than, or in addition to, development of cold hardiness.

In many woody plants, induction of dormancy and development of cold hardiness are initiated or accelerated by an SD photoperiod (Weiser, 1970; Vince-Prue, 1975; Nooden and Weber, 1978; Coleman et al., 1991). The possibility that genes encoding the chilling-responsive polypeptides identified in blueberry floral buds may be responsive also to short days cannot be absolutely ruled out at present. However, if the polypeptides are responsive only to short days and not to chilling, we would expect to find a gradual increase in the level of these polypeptides in field plants through about 600 to 700 CU (accumulated by about December-January), followed by a gradual decline thereafter. In contrast, the levels of these polypeptides increase most significantly within the initial 300 h of chilling and decrease to the 0-CU level with the initiation of budbreak. Furthermore, the levels of the three polypeptides remained constant in dormant plants kept in a heated greenhouse from November through January, although plants were exposed to natural lighting and, thus, to increasingly shorter photoperiods through about the end of December. Also, if plants are moved from a heated greenhouse to a cold room in January, the three polypeptides are induced (data not shown). This response is clearly different from that of a 32-kD BSP described by Coleman et al. (1993), which accumulates in bark of poplar in response to short days. If poplar plants are exposed to short days to induce dormancy and accumulation of the 32-kD BSP and then placed in cold storage, no further increase in the level of the 32-kD BSP is observed.

The 65-, 60-, and 14-kD polypeptides are inducible in leaves with cold treatment. The levels of the polypeptides are significantly higher after 340 h at 4°C than before cold treatment. Many cold-, ABA-, and dehydration-induced proteins have been shown to be water soluble and boiling stable (Close et al., 1989; Jacobsen and Shaw, 1989; Lin et al., 1990; Houde et al., 1992). Results indicate that the chilling-responsive polypeptides of blueberry are also present in the soluble protein fraction and stable to heat treatments of 95°C.

Purification and partial sequencing of the 65- and 60-kD polypeptides revealed that these polypeptides are very similar. Of two and three peptide fragments sequenced from the 60- and 65-kD polypeptides, respectively, one from each is identical and another is the same at six of eight amino acid residues. The sequenced peptide fragments are particularly rich in Gly. At present we cannot eliminate the possibility that the 60-kD polypeptide is a degradation product of the 65-kD polypeptide, or, conversely, that the 65-kD polypeptide is a precursor of the 60-kD polypeptide.

Results from our experiments revealed obvious similarities between the chilling-responsive polypeptides of blueberry and other stress-induced proteins, namely dehydrins, identified in other plants (Close et al., 1993a, 1993b). The most notable similarities are that they are water-soluble at 95°C, cold-inducible, and, from limited sequence information on the 65- and 60-kD polypeptides of blueberry, apparently Gly-rich. Furthermore, antiserum to the consensus sequence EKKGIMDKIKEKLPG of dehydrins cross-reacts with the three chilling-responsive polypeptides of blueberry. Consequently, we conclude that the three major chilling-responsive polypeptides of blueberry are dehydrins or dehydrin-like proteins, and, as has been suggested for annual plants, these proteins may play a role in protecting perennial plants from cold stress or dehydration stress related to cold stress, although as yet no causal relationship has been shown.

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