Protein Changes in the Flower Buds of Japanese Pear during Breaking of Dormancy by Chilling or High-temperature Treatment

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ABSTRACT. The dormancy of Japanese pear (*Pyrus pyrifolia* Nakai) floral buds was broken by prolonged chilling or short-term high-temperature treatment (45 °C for 4 hours). Changes in the protein profiles of the floral buds were studied using two-dimensional electrophoretic analysis (2-DE). The quantities of nine cold-induced proteins (CIPs) increased in the floral buds with increases in chill unit (CU) value, but did not change rapidly when bud dormancy was near completion. When dormancy of floral buds was broken by high-temperature treatment, nine heat-shock proteins (HSPs) accumulated. These HSPs were distinct from the CIPs. The isoelectric point of the 19-kDa CIP shifted to the basic side by high-temperature treatment as well as by chilling. These results suggest that the 19-kDa protein may be a usable marker to measure the degree of bud dormancy in Japanese pear.

Bud dormancy of deciduous fruit trees, such as Japanese pear, is broken by winter chilling (Saure, 1985; Westwood, 1978). The hormonal aspects of the mechanism of this phenomenon have been studied by many authors as reviewed by Powell (1987) and Dennis (1994). Guy (1990) reported that cold accumulation clearly induced some protein synthesis with the development of freezing tolerance in certain higher plants. In recent biochemical studies in woody plants, it was found that cold accumulation also induced some protein synthesis with the developments of freezing tolerance or endodormancy (Rowland and Arora, 1997). Lang and Tao (1991) observed a decrease in a 61-kDa protein in peach buds during final stages of dormancy, while Muthalif and Rowland (1994a, 1994b) and Arora et al. (1997) identified an increase of 65, 60, and 14-kDa proteins in dormant blueberry floral buds with chilling. Muthalif and Rowland (1994a, 1994b) and Arora et al. (1997) had also found these proteins were related to the dehydrin protein family (Close et al., 1993).

In previous studies (Tamura et al., 1993, 1995), bud dormancy of Japanese pears was broken not only by chilling, but also by a high-temperature treatment of 45 °C for 4 h. We also reported that the effect of high-temperature treatment was reversed by the presence of actinomycin D, which inhibits m-RNA synthesis. Therefore, we proposed that genes related to the breaking of bud dormancy were expressed during the high-temperature treatment.

The objective was to detect the protein changes associated with breaking of endodormancy in Japanese pear floral buds.

Materials and Methods

PLANT MATERIALS AND TREATMENTS. Forty trees of 4-year-old 'Nijisseiki' pear on seedling roots of *Pyrus betulaefolia* Bunge were grown in 20-L pots at the orchard of Tottori University, Tottori City, Japan. Twenty trees (greenhouse-cultured trees) were placed in a greenhouse (at 20 ± 2 °C) on 5 Nov. 1995 immediately after natural defoliation and twenty other trees (open-cultured trees) were left in the orchard. Two-year-old branches were sampled

periodically from these trees between October 1995 and February 1996. The percentage of budbreak and the protein profiles in these spur floral buds on the branches were determined as described below.

Two-year-old branches of 17-year-old 'Nijisseiki' pear trees on seedling roots of *P. betulaefolia* grown in the same orchard were collected at 200 and 400 chill units (CUs) and their base portions were submerged in distilled water in a plastic container. These branches were placed in a chamber at 45 ± 0.2 °C for 4 h in darkness. After treatment, percentage budbreak and protein profiles were determined.

BUDBREAK MEASUREMENT. These branches were pruned to 25 cm long with five floral buds, their vegetative buds were cut off, and their base portions submerged in a solution of 3.0% (w/v) 8-hydroxyquinoline citrate and 0.03% (w/v) aluminum sulfate in plastic containers. The branches were forced in a growth chamber at 23 ± 0.5 °C. Photoperiod was 14 h·d⁻¹ with an irradiance of 250 mmol·m⁻²·s⁻¹. Floral bud development was observed every 3 d and budbreak was defined when buds were swollen with green tip showing between scales (Tamura et al., 1993).

The percent budbreak was determined after 21 d of forcing in the growth chamber using eight single-branch replications for each treatment.

CU CALCULATION. To determine CU value at the sampled date, air temperatures were recorded by a thermograph in the orchard. The CU value was computed using the Saitama method (Asano and Okuno, 1990), where temperatures <10 °C are equally effective for budbreak, while temperatures above 15 °C reverse chilling effect. To determine the CU accumulation for a 24-h period, each 15-min temperature was converted to the equivalent values of chilling, then these values were added. Effective chilling hours were calculated just after the day when the largest negative accumulation was attained in November.

PROTEIN EXTRACTION AND ANALYSIS. Proteins were extracted using the method of Damerval et al. (1986). One gram of the floral buds without bud scales was frozen in liquid nitrogen, ground to a fine powder, and suspended in a solution of 10% trichloroacetic acid (w/v) in acetone containing 0.07% (v/v) 2-mercaptoethanol. The homogenates were kept at -18 °C for 45 min with shaking, then centrifuged at 35,000 g_n for 15 min at 0 °C. The precipitates were soaked with acetone containing 0.07% 2-mercaptoethanol for 1 h at -18 °C, then centrifuged at 35,000 g_n for 15 min at 0 °C.

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Table 1. Seasonal changes in percent floral budbreak on excised 'Nijisseiki' pear branches collected from open-cultured and greenhouse-cultured trees.

Date	Chilling unit value in open field	Budbreak (%) ^z	
		Open-cultured tree	Greenhouse- cultured tree ^y
10 Oct. 1995	0	20.0 d ^x	
20 Nov. 1995	98	5.0 c	5.0 a ^{NS}
6 Dec. 1995	202	2.5 e	5.0 a ^{NS}
15 Dec. 1995	414	5.0 e	5.0 a ^{NS}
23 Dec. 1995	602	5.0 e	2.5 a ^{NS}
4 Jan. 1996	800	35.0 c	5.0 a ^{**}
13 Jan. 1996	1002	62.5 b	2.5 a ^{**}
22 Jan. 1996	1198	100.0 a	2.5 a ^{**}
2 Feb. 1996	1410	100.0 a	2.5 a ^{**}

^zValue after 21 d after forcing at 23 °C.

^yTrees were put in a greenhouse at 20 ± 2 °C on 5 Nov. 1995.

^xMean separation within columns by Duncan's multiple range test at $P \leq 0.05$.

^{NS,**}Nonsignificant or significant between open-cultured trees and greenhouse-cultured one at $P \leq 0.01$, respectively.

The precipitates were dried under a vacuum and resuspended in 0.5 mL of a buffer containing 9 M urea, 0.8% ampholyte, pH 3.5 to 10.0 (Ampholine, Pharmacia-Biotechnology, Uppsala, Sweden), 2% (v/v) 2-mercaptoethanol, and 0.8 mM phenylmethylsulfonyl fluoride. The samples were stored at -80 °C until electrophoresis was performed.

Proteins in samples were determined using the method of Ramagli and Rodriguez (1985). Ten milliliters of 0.1 N HCl and 80 mL of distilled water were added to 10 mL of sample, and then 3.5 mL of Bradford reagent (Bio-Rad, Richmond, Calif.) was added to the solution with shaking. After 5 min at room temperature, the optical density at 595 nm was measured against the ovalbumin standard.

For first-dimensional electrophoresis, ready-made immobilized pH gradient (IPG) gels (Immobiline Dry Plate, 250 × 110 mm, pH 4.0 to 7.0 or 4.5 to 5.4, Pharmacia-Biotechnology) were used. Before isoelectric focusing (IEF), the gels were cut into 3.5-mm-wide strips with a paper cutter. The IPG gel strips were rehydrated overnight in a mold to the original gel thickness (0.5 mm). The rehydration solution contained 8 M urea, 0.5% (v/v) Nonidet P-40, 10 mM dithiothreitol (DTT), 0.002% (w/v) orange G, and 0.5% (v/v) ampholyte, pH 3.5 to 10.0 (Ampholine, Pharmacia-Biotechnology). A 20-mg sample of proteins was applied on each of 20 rehydrated IPG gel strips, and the strips were placed on the flat bed of a horizontal electrophoresis system, Multiphor II (Pharmacia-Biotechnology) at 15 °C. Voltage, current, and power were set at 300 V, 1 mA, and 5 W, respectively, for the first 60 min. Then IEF continued at a maximum setting of 3500 V, 1 mA, and 5 W until a constant focusing pattern was obtained.

Before second-dimension (2-D) sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), the IPG gel strips were equilibrated for 20 min in 20 mL of equilibrate solution of 50 mM Tris-HCl (pH 6.8) with 6 M urea, 30% (v/v) glycerol, 1% (w/v) SDS, 0.25% (w/v) DTT, and 0.002% (w/v) bromophenol blue. The equilibrated gel strips were then laid on horizontal laboratory-made SDS gels. The gels were made with 6% (w/v) and 12% to 15% (w/v) linear gradient polyacrylamide. Electrophoresis was performed using the horizontal electrophoresis system described above according to the method of Grög et al. (1988) at 20 °C with a constant power of 40 W until bromophenol blue reached the end of the gel.

Two-dimensional gels were silver-stained according to Blum et al. (1987) at room temperature.

The numbers of spots and intensity, isoelectric point, and molecular weight of each spot were analyzed with the Densitograph AE-6920M (Atto, Tokyo, Japan), a gel analysis system equipped with a personal computer with hard disk drive, a linear CCD scanning camera, and spot analysis software (Atto).

Results

In the open-cultured trees, percent floral budbreak increased after 800 CU, reaching complete budbreak at 1200 CU (Table 1). Protein concentration of floral buds increased rapidly upon the accumulation of CU and became stable after 600 CU (Fig. 1). In the greenhouse cultured trees, no significant increases in budbreak or protein concentration were observed in floral buds over the course of the experiment.

For each of the variously chilled floral buds, ≈3000 protein spots were resolved on a 2-D gels (Fig. 2). No significant changes in the number of proteins were detected among the chilled buds. Eleven proteins [Fig. 2; spot A (30-kDa), B (29-kDa), C (28-kDa), D (28-kDa), E (27-kDa), F (27-kDa), G (22-kDa), H (22-kDa), I (19-kDa), J (16-kDa), and K (14-kDa)] appeared to increase with increasing chilling accumulation. Especially, as shown in Fig. 3, the protein spot of 19-kDa (spot I) showed a distinctive change with more chilling. At each later sampling time, the location of the 19-kDa gradually shifted to the basic side of the former spot with the similar shape of streamline (Fig. 3).

Comparing to the protein profiles of the greenhouse-cultured trees, nine proteins (Fig. 4; spots C to K) were present at higher levels in the open-cultured trees. Spots A and B were of similar intensity.

High-temperature treatment increased the percent budbreak (Table 2). Protein profiles changed as well (Fig. 5). The levels of nine proteins [Fig. 5; spot L (25-kDa), M (22-kDa), N (22-kDa), O (22-kDa), P (21-kDa), Q (21-kDa), R (21-kDa), S (21-kDa), and T (20-kDa)] increased with the heat treatments while two proteins

Fig. 1. Seasonal changes in protein content (fresh mass basis) of floral buds of 'Nijisseiki' pear collected from open-cultured (●) and greenhouse-cultured (○) trees. Green house-cultured trees were put in a greenhouse at 20 ± 2 °C on 5 Nov. 1995. Each value represents the mean of three replications and vertical bars represent ± SD.

Fig. 2. Seasonal changes in two-dimensional (2-D) electrophoretic patterns of total protein isolated from floral buds on open-cultured 'Nijisseiki' pear trees (a-d) and close-up section (e) of 2-D gel in d. Each small letter indicates sampling date: a = 20 Nov., b = 15 Dec., c = 4 Jan., d and e = 2 Feb. First dimension: immobilized pH gradient 4.0 to 7.0. Second dimension: horizontal SDS pore gradient gel (4% C, 12% to 15% T). Twenty milligrams of total protein was loaded onto each gel. Arrowheads indicate spots of higher intensity when compared to the same spots on the other gels.

[Fig. 5; spot U (20-kDa) and V (15-kDa)] decreased. Protein spots A to K, which increased with chilling, showed no significant changes with the heat treatment. The protein spot of 19-kDa (spot I) disappeared and a similar spot appeared on the basic side of the former one after the high-temperature treatment (Fig. 6).

New proteins were found in higher plants with cold accumulation (Guy, 1990). We found, using 2-DE, the levels of 11 proteins increased in open-cultured pear floral buds with increased chilling accumulation. Additionally, nine of the eleven proteins increased in the open-cultured pear floral buds in comparison to the greenhouse-cultured trees. Thus, the nine spots, C to K, are regarded as cold-accumulation-induced proteins (CIPs). However, these nine spots did not show a rapid increase either immediately before or after breaking dormancy. Muthalif and Rowland (1994a, 1994b) and Arora et al. (1997) studied blueberry buds and observed changes in three major proteins of 65, 60, and 14-kDa following accumulation of chilling. Arora et al. (1997) suggested that three dehydrin-like proteins of 65, 60, and 14-kDa accumulated with chilling in blueberry flower buds were more closely associated with cold hardness than with dormancy transitions. It remains uncertain whether the CIPs in the Japanese pear floral bud were related to breaking dormancy or cold tolerance.

Heat-shock proteins (HSPs) have been found in many higher plants and, in some cases, have been shown to increase with chilling (Krishina et al. 1995). In the present experiment, exposure to 45 °C induced budbreak and accumulation of nine proteins (spots L to T) in pear floral buds. Thus, the nine spots L to T, are regarded as a kind of HSPs. However, these HSPs spots did not coincide with the CIPs (spots C to K) that increased with chilling accumulation. Thus, due to the quantitative rather than qualitative changes, it is suggested that the CIPs and the HSPs are not suitable markers for marking satisfaction of chilling requirement in the Japanese pear floral bud.

The cause of the changes in the 19-kDa protein (spot I in Figs. 3 and 6) may be 1) the continued synthesis of new proteins accompanied by disappearance of a previous one or 2) a gradual change in a protein structure such as substitution of amino acid. The streamlined shape of the spot indicates that the spot consisted of several proteins which were slightly different in structure. Thus, it is concluded that a 19-kDa protein gradually changed in structure during accumulation of chilling. The same change in protein structure was also induced by high temperature. These results suggest that protein spot I could be a useful indicator of the depth of floral bud dormancy in the Japanese pear.

'Nijisseiki' pear floral buds showed no further increase in cold hardness from mid-December to mid-January when floral buds are going from their deepest phase into final phase of endodormancy in Tottori (Liao et al., 1996). The change in isoelectric point of the protein spot I occurred when chilling accumulation started, thereafter continued until end of endodormancy. Therefore, the changes of 19-kDa protein may be more associated with breaking bud dormancy than with cold hardness.

Fig. 3 (right). Magnified pictures of spot I (arrowheads) shown on Fig. 2. See legend in Fig. 2 for details.

Fig. 4 (below). Close up sections of two-dimensional gels from (a) floral buds of open-cultured and (b) greenhouse-cultured 'Nijisseiki' pear trees. Buds were collected on 22 Jan. First dimension: immobilized pH gradient 4.0 to 7.0. Second dimension: horizontal SDS pore gradient gel (4% C, 12% to 15% T). Twenty milligrams of total protein was loaded onto each gel. Arrowheads indicate spots of different intensity when compared to the same spots in the greenhouse.

IPG →

S
D
S
↓

Table 2. Effect of heat (45 °C) treatment for 4 h on floral budbreak of partially chilled 'Nijisseiki' pear branches.

Treatment	Budbreak(%) ^z	
	Chilling unit value	
	200	400
45 °C for 4 h	82.5	90.0
Control	2.5	5.0
Significance	**	**

^zValue after 21 d after forcing at 23 °C.
**Significant at $P \leq 0.01$.

IPG →

Fig. 5 (above). Close-up sections of two-dimensional gels from floral bud of 'Nijisseiki' pear collected before (a) and after (b) high-temperature treatment (45 °C for 4 h on 20 Nov.). First dimension: immobilized pH gradient 4.0 to 7.0. Second dimension: horizontal SDS pore gradient gel (4% C, 12% to 15% T). Twenty milligrams of total protein were loaded onto each gel. Arrowheads indicate spots of different intensities with the two treatments.

Fig. 6. Magnified pictures of spot I (arrowhead) on two-dimensional gels from floral bud of 'Nijisseiki' pear collected before (a) and after (b) high-temperature treatment (45 °C for 4 h on 20 Nov.) First dimension; immobilized pH gradient 4.5 to 5.4. Second dimension; horizontal SDS pore gradient gel (4% C, 12% to 15% T). Twenty milligrams of total protein were loaded onto each gel.

Literature Cited

- Arora, R., L.J. Rowland, and G.R. Panta. 1997. Chill-responsive dehydrins in blueberry: Are they associated with cold hardiness or dormancy transitions? *Physiol. Plant.* 101:8–16.
- Asano, S. and T. Okuno. 1990. Period of breaking the rest and the quality of chilling requirement of 'Kosui' and 'Hosui' Japanese pears. *Bul. Saitama Hort. Expt. Sta.* 17:41–47 (in Japanese with English summary).
- Blum, H., H. Beier, and H.J. Gross. 1987. Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. *Electrophoresis* 8:93–99.
- Close, T., R.D. Fenton, and F. Moonan. 1993. A view of plant dehydrins using antibodies specific to the carboxy terminal peptide. *Plant Mol. Biol.* 23:279–286.
- Damerval, C., D. Vienne, M. Zivy, and H. Thiellement. 1986. Technical improvements in two-dimensional electrophoresis increase the level of genetic variation detected in wheat-seedling proteins. *Electrophoresis* 7:52–54.
- Dennis, Jr., F.G. 1994. Dormancy—What we know (and don't know). *HortScience* 29:1249–1255.
- Grög, A., W. Postel, and S. Gunther. 1988. The current state of two-dimensional electrophoresis with immobilized pH gradients. *Electrophoresis* 9:531–546.
- Guy, L.C. 1990. Cold accumulation and freezing stress tolerance: role of protein metabolism. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 41:187–223.
- Krishina, P., M. Sacco, J.F. Cherutti, and S. Hill. 1995. Cold-induced accumulation of hps 90 transcripts in *Brassica napus*. *Plant Physiol.* 107:915–923.
- Lang, G.A. and J. Tao. 1991. Dormant peach flower bud proteins associated with chill unit accumulation or negation temperatures. *HortScience* 26:117. (Abstr.)
- Liao, R., K. Tanabe, F. Tamura, and A. Itai. 1996. Seasonal variations in fatty acid composition and cold hardiness of Japanese pear in response to air temperature and growing stages. *Environ. Control Biol.* 34:303–311.
- Muthalif, M.M. and L.J. Rowland. 1994a. Identification of dehydrin-like proteins responsive to chilling in floral buds of blueberry (*Vaccinium secction Cyanococcus*). *Plant Physiol.* 104:1439–1447.
- Muthalif, M.M. and L.J. Rowland. 1994b. Identification of chilling responsive proteins from floral buds of blueberry. *Plant Sci.* 101:41–49.
- Powell, L.E. 1987. Hormonal aspects of bud and seed dormancy in temperate-zone woody plants. *HortScience* 22:845–850.
- Ramagli, L.S. and L.V. Rodriguez. 1985. Quantitation of microgram amounts of protein in two-dimensional polyacrylamide gel electrophoresis sample buffer. *Electrophoresis* 6:559–563.
- Rowland, L.J. and R. Arora. 1997. Proteins related to endodormancy (rest) in woody perennials. *Plant Sci.* 126:119–144.
- Saure, M.C. 1985. Dormancy release in deciduous fruit trees. *Hort. Rev.* 7:239–300.
- Tamura, F., K. Tanabe, K. Banno, and T. Ikeda. 1993. Effect of high temperature treatment on breaking of bud dormancy in Japanese pear 'Nijisseiki'. *J. Jpn. Soc. Hort. Sci.* 62:41–47 (in Japanese with English summary).
- Tamura, F., K. Tanabe, and A. Itai. 1995. Effect of interruption of chilling on budbreak in Japanese pear. *Acta Hort.* 395:135–140.
- Westwood, M.N. 1978. Temperate-zone pomology. W.H. Freeman. New York.