

Protein Changes in Peach Seeds during Chilling Are Not Associated with Breaking Dormancy

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Abstract. Siberian C peach (*Prunus persica* L.) seeds were stratified at 5 and 20°C. DWs and soluble protein content remained constant regardless of stratification temperature and duration. Seed extracts subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis revealed a decrease in the intensity of nine polypeptides in the cotyledons of seeds held at 5°C during weeks 5 through 8, coinciding with an increase in germination capacity. These changes were confined to cotyledons held at 5°C, and were observed only when the seeds were able to germinate. The effects of stratification and the imbibition degree on changes in the protein content of seeds of two additional peach biotypes ('Farouki' and 'Maloussi') were also evaluated. Germination of fully imbibed seeds at 20°C increased steadily as stratification time at 5°C increased. Partially imbibed seeds (25 % or 50% of full imbibition) did not germinate regardless of stratification time. However, when these seeds were soaked in water after stratification, their germination paralleled that of fully imbibed seeds. Thus, dormancy was broken, even though the seeds could not germinate. Changes in protein profiles in fully imbibed seeds confirmed those previously reported for Siberian C seeds. Similar changes occurred in cotyledons of partially imbibed seeds during stratification at 5°C, but at a slower rate. Those changes were, however, delayed by partial imbibition, whereas germination capacity (ability to germinate when fully imbibed) was not. Changes in cotyledon protein profiles were not affected by removing the embryonic axis before stratification, a result indicating that such changes are not controlled by the axis. Gibberellic acid (GA_3) induced 35 % to 40% germination of nonchilled seeds. It hastened the loss of protein band intensity in 'Farouki' but not in 'Maloussi'. However, GA_3 -treated seeds germinated before any visible changes occurred in protein profiles. We conclude that the effects of chilling on breaking dormancy are independent of its effects on the protein changes observed in this study.

Dormant seeds of many species, such as peach, must be exposed to low temperatures (stratified) before they can germinate. Much effort has been devoted to understanding the action of low temperatures in breaking dormancy. Several studies have emphasized protein changes associated with chilling.

When *Heracleum sphondylium* L. seeds were held moist at 2°C, reserve proteins were hydrolyzed in the endosperm and transferred to the embryo; this did not occur in seeds kept at 15°C (Stokes, 1952). In apple (*Malus domestica* Borkh.) embryos, however, reserve protein hydrolysis occurred at 5 and 20°C (Bouvier-Durand et al., 1983). Further, proteolysis was absent in seeds held in the fruit at 0°C, although this treatment broke embryo dormancy.

Changes in total soluble protein content during chilling were insignificant in pear (*Pyrus communis* L.) embryos (Alscher-Herman and Khan, 1980) and in apple axes and cotyledons (Eichholtz et al., 1983). Although the concentrations of four polypeptides increased in the axes of apple seeds held at 5°C, no changes were apparent at 20°C or in cotyledons held at either temperature (Eichholtz et al., 1983). These authors suggested that protein reserves may be mobilized to the axes when dormancy is broken.

In sugar pine (*Pinus lambertiana* Dougl.), four protein bands were consistently more intense in extracts of embryos from seeds held at 25°C than in those held at 5°C, although dormancy was broken only at 5°C (Noland and Murphy, 1986). These authors speculated that such proteins may inhibit germination in dormant seeds.

Callaway (1988) followed protein changes in peach buds and seeds during chilling. Response varied with protein band, some increasing in intensity with chilling and some decreasing relative to controls held at 25°C. Radiolabeling with ³⁵S-methionine gave

similar results. Callaway suggested that proteins that accumulated at 25°C inhibited, while those that accumulated at 4°C stimulated, breaking dormancy.

Lang and Tao (1991), working with peach flower buds, observed a substantial decrease in a 61-kDa protein during the final stages of chilling. Its role in dormancy has yet to be determined.

The objective of our study was to assess the relationship between protein changes and breaking dormancy in peach seeds.

Materials and Methods

Plant material. Siberian C peach pits were obtained from Hilltop Nurseries, Hartford, Mich., and 'Farouki' and 'Maloussi' (local Moroccan seedlings used as rootstock) were purchased from seed collectors in the Missouri region of Morocco. All seeds were from open-pollinated flowers. After pericarp removal, the pits were washed, dried at 20°C, and stored at 5°C until used.

Stratification, germination, and fresh and dry weights (DWs). Seeds were removed from the pits with a mechanical cracker and soaked in *N*-(trichloromethyl) thio-4-cyclohexene-1,2-dicarboximide (captan) solution (0.03%) for 72 h. The seeds were stratified on sterile filter paper moistened with captan in Petri dishes held in darkness at either 5°C or 20°C for 1 to 10 weeks. To evaluate germination capacity, three replications of 10 seeds each were held in Petri dishes at 20°C in darkness. The seeds were considered to have germinated when the radicle had elongated 3 mm or more after 14 days. Thirty seeds from each treatment were dissected into cotyledons and embryonic axes to determine fresh and dry weights. Fresh weights were recorded immediately after dissection and DWs after 72 h at 90°C.

Protein content analysis. To determine soluble protein content, 10 embryonic axes were extracted in 0.5 ml of 62.5 mM tris-HCl (pH 6.8) in a small mortar. The homogenate, along with two 0.25-ml rinses, was left overnight at room temperature (≈ 20°C) and cleared by centrifugation at 23,000 × *g* for 20 min. The supernatant

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was filtered through Whatman no. 1 filter paper and stored in a freezer. One cotyledon was removed from each of eight seeds. The eight cotyledons were homogenized in 2 ml of the same buffer in a small mortar. The homogenate, with two 1-ml rinses, was treated as above.

Protein content was determined according to Bradford's (1976) method. This assay is based on the shift in absorbance for an acidic solution of Coomassie Brilliant Blue G-250 from 465 nm to 595 nm when protein binding occurs.

For sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), samples were extracted as described above with 62.5 mM tris-HCl containing SDS (2%), glycerol (10%), 2- β -mercaptoethanol (5%), and bromophenol blue (0.002%, as a tracking dye). Electrophoresis was performed according to Laemmli (1970), with 12% (w/v) polyacrylamide on 1.5-mm slab gels for separating and 4% (w/v) for stacking gels at constant temperature (20C) and constant current of 20 mA. Fifty μ g of protein was loaded per well. Gels were stained with Coomassie Brilliant Blue R-250 in 40% methanol plus 10% glacial acetic acid for 4 h, then destained for 12 h. Three replicate gels were run for each sampling date.

The relative nobilities and molecular weights (MWs) of the proteins were determined by comparing them with those of proteins of known MWs. Low-range MW standards (Bio-Rad, Richmond, Calif.) were used in each electrophoretic run. The six standards and their MWs (in kiloDaltons) were rabbit muscle phosphorylase b, 97.4; bovine serum albumin, 66.20; hen egg white ovalbumin, 42.699; bovine carbonic anhydrase, 31.0; soybean trypsin inhibitor, 21.5; and hen egg white lysozyme, 14.4.

Stratification time and temperature. Siberian C seeds were stratified for 8 weeks at 5C or 20C and samples were removed weekly.

Three remaining experiments were conducted concurrently using one set each of intact, fully imbibed 'Farouki' and 'Maloussi' seeds as controls. Results are presented only for 'Maloussi', as the two biotypes responded similarly in all but a few instances.

Imbibition degree. The seeds were soaked in distilled water for 5, 15, or 72 h, corresponding to 25%, 50%, and 100% of full imbibition, respectively, as determined by water content. The seeds were wrapped in a piece of cheesecloth, which was attached to the top of a 250-ml jar containing 25 ml of captan solution. The jar was tightly sealed with a cap and Parafilm (American Can Co., Greenwich, Conn.), then held at 5C for 2 to 10 weeks.

Stratification of cotyledons separately from embryonic axis. Seeds that had been imbibed for 72 h were cut transversely with a razor. The chalazal and micropylar ends of the seeds were stratified independently at 5C. The cotyledons remaining in the chalazal end represented 94% to 97% and 89% to 95% fresh weight of the intact cotyledons, respectively, for 'Farouki' and 'Maloussi'. After 0 to 8 weeks, the embryonic axis was dissected out. Fresh and dry weights of the cotyledons and the axis were recorded, but germination capacity of the axis was not tested.

Gibberellic acid. GA₃ stimulates germination in partially chilled peach seeds (Donoho and Walker, 1957). To determine if protein hydrolysis was also hastened by GA₃, 'Farouki' and 'Maloussi' seeds were soaked in distilled water for 48 h, then some of the seeds were transferred to a GA₃ solution (500 ppm) for the remaining 24 h of a 72-h imbibition period. Control seeds were held for 72 h in water.

For each treatment, the following data were recorded at 2-week intervals: fresh and dry weights; germination at 20C with and without additional imbibition (three replications of 10 seeds each per treatment); and soluble protein content and protein profiles for axes and cotyledons.

Results

Stratification time and temperature. Siberian C seeds held at 20C did not germinate. Those held at 5C began to germinate after 5 weeks, and germination percentage increased with time. Germination percentages for weeks 5 through 8 were 20%, 45%, 75%, and 94%, respectively (all significantly different at $P \leq 0.05$ by analysis of variance and Duncan's multiple range test). Fresh weights and water content of cotyledons and embryonic axes increased with time at 5C and 20C, with a greater rate of increase at 5C (data not shown). DWs remained constant at ≈ 0.65 and 260 mg, for embryonic axes and cotyledons, respectively. Soluble protein content remained unchanged at 26% to 28% DW (cotyledons) and 14% to 16% (embryonic axes), regardless of temperature or stratification time (data not shown).

SDS–PAGE revealed a decrease in the concentrations of nine polypeptides (apparent MWs of 66, 54, 48, 47, 41, 39.5, 36, 35, and 33.5 kDa) in the cotyledons of seeds held at 5C during weeks 5 through 8 (Fig. 1), coinciding with an increase in germination capacity. These decreases were accompanied by the appearance of several new low-MW (10 to 14 kDa) polypeptides. None of these changes occurred in the embryonic axes of these seeds (Fig. 1) or in cotyledons or axes of seeds held at 20C.

Imbibition degree. Germination of fully imbibed 'Farouki' and 'Maloussi' seeds at 20C increased steadily as stratification time at 5C increased (Table 1). Partially imbibed seeds did not germinate regardless of stratification time. However, if these seeds were fully imbibed after stratification, their germination paralleled that of seeds that were fully imbibed before stratification (Table 1). Thus after-ripening occurred even though the seeds could not germinate.

Fresh weight and water content of cotyledons and embryonic axes increased with stratification time of fully imbibed seeds, but the rate of increase was greater at 5 than 20C (data not shown). Data for partially imbibed seeds held at 5C paralleled those of fully imbibed seeds, but all values were lower, as might be expected. Thus, after 8 weeks of stratification, the water content of seed parts of partially imbibed seeds was equal to or less than that of seed parts of fully imbibed but nonstratified seeds. None of the treatments affected axis or cotyledon DWs (data not shown).

Soluble protein content varied from 19% to 24% (cotyledons) or 9% to 12% (embryonic axes) and was not affected by cultivar, moisture content, or stratification time (data not shown). Changes in protein profiles in fully imbibed seeds of both biotypes, exemplified by 'Maloussi' (Fig. 2), confirmed those previously observed in Siberian C seeds. Decreases in the intensity of nine bands were evident in extracts of the cotyledons after 2 weeks of stratification and the decreases continued until the end of the experiment (8 weeks). Again, two low-MW bands appeared at the same time. However, none of these changes occurred in axes of these seeds or in axes or cotyledons of fully imbibed seeds kept continuously at 20C (could not germinate) (Fig. 2). Although the concentrations of polypeptides 5 and 7 (41 and 36 kDa, respectively) decreased within 1 week in axes of seeds held at both temperatures, levels of the same polypeptides, or ones of similar MWs, in cotyledons decreased only at 5C. Similar changes occurred in cotyledons of partially imbibed seeds during stratification at 5C, but at a slower rate (Fig. 3). Thus the intensities of bands 5 and 7 in partially imbibed seeds stratified for 10 weeks were about equal to those of the same bands in fully imbibed seeds stratified for only 3 weeks. In 'Farouki' seeds, low-MW bands also were slower to appear, especially in seeds imbibed for only 5 h.

Stratification of cotyledons separately from embryonic axis. Fresh weights and water content of embryonic axes and cotyledons

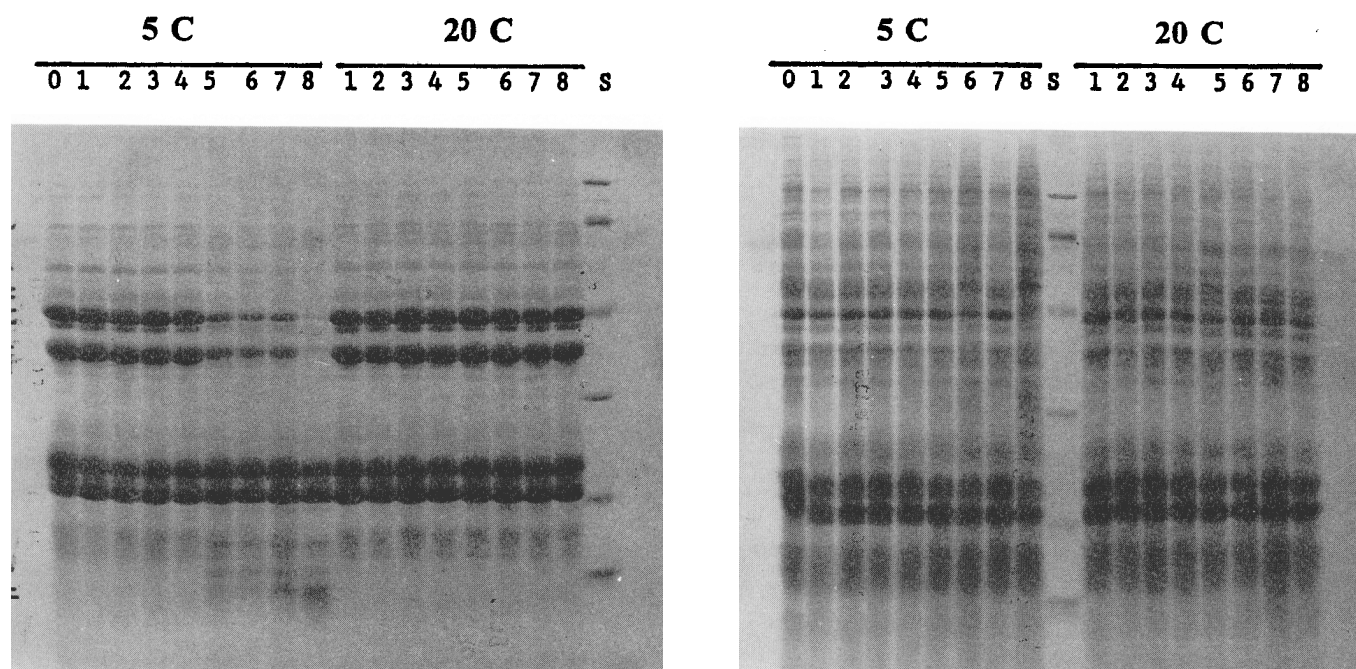


Fig. 1. Protein profiles of cotyledons (left) and embryonic axes (right) of Siberian C peach seeds stratified for 0 to 8 weeks at 5 or 20C. Numbers above figure indicate weeks of stratification. Lines at left indicate protein bands that changed in intensity during stratification. 0 = Control (imbibed for 72 h, no chilling); S = low-range molecular weight standards (see text for identities of proteins), values (kDa) in parentheses: 1) RMPb (97.4), 2) BSA (66.2), 3) HEWO (42.699), 4) BCA(31.0), 5) STI (21.5), 6) HEWL (14.4).

increased with time at 5C. 'Farouki' and 'Maloussi' cotyledon DWs were $\approx 78\%$ and 88% , respectively, of those of intact seeds because some portions were removed when the seeds were cut. DWs and total soluble protein content were again not affected by time at 5C (data not shown). Protein profiles in the cotyledons were unaffected by removing the embryonic axis before stratification; the two bands at 41 and 36 kDa decreased in intensity at the same rates, and new highly mobile bands appeared as the larger polypeptides disappeared (Fig. 4). Protein profiles of axes showed no visible changes except the decrease in band intensity at 41 and 36 kDa observed earlier at 5 and 20C (data not shown).

Gibberellic acid. After treatment with GA_3 , 35% to 40% of the seeds germinated without chilling, whereas, none of the control seeds germinated. Chilling further stimulated germination, which reached 90% to 95% after 4 weeks, whereas 8 weeks of chilling was required to attain similar germination percentages in the absence of GA_3 .

GA_3 did "not affect total soluble protein content (data not shown). It hastened the loss of protein band intensity in 'Farouki' (Fig. 5) but not in 'Maloussi' cotyledons. However, GA_3 -treated seeds germinated before any major changes occurred in proteins. The two prominent bands at 41 and 36 kDa remained essentially unchanged in both cultivars after 1 week of chilling (Fig. 5), yet germination capacity was 50% at this time. No consistent changes in protein profile were evident in embryonic axes as a result of GA_3 .

Discussion

Our results can be summarized as follows: a) the concentrations of certain soluble proteins in the cotyledons decline at 5C but remain constant at 20C; b) similar changes do not occur in the embryonic axes; c) the presence of the embryonic axis is not required for these changes to occur; and d) these changes are not

Table 1. Effects of imbibition degree and stratification duration at 5C on germination (%) at 20C of peach seeds of two Moroccan biotypes ('Farouki' and 'Maloussi').

Time (wks) at 5C	Imbibition (%)		
	25	50	100
<i>Farouki</i>			
0	0 g ^a	0 g	0 g
2	20 f	20 f	15 f
4	40 d	50 d	30 e
6	75 b	77 b	60 c
8	90 a	93 a	90 a
<i>Maloussi</i>			
0	0 i	0 i	0 i
2	20 g	30 f	14 h
4	30 f	40 e	45 de
6	40 e	50 d	67 c
8	70 c	87 b	97 a

^aFully imbibed seeds held continuously at 20C failed to germinate and partially imbibed seeds held at 5C germinated only after additional imbibition.

^bMean separation within biotypes by analysis of variance of arcsin-transformed data and Duncan's multiple range test at $P \leq 0.05$.

related to germination, because germination can be prevented by axis removal or partial imbibition or hastened by GA_3 without markedly affecting the protein degradation pattern.

Presumably the critical metabolic changes involved in breaking dormancy occur in the embryonic axis or are controlled by the axis. The axis could send a chemical messenger to other parts of the seed to induce hydrolytic enzyme synthesis, as is the case in barley (*Hordeum vulgare* L.) (Varner et al., 1964). Such a system does not seem to be involved in the protein changes observed in peach cotyledons because the process occurs without the axis.

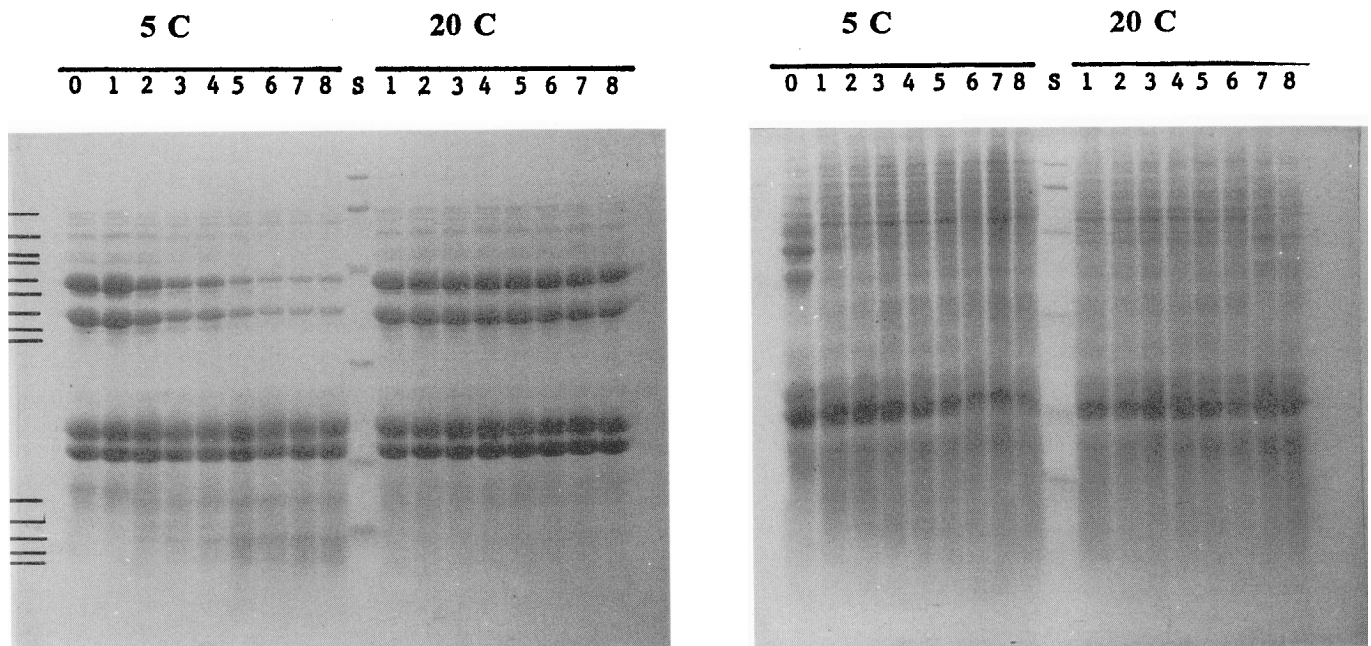


Fig. 2. Protein profiles of 'Maloussi' peach cotyledons (**left**) and embryonic axes (**right**) stratified for 0 to 8 weeks at 5C or 20C. Numbers above figure indicate weeks of stratification at 5C. Lines at left indicate bands that changed in intensity during stratification. 0 = Control (imbibed for 72 h, no chilling); S = low-range molecular weight standards (see text for identities of proteins), values (kDa) in parentheses: 1) RMPb (97.4), 2) BSA (66.2), 3) HEWO (42.699), 4) BCA (31.0), 5) STI (21.5), 6) HEWL (14.4).

IMBIBITION (HR)

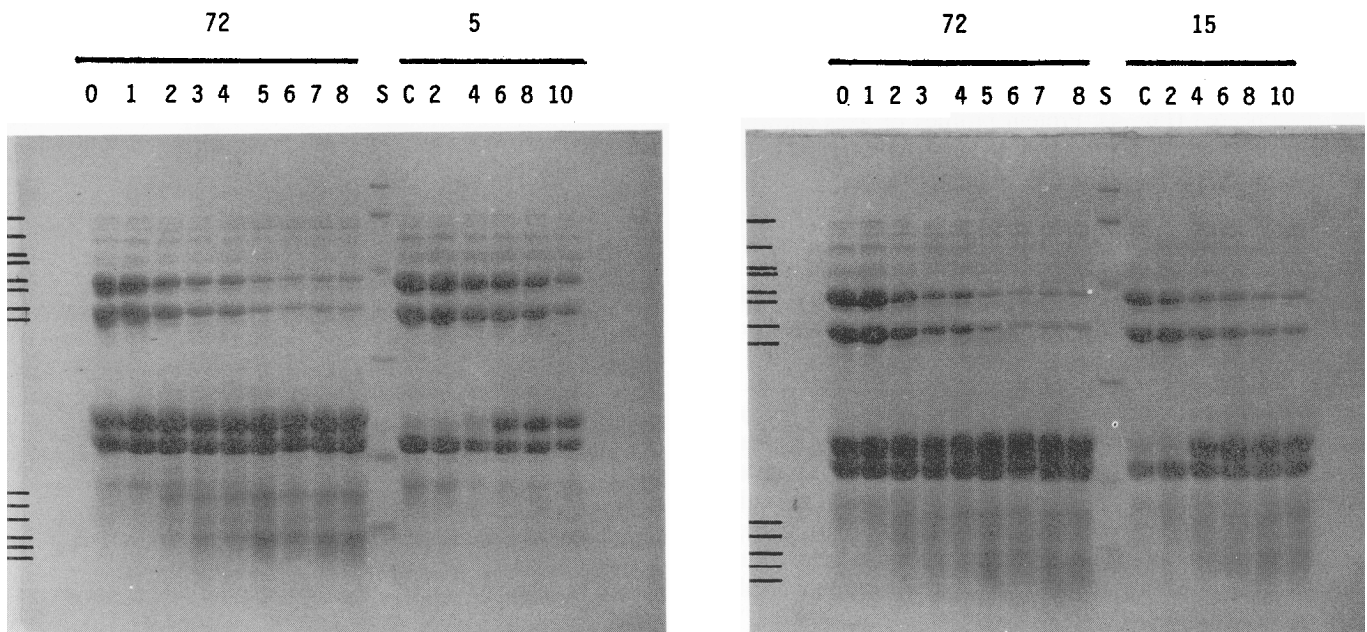


Fig. 3. Protein profiles of 'Maloussi' peach cotyledons from seeds imbibed for 5, 15 or 72 h (full imbibition) before stratification at 5C for 0 to 8 weeks. Numbers above figure indicate weeks of stratification. Lines at left indicate protein bands that changed in intensity during stratification. 0 = Control (imbibed for 72 h, no chilling); C = control (imbibed for 5 h, no chilling); S = low-range molecular weight standards (see text for identities of proteins), values (kDa) in parentheses: 1) RMPb (97.4), 2) BSA (66.2), 3) HEWO (42.699), 4) BCA (31.0), 5) STI (21.5), 6) HEWL (14.4).

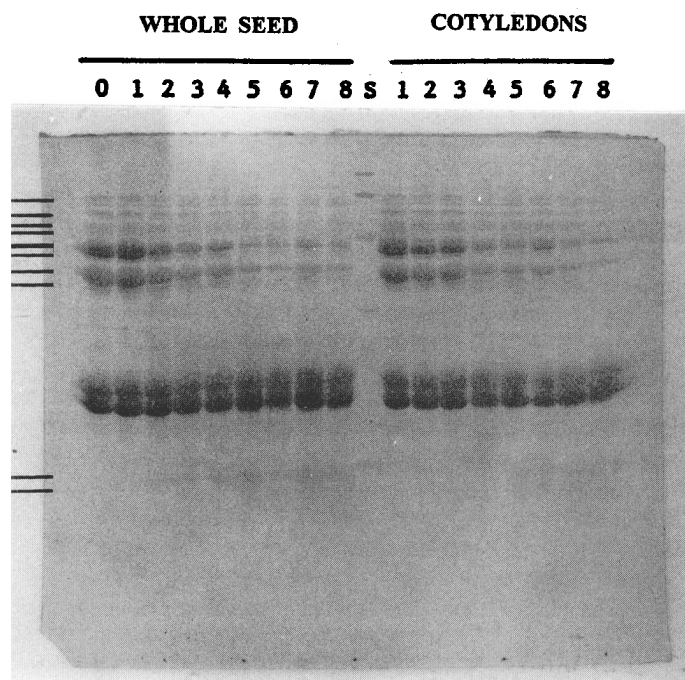


Fig. 4. Protein profiles of 'Maloussi' peach cotyledons stratified for 0 to 8 weeks in the presence (left) or the absence (right) of embryonic axes. Numbers above figure indicate weeks of stratification at 5C. Lines at left indicate protein bands that changed in intensity during stratification. 0 = Control (imbibed for 72 h, no chilling); S = low-range molecular weight standards (see text for identities of proteins), values (kDa) in parentheses: 1) RMPb (97.4), 2) BSA (66.2), 3) HEWO (42.699), 4) BCA (31.0), 5) STI (21.5), 6) HEWL (14.4).

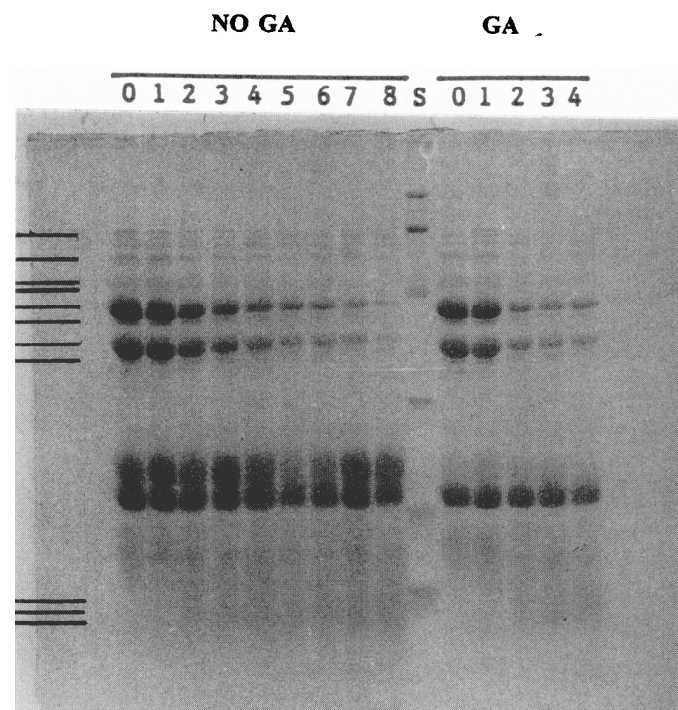


Fig. 5. Effects of gibberellic acid (GA, at 500 ppm for 24 h before stratification) on protein profiles of 'Faronki' peach cotyledons from seeds stratified for 0 to 8 weeks. Numbers above figure indicate weeks of stratification at 5C. Lines at left indicate protein bands that changed during stratification. No GA, 0 = control (imbibed for 72 h, no chilling); GA, 0 = control (imbibed for 48 h, then soaked in GA₃ solution for 24 h, no chilling); S = low-range molecular weight standards (see text for identities of proteins), values (kDa) in parentheses: 1) RMPb (97.4), 2) BSA (66.2), 3) HEWO (42.699), 4) BCA (31.0), 5) STI (21.5), 6) HEWL (14.4).

Processes involved in breaking dormancy are often difficult to separate from those occurring during germination. The changes observed in this study were clearly separated from germination in that a) the embryonic axis was not essential; b) the changes occurred, although at a slower rate, in partially imbibed seeds that could not germinate; and c) germination induced by GA₃ did not induce protein changes in the cotyledons. These facts indicate that the protein changes were the result of chilling, *per se*, rather than of processes associated with germination.

One-dimensional SDS-PAGE was used in this work; this method separated some 30 or 40 individual proteins. However, plants contain thousands of proteins; therefore, proteins specific to either dormant or nondormant seeds could easily have been overlooked. Incubating seeds with ³⁵S-labeled amino acids to label newly synthesized proteins is a much more powerful technique for detecting protein differences in response to chilling. Recent experiments with sugar maple (*Acer saccharum* Marsh.) (Hance and Bevington, 1991) and pear (*Pyrus serotina* Rehd.) (Lin et al., 1991) seeds indicate that dormancy release by chilling is associated with alterations in polypeptide constituents. In sugar maple, two proteins present in cotyledons of nonchilled seeds were absent from those of chilled seeds. In pear seeds, changes occurred during chilling in both cotyledons and axis proteins, some increasing, some decreasing, and others appearing *de novo*. Further research using cloned genes of specific proteins could provide new insight into the differences between dormant and nondormant tissues.

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