RESEARCH ARTICLE

Protein changes between dormant and dormancy-broken seeds of *Prunus campanulata* Maxim

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Seed dormancy is regulated by complex networks in order to optimize the timing of germination. However, the biochemical basis of the regulation of seed dormancy is still poorly understood. Many temperate timber species, which are of ecological and/or economic interest, are deeply dormant in seeds, such as *Prunus campanulata*. Freshly harvested seeds require warm plus cold stratification to break dormancy before they can begin to germinate. According to the results of germination, both warm and cold stratifications are the critical influences for breaking seed dormancy. Significant variations in seed proteins were observed by 2-DE before and after the breaking of seed dormancy. Among the 320, 455, and 491 reproducibly detected spots on the cotyledons, embryos, and testae, respectively, 71 dramatic changes in abundances were observed following warm and/or cold stratification. Among these protein spots, dehydrin, prunin 1 precursor, prunin 2 precursor, and prunin 2 were identified by MS and sequence comparison. The implications of protein changes in relation to the breaking of seed dormancy and germination are discussed. This is the first report of a proteomic analysis of dormancy breaking in woody plant seeds.

Keywords:
Dehydrin / Dormancy / *Prunus campanulata* / Prunin / Stratification

1 Introduction

Seed dormancy and germination, complex adaptive traits of higher plants, are influenced by a large number of environmental factors. Dormant seeds are well equipped to survive for extended periods in unfavorable climates [1]. Seed dormancy, discussed here, is defined as the failure of an intact viable seed to complete germination under favorable conditions. Genetic analysis has identified the crucial role of abscisic acid (ABA) in seed dormancy, as well as the requirement of gibberellins (GAs) for germination [2]. GAs and ABA can act antagonistically and be influenced by environmental factors, such as temperature and light [3, 4]. Germination results from a combination of many cellular and metabolic events, coordinated by a complex regulatory network that includes seed dormancy, an intrinsic ability to temporarily block radicle protrusion in order to optimize the timing of germination [5]. From a biochemical and molecular point of view, because a population of seeds does not complete the process synchronously, studying seed germination and dormancy is difficult, but is central to plant community development [6]. Although over 5000 publications on seed germination or dormancy have appeared in the last a few decades, the underlying molecular regulatory mechanisms are not fully understood.

The emergence of proteomics has been inspired by the realization that the final product of a gene is inherently more complex and closer to its function than the gene itself [7]. Proteomic tools offer other ways to analyze net-

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Abbreviations: **ABA**, abscisic acid; **GA**, gibberellic acid; **MGT**, mean germination time

works of proteins that control important physiological reactions involved in seed germination. Studies of plant proteomic analyses used so far have been reported [8]. Due to the availability of complete genomic sequence information, a lot of recent proteomic analysis studies have focused on *Arabidopsis thaliana*. However, the subjects of these studies were concentrated not on seed dormancy but on germination [9, 10], probably due to the weak dormancy of *A. thaliana* seeds. Many seeds of woody plants are deeply dormant and germinate poorly before a period of dormancy breaking. They include *Prunus spp.*, *Elaeocarpus sylvestris*, *Fagus silvatica*, and so on [11], which are of ecological and/or economic interest [8, 12]. *Prunus campanulata* Maxim., distributed from Japan's Ryukyu Islands and Taiwan to southern China, is a deciduous and outstanding flowering tree with dense branching [13]. This tree grows on mountains at 500–2500 m and flowers in January to March each year. Freshly harvested seeds are strongly dormant and require warm plus cold stratification for complete dormancy breaking [14, 15].

As the first step to identifying proteins related to the regulation of seed dormancy and germination, we analyzed the temperature effect on *P. campanulata* seeds. The aim of the present study was to find the proteins or peptides that dramatically change during dormancy breaking, identify them and discuss their potential roles.

2 Materials and methods

2.1 Materials

The IPG strips (13 cm, pH 3–10 NL), Pharmalytes, the protein marker kit, silver-staining kit, and glycerol were purchased from Amersham Biosciences (Uppsala, Sweden). The RC DC protein assay kit was obtained from BioRad (Richmond, CA, USA). Sequencing-grade modified trypsin was obtained from Promega (Mannheim, Germany). All other chemicals were the highest purity grade and were obtained commercially from Sigma-Aldrich (Deisenhofen, Germany).

2.2 Seed harvesting and stratification treatments

Fruits of *P. campanulata* were harvested from Ali Mt. (23°32'N, 120°47'E) at an elevation of 2000 m, Chiayi County, central Taiwan in early May 2004. The seeds were extracted by removing the pulp in water, and filled, sunken seeds were used for subsequent treatments. Warm stratification was carried out with seeds stratified at alternating temperatures of 30/20°C with 12 h fluorescent light (80–100 $\mu mol \cdot m^{-2}s^{-1}$, 400–700 nm). Cold stratification was carried out with seeds stratified at 4°C in the dark. For warm plus cold stratification, fresh seeds were stratified at 30/20°C for 6 wk, followed by cold stratification for 6 or 8 wk. Each treatment consisted of three replicates of 50 seeds each. The 50 clean seeds were mixed with moist sphagnum in a polyethylene bag (0.05 mm in thickness). Excess water in the sphagnum was removed to obtain a water content of about 400% by mass (on a dry-weight basis). Germination tests were performed at alternating temperatures of 30/20°C (12/12 h) with a 12-h daily photoperiod (80–100 $\mu mol \cdot m^{-2}s^{-1}$, 400–700 nm) during the 30°C treatment. Germination, judged by radicle protrusion of at least 5 mm, was recorded weekly, and results were expressed as percent germination and mean germination time (MGT) in days [16]. $MGT = (\Sigma n_i t_i)/N$, where n_i is the number of seeds germinated in t_i days from the beginning of the test, and N is the total number of germinated seeds at the end of the test. The value of MGT gives an indication of how fast germination takes place and how narrow the germination peak is. Fresh and treated seeds were lyophilized and stored at −80°C for proteomic analysis.

2.3 Sample preparation and 2-DE

Seeds were separated into four parts, including the endocarp, testa, cotyledon, and embryo using hammer and tweezers. The hard endocarp was opened by hammer and the testa, cotyledon, and embryo were separated by tweezers. The tissues can be distinguished easily by naked eyes and stratified seeds were more easily to be separated than fresh seeds because they grew into independent parts. Extracts were homogenized with PRO 250 homogenizer (PRO Scientific, Oxford, CT, USA) containing 125 mM sucrose, 10% v/v glycerol, 1 mM DTT, 1 mM EDTA, 1 mM PMSF, and 10 mM Tris-HCl at pH 7.4. The suspension was centrifuged for 30 min at 10 000 × *g* at 4°C, and the resultant supernatant liquid was centrifuged for 120 min at 32 000 × *g* at 4°C. The final supernatant liquid was used for 2-DE. Protein concentrations of extracts were estimated on the basis of an RC DC protein assay kit from BioRad. 2-DE was performed by a commercially available Ettan IPGphor IEF system and Hoefer SE600 Ruby (gel size 13 cm × 15 cm) from Amersham. Proteins from the various extracts were separated using gel strips and formed an immobilized nonlinear pH gradient from 3 to 10 (Immobiline DryStrip, pH 3–10 NL, 13 cm; Amersham). Analytical IPG strips were rehydrated for 13 h at 20°C with 250 μL of the rehydration buffer including 200 μg of sample proteins. IEF was performed at 20°C in the Ettan IPGphor system (Amersham) for 1 h at 500 V, 1 h at 1000 V, 1 h at 4000 V, and 3 h at 8000 V. Prior to the second dimension, the strips were equilibrated for 2 × 15 min in equilibration solution containing 6 M urea, 30% v/v glycerol, 2% w/v SDS, 0.5 M Tris-HCl (pH 8.8). DTT (1% w/v) was added to the first equilibration solution and 2.5% w/v iodoacetamide was added to the second one. For the second dimension, the strips were transferred onto SDS polyacrylamide gels (12.5%) with a run of 50 mA *per* gel for 4–5 h at 4°C. The 2-DE gels were made in triplicate and sample proteins were from two independent extractions.

2.4 Protein staining and analysis of 2-DE gels

After electrophoresis, proteins were visualized by a modified silver-staining kit [17]. Digital images of the gels were obtained by using an ImageScanner and were analyzed using ImageMaster 2D elite software (Amersham). The spots were detected and the background was subtracted (mode: average on boundary), and the 2-DE gels were aligned and matched. A quantitative determination of the spot volumes was performed (mode: total spot volume normalization). Specific spots were described during different treatments when their volumes significantly differed (at least ten-fold in relative abundance). The interesting proteins were identified by MALDI-TOF MS/MS or ESI-MS/MS analysis.

2.5 Protein identification by MS

For MS analysis, protein spots were excised from the gel and digested with trypsin according to published procedures [18]. Proteins were identified by searching the protein databases (Swiss-Prot, OWL, TrEMBL, and NCBInr) using MASCOT (http://www.matrixscience.com). To denote a protein as unambiguously identified, the Mowse scoring algorithms were used. Only proteins whose score exceeded the significance threshold (probability, which can be ambiguous if smaller than 0.05) are discussed.

3 Results and discussion

3.1 Analysis of the effects of various treatments on the protein patterns of *P. campanulata* seeds

Proteins were extracted from the various seed samples (freshly harvested, warm stratification for 6 wk, cold stratification for 6 wk, and warm stratification for 6 wk plus cold stratification for 8 wk) and analyzed by 2-DE as described in Section 2. For analysis of each treatment, 2-DE gels were made in triplicate from two independent protein extractions and were highly reproducible (using ImageMaster 2D elite software (Amersham) as described in Section 2.4). In total, 320, 455, and 491 reproducible spots from cotyledons, embryos, and testae were, respectively, detected and 71 dramatic changes in abundances were observed following warm and/or cold stratification. Different tissues of the seed have individual importance to dormancy because of their functions. It is well known that cotyledons of the seed provide nourishment for the embryonic axis, which is the tissue that directly elongates to establish a new plant. The testa of higher plant seeds is a multifunctional tissue that protects the embryo against adverse environmental conditions, and it may also interfere with dormancy [19]. To compare the different protein patterns of seeds in various tissues (cotyledon, embryo, and testa), it was shown that various proteins were located in different parts of the seed. The protein patterns of the cotyledon (Fig. 1) were similar to those of the embryo

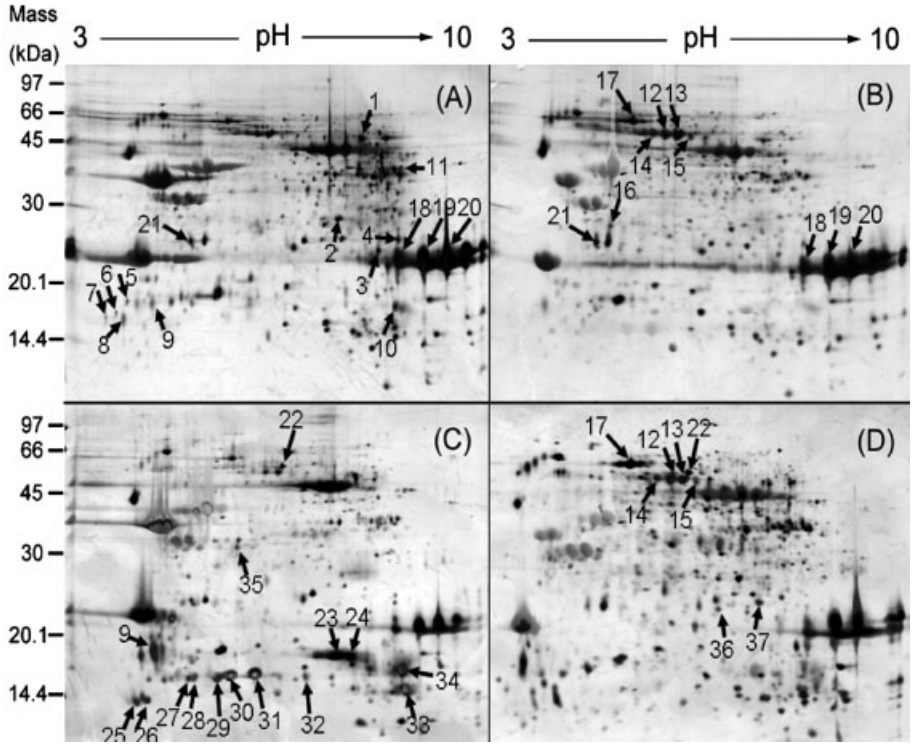

Figure 1. Protein patterns of cotyledons following different treatments for the breaking of seed dormancy. (A) Fresh (untreated); (B) warm stratification for 6 wk; (C) cold stratification for 6 wk; (D) warm stratification for 6 wk plus cold stratification for 8 wk. Labeled spots of the gels are those differentially expressed by at least ten-fold during the various treatments.

(Fig. 2). However, protein patterns of the testa significantly differed from those of the cotyledon and embryo (Fig. 3). This suggests that some proteins may have position specificity. Consistant with the position specificity, it was found that high ABA contents were detected in the testa but not in the embryo and cotyledon, and reduced 6–12 times during warm and/or cold stratification. Furthermore, a higher concentration of GA_4 was detected in embryo and cotyledon of dormancy-breaking seed (data not shown). The different features between testa and embryo/cotyledon suggested that testa and embryo/cotyledon may play specific roles in seed dormancy and germination, respectively. Germination experiments also reveal that the removal of testa of *P. campanulata* seed eliminates its dormancy effect (data not shown).

According to the germination experiments (Table 1), most of seeds do not germinate under either warm stratification or cold stratification, but warm plus cold stratification increases seed germination significantly. It indicated that a combination of warm and cold stratification is necessary for releasing dormancy completely. A small amount of seeds (sometimes over 10% depend upon the year and the location of the seed harvested [14, 15]) germinated following only cold stratification. It is likely that some seed had experienced "warm treatment" before they were delivered from the field to the laboratory or it may just reflect the heterogeneity of the seeds.

When comparing the different protein pattern of seeds at different treatment stages, some protein spots with significant changes (of at least ten-fold) in the 2-DE gels are shown in Figs. 1–3. The protein spots whose abundances changed could be significantly divided into four groups: (1) proteins which changed after each treatment; (2) proteins which changed only after warm stratification; (3) proteins which changed only after cold stratification; and (4) proteins which changed only after warm plus cold stratification. These protein spots are summarized in Table 2. In the first group, proteins which changed were apt to change similarly after each treatment. It is possible that those proteins were changed due to imbibition. Proteins of nos. 1, 2, 3, 4, 5, 6, 7, 8, 10,

Table 1. Germination of *P. campanulata* seeds following warm and/or cold stratification[a)]

Treatment	% Germination[b)] (MGT[c)])
Freshly harvested seeds	0
2 wk warm	2.7 e
4 wk warm	5.3 de (99.1)
6 wk warm	6.0 de (86.0)
5 wk cold	5.3 de (76.6)
6 wk cold	8.7 cd (49.7)
8 wk cold	12.0 c (31.6)
6 wk warm + 6 wk cold	72.0 b (30.5)
6 wk warm + 8 wk cold	98.0 a (7.9)

a) Germination, judged by radicle protrusion of at least 5 mm, was recorded weekly following each treatment for a total of 16 wk. The conditions of germination were at 30/20°C the same as those for warm stratification.

b) Means ($n = 3$) with the same letter do not significantly differ ($p = 0.05$) by the LSD test.

c) Values in parentheses represent the MGT in days. In other words, germination rate increased rapidly after warm stratification for 6 wk plus cold stratification for 8 wk.

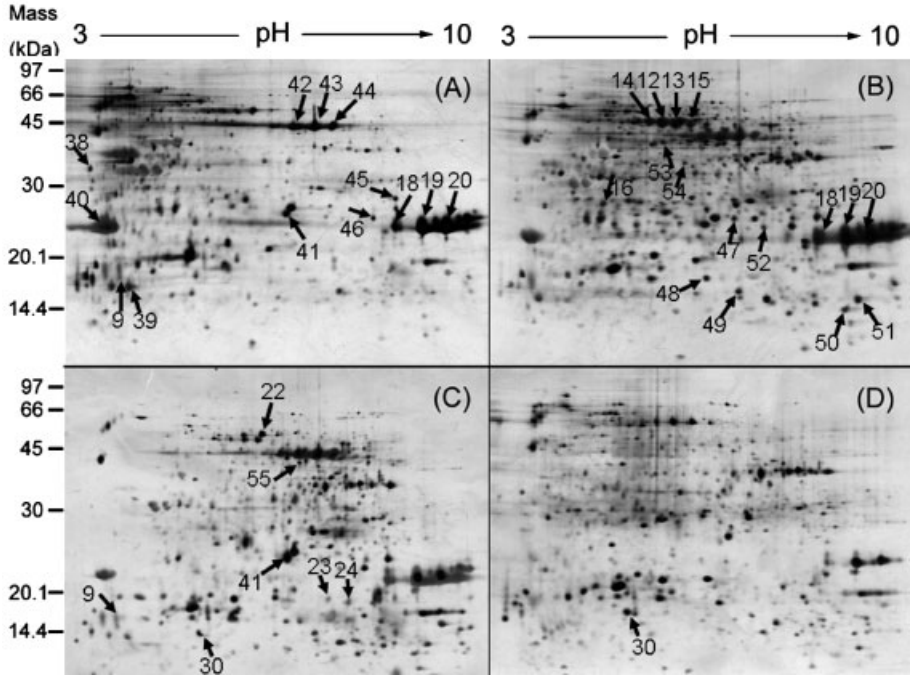

Figure 2. Protein patterns of embryos with different treatments for the breaking of seed dormancy. (A) Fresh (untreated); (B) warm stratification for 6 wk; (C) cold stratification for 6 wk; (D) warm stratification for 6 wk plus cold stratification for 8 wk. Labeled spots of the gels are those differentially expressed by at least ten-fold during the various treatments.

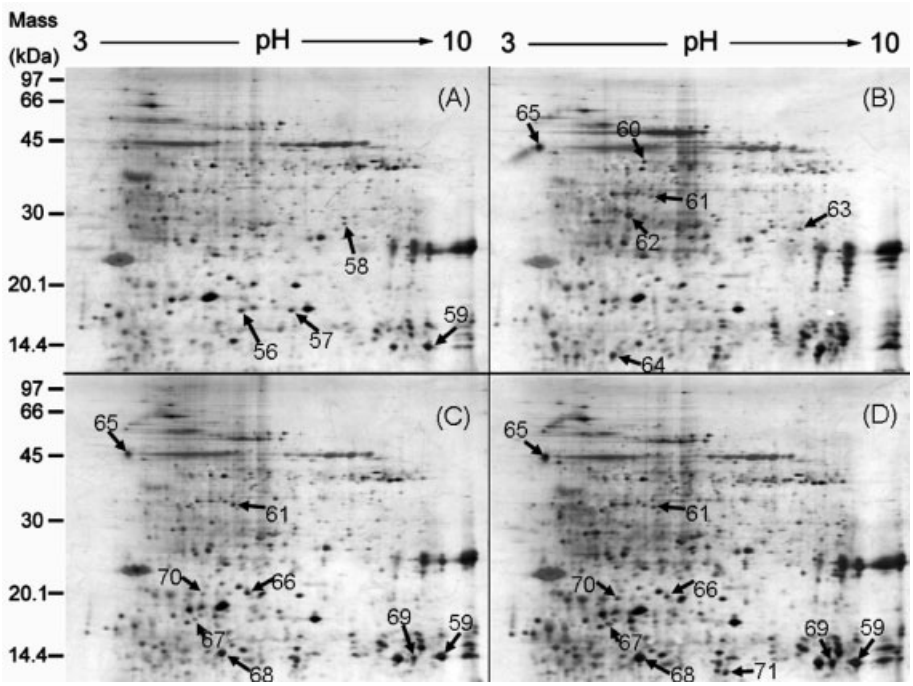

Figure 3. Protein patterns of testae with different treatments for the breaking of seed dormancy. (A) Fresh (untreated); (B) warm stratification for 6 wk; (C) cold stratification for 6 wk; (D) warm stratification for 6 wk plus cold stratification for 8 wk. Labeled spots of the gels are those differentially expressed by at least ten-fold during the various treatments.

Table 2. Changes in *P. campanulata* seed proteins following various stratification treatments[a)]

Treatment	Decreased protein spot nos.			Increased protein spot nos.		
	Cotyledon	Embryo	Testa	Cotyledon	Embryo	Testa
Warm or cold treatment[b)]	**1**, 2, 3, 4, 5, 6, 7, 8, 10, 11	38, 39, 40, 42, 43, 44, 45, 46	56, 57, 58	–	–	61, 65
Warm stratification only[c)]	9	9, 41	59	12, 13, 14, 15, 16, 17, 18, 19, 20, 21	12, 13, 14, 15, 16, 47, 48, 49, 50, 51, 52, 53, 54	60, 62, 63, 64
Cold stratification only[d)]	16, 18, 19, **20**, 21	18, 19, **20**	–	9, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35	22, 23, 24, **30**, 55	66, 67, 68, **69**, 70
Warm plus cold treatment[e)]	–	–	–	36, 37	–	71

a) Spot changes were obtained by comparing protein patterns of fresh seeds and treated seeds (warm for 6 wk, cold for 6 wk, and warm for 6 wk plus cold for 8 wk) as shown in Figs. 1–3.
b) These spots change their abundance following either warm or cold stratification.
c) These spots change their abundance only after warm but not after cold stratification.
d) These spots change their abundance only after cold but not after warm stratification.
e) These spots change their abundance only after warm plus cold treatment. Warm or cold stratification alone cannot induce their changes.

11, 35, 39, 40, 42, 43, 44, 45, 46, 56, 57, and 58 decreased, while only nos. 61 and 65 increased after the uptake of water regardless of temperature. Changes in proteins probably indicate biochemical activities of the seed after the uptake of water. It is unlikely that this group of proteins directly involve in the dormancy-breaking since warm and cold stratification are both required to completely induce seed germination (Table 1). In the second group, proteins changed only with warm stratification. In testa, the proteins (nos. 60, 62, 63, and 64 in Fig. 3B) could not be induced through cold stratification (Fig. 3C) and did not change back to their original fresh state (Fig. 3A) even following cold stratification (Fig. 3D).

Most of the proteins induced following warm stratification (Fig. 2B) change continuously in embryo following cold stratification (Fig. 2D), and they did not change back to their original fresh state (Fig. 2A) either. As shown in Table 2, many proteins (23 different spots) increase their abundance in cotyledon, embryo, and testa following warm stratification. Only three proteins, nos. 9, 41, and 59, decrease in their abundance. Proteins in this group could be turned on or off under favorable conditions for preparing seed germination regardless of the chilling circumstances. The functions of these proteins are interesting, but unclear so far. Some proteins in this group may involve in the postharvested maturation of seed. However, a mechanism that inhibits the seed germination before chilling treatment is also needed. In the third group, proteins changed with cold stratification regardless of whether seeds had been treated by warm stratification or not. Five proteins which decreased included nos. 16, 18, 19, 20, and 21. As shown in Table 2, many proteins (21 different spots) increase their abundance, especially in cotyledon (15 spots). Some of the proteins in this group may be responsible for triggering the dormancy-broken effect. It is also possible that inhibitors of germination (or inducer of dormancy) produced during warm stratification are removed following cold stratification. In the forth group, the proteins changed following warm plus cold treatment, and produced only two protein spots, nos. 36 and 37. This probably indicates that most of the proteins change in seed can be regulated independently by temperature. But why these two proteins induced after cold stratification requires a previous warm stratification is an interesting question.

Groups 2 and 3 protein spots (Table 2), whose changes in abundance directly link to the variation of temperature, are more likely to involve in the regulation of seed dormancy and germination. The results of germination in the various treatments implied that some inhibitors of germination were probably increased or induced during warm stratification, and were decreased or removed during cold stratification. In addition, enhancers of germination probably increased during cold stratification. Proteins nos. 20, 30, and 69 were identified as prunin-related proteins. They are discussed below.

3.2 MS

A total of 71 interesting spot proteins were excised from preparative 2-DE gels. After trypsin in-gel digestion, the proteins were analyzed by ESI-Q-TOF-MS. Among the cold-changed proteins, four spots showed significant matches (Table 3). MS data including peptide masses and putative amino acid sequences for spots of interest are summarized in Supplementary Tables 1, 2. It seems important to discuss the functions of the identified proteins and related metabolic pathways involved in breaking dormancy.

3.3 The relationship of identified proteins to seed dormancy and germination

Warm plus cold stratification treatment effectively broke the dormancy of *P. campanulata* seeds, and the change in proteins following this treatment may be important factors in seed dormancy. The labeled protein spots identified by MS are listed in Table 3. Three proteins which exhibited dramatic changes after cold treatment were identified as prunin 1, prunin 2, and dehydrin. Prunins, are known as globulins of the genus *Prunus*, which comprise the main family of storage proteins synthesized in seeds during embryogenesis [20].

Table 3. Proteins of *P. campanulata* seeds[a)]

Spot number	Protein name	Identified sequence	Protein MW (kDa)/p*I*		Accession No.	Species
			Experimental	Theoretical		
1	Dehydrin	TTGAYGGAGYTGDDTR	45.0/7.5	28.70/7.05	T52421	*P. persica* [34]
20	Prunin 1 precursor	QQQGEQLMANGLEETFCSLR LKENIGNPER ADIFSPR ISTLNSHNLPILR ALPDEVLANAYQISR YNRQETIALSSSQQR QETIALSSSQQR	23.0/8.8	63.05/6.41	S51941	*P. dulcis* (almond) [20]
30	Prunin 2 precursor	VQGQLDFVSPFSR	16.0/5.9	57.02/5.53	S51942	*P. dulcis* (almond) [20]
69	Prunin 2	LLSATSPPR TDENGFTNTLAGR	14.4/8.4	57.02/5.53	CAA55010	*P. dulcis* (almond) [20]

a) Protein spots labeled with the same number in different gels indicate the same protein by analysis of Image Master 2D elite software. Except for spot nos. 1, 20, 30, and 69, other spots showed no good matches. Spot no. 20 was identified by MALDI-TOF/TOF MS; spots nos. 1, 30, and 69 were identified by ESI MS/MS.

According to the results from the 2-DE gels for both cotyledons and embryos, prunins were fragmented during cold stratification and very weakly fragmented during fresh or warm stratification. In the poplar (*Populus deltoides*), degradation of the bark storage proteins was observed only in plants with buds that either had not initiated dormancy or whose dormancy had been overcome by low-temperature or H_2CN_2 treatment [21]. The hydrolysis which occurs to bark storage proteins may be similar to that which occurs in seeds. Furthermore, in rice (*Oryza sativa*), cysteine proteinase is responsible for digestion of the seed storage proteins. The protease encoded by OsEP3A may play a role in various physiological responses and processes, including nitrogen regulation of β-glucuronidase expression [22]. The expression of the gene for REP-1 (a cysteine proteinase) is induced by GAs and repressed by ABA [23]. Hence, the fragmentation of prunin may possibly be used as an index of seed germination. In other words, degradation of prunin which occurred during cold stratification is probably related to GA induction. Previous studies showed that the GA contents of seeds were increased during chilling. For example, cold stratification induced a rise in GA in peach (*Prunus persica*) seeds [24], in *Corylus avellana* seeds [25], and in apple seeds [26]. Induction of enzymes by chilling in GA biosynthesis was found as well [27]. It will be interesting to study the effect of the degradation of prunin during the breaking of seed dormancy.

Another protein, dehydrin, abounded in cotyledons of fresh seeds. Dehydrins are considered to be stress proteins which are involved in the formation of plant protective reactions against dehydration [28] and also confer freezing protection activity [29, 30]. Dehydrins play an important role in seed desiccation during the maturation period, and so they have been pooled into an independent group of proteins [28]. This may be the reason why dehydrins are found in abundance in cotyledons. It is also known to be a positive regulator of the promoter of a barley dehydrin gene which is upregulated by ABA [31]. We found that the reduction of ABA level during warm or cold stratification coincided with the decrease of dehydrin level. Therefore, prunin digestion and dehydrin disappearance provided evidence that the germination results from a combination of complex regulatory networks are possibly associated with GAs and ABA. Although cold stratification alone may increase GAs contents and decrease the ABA level, germination was not promoted greatly. It is likely that seeds requiring a warm period before cold stratification enable immature embryos to develop further [32]. However, the direct connection between these factors and the regulation of seed dormancy still needs to be elucidated. The results of the proteomic analysis have provided important clues on which we can continue to build.

The testa of higher plant seeds protects the embryo against adverse external conditions, and is assumed to mainly control germination through dormancy imposition [19]. The protein patterns of the testa greatly differed from those of embryos and cotyledon (Fig. 3). The only protein spot which exhibited dramatic changes was identified as prunin 2. It showed that either the hydrolysis of storage proteins occurred in different tissues of the seed after cold treatment or the fragments of prunin moved to different tissues for some purposes.

4 Concluding remarks

Results of our analysis provide evidences of the involvement of prunin and dehydrin in the response to warm and chilly conditions. The study of the effect of warm and cold treatment on the proteomes of tree seeds will contribute to an understanding of the molecular basis of the response to a warm or chilly environment during the breaking of seed dormancy. This is the first reported proteomic analysis to study the dormancy breaking of woody plant seeds. Further investigations of other tree species are required to elucidate species-specific questions like dormancy as the entire genomic information of the poplar is available [33].

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