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Differential regulation of two dehydrin genes from peach (*Prunus persica*) by photoperiod, low temperature and water deficit

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Summary Dehydrins are one of several proteins that have been specifically associated with qualitative and quantitative changes in cold hardiness. Recent evidence indicates that the regulation of dehydrin genes by low nonfreezing temperature (LT) and short photoperiod (SD) can be complex and deserves more detailed analysis to better understand the role of specific dehydrin genes and proteins in the response of woody plants to environmental stress. We have identified a new peach (*Prunus persica* (L.) Batsch) dehydrin gene (*PpDhn2*) and examined the responses of this gene and a previously identified dehydrin (*PpDhn1*) to SD, LT and water deficit. *PpDhn2* was strongly induced by water deficit but not by LT or SD. It was also present in the mature embryos of peach. In contrast, *PpDhn1* was induced by water deficit and LT but not by SD. We conducted an in silico analysis of the promoters of these genes and found that the promoter region of *PpDhn1* contained two dehydration-responsive-elements (DRE)/C-repeats that are responsive to LT and several abscisic acid (ABA)-response elements (ABREs). In contrast, the promoter region of *PpDhn2* contained no LT elements but contained several ABREs and an MYCERD1 motif. Both promoter analyses were consistent with the observed expression patterns. The discrepancy between field-collected samples and growth-chamber experiments in the expression of *PpDhn1* in response to SD suggests that SD-induced expression of dehydrin genes is complex and may be the result of several interacting factors.

Keywords: cold acclimation, dormancy, drought, PCA60, stress response, water deficit.

Introduction

Cold acclimation in temperate woody plants is a complex phenomenon (Wisniewski and Arora 2000, Artlip and Wisniewski 2001, Welling et al. 2002, Wisniewski et al. 2003). Mechanisms of cold hardiness in fruit trees rely on biophysical (deep supercooling) and biochemical adaptations. Factors involved include short photoperiod (SD), low nonfreezing temperature (LT), cellular dehydration and abscisic acid (ABA) which interact to induce maximum cold hardiness (Junttila et al. 2002).

As in herbaceous plants (Guy 1990), specific genes are either up- or down-regulated during cold acclimation of woody plants (Wisniewski et al. 2003, Nanjo et al. 2004, Welling et al. 2004). A transcription factor (CBF) induced by LT has been shown to regulate the expression of several genes associated with cold acclimation in herbaceous and woody plants (Thomashow 1999, Thomashow et al. 2001, Owens et al. 2002, Chinnusamy et al. 2004, Puhakainen et al. 2004). The abundance of dehydrins is associated with extent of cold hardiness (Wisniewski and Arora 2000, Artlip and Wisniewski 2001, Wisniewski et al. 2003) and seasonal patterns of dehydrin expression appear to be a common feature in woody plants (Wisniewski et al. 1996, Sauter et al. 1999).

The specific regulation of dehydrin genes by either SD or LT can be complex (Welling et al. 1997, Rinne et al. 1998, Welling et al. 2002, Puhakainen et al. 2004, Welling et al. 2004). In birch, it appears that LT induction of some dehydrin genes is potentiated by prior exposure to SD whereas other dehydrin genes are primarily responsive to LT. Although we have demonstrated seasonal, water deficit and ABA regulation of a dehydrin gene (*PpDhn1*) in peach bark tissues, we have not determined the specific effect of LT or SD on the expression of this gene. Nor have we characterized the regulatory elements in the promoter region. This information is critical to understanding the role of dehydrins in cold acclimation in fruit trees.

In this paper, we describe a new dehydrin gene, *PpDhn2*, in peach and compare seasonal expression patterns of *PpDhn1* and *PpDhn2* and their responses to SD and LT, water deficit and prolonged exposure to an SD + LT regime. We also provide the sequence and describe the structural organization of the UTR of both genes. Lastly, we report on microsynteny between peach and *Arabidopsis* in to the organization of *PpDhn1* and *PpDhn2* and of *Xero1* and *Xero2*.

Materials and methods

Plant material

To characterize seasonal patterns of expression of two dehydrin genes (*PpDhn1* and *PpDhn2*) and compare seasonal responses with responses to controlled environmental conditions, current-year shoots from mature 'Loring' peach (*Prunus persica* (L.) Batsch) trees were collected monthly at the Appalachian Fruit Research Station (Kearneysville, WV, USA). The bark was scraped from current-year shoots, plunged into liquid nitrogen (N_2) and stored at −80 °C. To determine whether either dehydrin gene was expressed in mature embryos, mature unstratified seeds of 'Loring' peach were collected in August and the embryos dissected from the endosperm and stored for subsequent analysis in the same way as the bark tissues.

To determine the response of peach bark tissues to controlled photoperiod and temperature, one-year-old dormant trees of stem-grafted 'Canadian Harmony' peach on 'Tennessee Natural' rootstock seedlings were planted in 10-l pots in Metromix 510 (Scotts – Sierra Horticultural Products Co., Marysville, OH) which consists of horticultural vermiculite, Canadian Sphagnum peat moss, processed bark ash, composted pine bark and washed sand. For the water deficit and dormancy-induction experiments, one-year-old dormant trees of stem-grafted 'Early Loring' peach on 'Tennessee Natural' rootstock seedlings were planted in 12-l pots of Metromix 510. Sierra 17,6,10 Slow Release (5 months at 30 °C) fertilizer with 17% total N,5% available P,10% K (soluble potash) (Scotts−Sierra Horticultural Products Co.) was added to the pots at a rate of 10 g l^{-1} of potting mix. The trees were grown in a glasshouse without supplemental lighting. Temperature in the glasshouse ranged between 20 and 30 °C. Trees were watered daily and fertilized again after 5 months. Several trees were destructively harvested at the attainment of full leaf stage and bark and leaf tissues were collected to provide a time zero control for the various treatments. As an additional control, bark tissue samples were obtained from three dormant trees (over wintered and stored at 4 °C) before the trees were potted (Pre-pot). The cultivars used for these experiments are commonly planted in West Virginia and are considered comparable to one another in most characteristics including chilling requirements (Okie 1998).

Photoperiod and temperature treatments

Fourteen trees of 'Canadian Harmony' peach stem-grafted on 'Tennessee Natural' rootstock were placed in a Conviron CMP4030 growth chamber (Winnipeg, Manitoba, Canada) at either 5 °C or 25 °C with 8 h light/16 h dark cycles. Photosynthetic photon flux (PPF) was approximately 500 µmol photons m^{-2} $s^{-1.}$ Each day, 8 h into the dark period, the trees were covered with light-impermeable black plastic and the LD group exposed to 15 min of white light (4 × 150 W incandescent bulbs) beneath the plastic to reset the P_f/P_{fr} ratio and thus, simulate a long-day photoperiod (Zhu and Coleman 2001, Karlson et al. 2003). Confirming the efficacy of the day length extension treatment, trees formed buds in the SD treatment but not in the LD treatment. Trees were subjected to these photoperiods and temperatures for 5 weeks and bark and leaves were obtained from destructively harvested trees at 3 and 5 weeks (three trees at three weeks and four trees at five weeks). Time zero trees were destructively harvested immediately prior to treatment trees being placed in the chamber. Bark and leaf samples were immediately frozen in liquid N_2 and stored at −80 °C.

An additional, prolonged dormancy-induction experiment was conducted, beginning soon after the summer solstice, with ten 'Early Loring' peach trees on 'Tennessee Natural' rootstock being placed in the Conviron growth chamber at 25 °C and a 10 h light/14 h dark regime. After four weeks, five trees were harvested and the chamber temperature was then lowered to 4 °C but with the same photoperiod. After an additional four weeks, the remaining five trees were harvested. Leaves were senescent at this time and so were not collected. Bark and leaf samples were immediately frozen in liquid N_2 and stored at −80 °C.

Water deficit treatments

Fifteen 'Early Loring' peach on 'Tennessee Natural' rootstock seedlings trees were used in a water deficit experiment similar to that described by Artlip and Wisniewski (1997). Briefly, water deficit was imposed on six trees by withholding water for 5 days, by which time pot mass had fallen to 45% of the saturated mass. Pot mass was then maintained at 45% of the saturated mass for one week. The pot mass of six control trees was maintained at 100% of the saturated mass by daily watering. From earlier research (Artlip and Wisniewski 1997) we infer that the water potential of the water-stressed trees was approximately −2.0 MPa versus −0.02 MPa for the control trees. At the end of the water stress treatment three trees were destructively harvested and the three remaining water-stressed trees were watered daily for one week and then harvested. At the time of each harvest, three well-watered control trees were also harvested. At each harvest, bark tissue samples were obtained as described above. All tissue samples were immediately frozen in liquid N_2 and stored at −80 °C.

Genomic library construction and isolation of G10a

A genomic library of peach was constructed from leaf DNA as described by Artlip et al. (1997). To obtain the genomic clone G10a containing a dehydrin gene, the library was screened using a clone of *PpDhn1,* also as described by Artlip et al. (1997). G10a was sequenced in its entirety.

RNA isolation

Total RNA was extracted from bark and leaf samples as described by Artlip et al. (1997), whereas RNA was extracted from embryos with the Purescript Kit (Gentra, Minneapolis, MN).

Sequencing

Sequencing reactions were performed with the ABI BigDye Terminator kit (Applied Biosystems, Foster City, CA) and analyzed on an ABI 310 sequencer (Applied Biosystems).

RT-PCR analysis

Total RNA from the tissues (1.0 µg) was subjected to a reverse-transcriptase polymerase chain reaction (RT-PCR) as described by Bassett et al. (2002), with the PCR performed with *PpDhn1* and *PpDhn2* specific primers (Table 1). Input cDNAs were normalized to control samples within the respective experimental and tissue groups. A parallel PCR was performed with primers specific to *Prunus persica* 26S rRNA (GenBank Accession no. BF717169; Table 1) as an additional normalization control. Previous work in our laboratory indicated that the expression of this gene is relatively invariant across peach tissues and conditions. No-RT control reactions were performed with primers specific to *PpDhn2*, including a primer that anneals to the intron within the coding region (G10F3 and G10R3; Table 1). The reactions failed to display any product, an indication that contaminating genomic DNA was not detectable in the samples (data not shown). All of the cDNA and RT-PCR reactions were fractionated by agarose gel electrophoresis, stained with SYBR Gold (Invitrogen, Carlsbad, CA), scanned and quantified on a STORM 860 Gel and Blot Imaging System (Amersham, Piscataway, NJ). Quantification of amplification products across a range of cycles empirically established the nonsaturation ranges for *PpDhn1*, *PpDhn2* and 26S rRNA amplification.

Abscisic acid extraction and purification and preparation

One gram samples were extracted overnight at −20 °C with 80% methanol (supplemented with stable isotope, 3′,5′,5′, 7′,7′,7′-d6 ABA, butylhydroxytoluene and ascorbic acid). Samples were centrifuged, decanted, filtered, re-extracted and the supernatants pooled. The extract was rotary flash evaporated, chilled to 0 °C, decanted and passed through a column of insoluble polyvinylpolypyrrolidone. Extracts were adjusted to pH 3, passed through C18 columns, eluted with 80% methanol and dried. The extracts were then methylated with ethereal diazomethane and quantified by gas chromatography–mass spectrometry, correcting for losses with the internal standard. Abscisic acid was analyzed by gas chromatography (5890 Series, Hewlett Packard) equipped with a 30 m × 0.320 mm × 0.25 µm column (DB5, J&W Scientific) and a mass selective detector (5971, Hewlett Packard). The chromatographic conditions were set starting with the injector at 250 °C, the detector at 315 °C and the oven from 60 to 200 °C (5 °C min^{-1}), 200 to 300 °C (30 °C min^{-1}) and then held at 300 °C for 10 min, and finally 300 to 60 °C (50 °C min^{-1}). The ABA eluted at 29.6 min and quantification was accomplished by monitoring authentic ABA (m/z 190) and D6-ABA (m/z 194) with selective ion monitoring (100 ms dwell per ion). The limit of ABA quantification was 156 pg and the mean recovery was 45%.

Results

The genomic subclone G10a is 5.6 kb long. It consists of the promoter, coding and 3′ UTR regions of *Ppdhn1* followed by the promoter, coding and 3′ UTR regions of *Ppdhn2* (data not shown). The coding region of *PpDhn1* has been reported previously (Artlip et al. 1997; GenBank Accession nos. U34809 and U62486). The gene encoding *Ppdhn2* is 952 nucleotides long and in contrast to *PpDhn1*, contains a 342 nucleotide intron (Figure 1A; GenBank Accession no. AY465376). The intron occurs within a predicted S-tract of *Ppdhn2*, and was confirmed by cloning and sequencing an RT-PCR synthesized cDNA (data not shown). The predicted amino acid sequence of *PpDhn2* is 202 amino acids long, with a putative M_r of 21.4 kDa.

The predicted translation indicates that *PpDhn2* has a Y_2SK_3 pattern (Figures 1A and 1B) whereas the predicted amino acid sequence of *PpDhn1* has a Y_2K_9 arrangement (Figure 1B). A BLAST search (Altschul et al. 1997) indicated that *Ppdhn2* is related to the *Arabidopsis thaliana* (L.) Heynh. dehydrin *Xero1*, located on chromosome III. The introns in *PpDhn2* and *Xero1* are located at the same location (Figure 1B). Upstream of *Xero1* is another dehydrin, *Xero2*, that has a predicted K_6 configuration and some homology to *PpDhn1*. The BLAST analysis indicated that *PpDhn2* is nearly identical at the nucleotide and amino acid levels (98 and 97%, respectively) to a dehydrin from almond (*Prunus amygdalus* Batsch.) termed *Parab21* (Campalans et al. 2000; GenBank Accession no. AF172263). This similarity extends to gene copy number as well. Just as for *Parab21* in *Prunus amygdalus* (Campalans et al. 2000), the DNA blot analysis indicated that a single copy of *PpDhn2* exists in *Prunus persica* (data not shown). Like *Parab21* (Campalans et al. 2000), *PpDhn2* is also expressed in embryos (Figure 2A). Compared to *PpDhn1*, *PpDhn2* transcripts are more abundant in embryo tissues than in leaf and bark tissues.

Table 1. Primers and their sequences used in the RT-PCR experiments.

Primer Name	Gene	Sequence
Ppdhn1proF4	*PpDhn1*	5′-CATCACTTCATCCCAAACCAAAGC-3′
Ppdhn1codRup	*PpDhn1*	5′-GGTGGTGGTGTGATGAACCG-3′
Ppdhn2proF4	*PpDhn2*	5′-CCGTTTCTCATTTCAAATACATCAAATCCC-3′
Ppdhn2codRup	*PpDhn2*	5′-CCCTTTCCTGTCATAGTCGTGACC-3′
G10F3	*PpDhn2*	5′-ATGGCGAGCTATGAGAAGCAG-3′
G10R3	*PpDhn2* (intron specific)	5′-TGTGATCCGTGAGACTTGACAG-3′
26S rRNA5′ (Forward)	26S rRNA	5′-GCAGCCAAGCCTTCATAGCG-3′
26S rRNA3′ (Reverse)	26S rRNA	5′-GTGCGAATCAACGGTTCCTC-3′

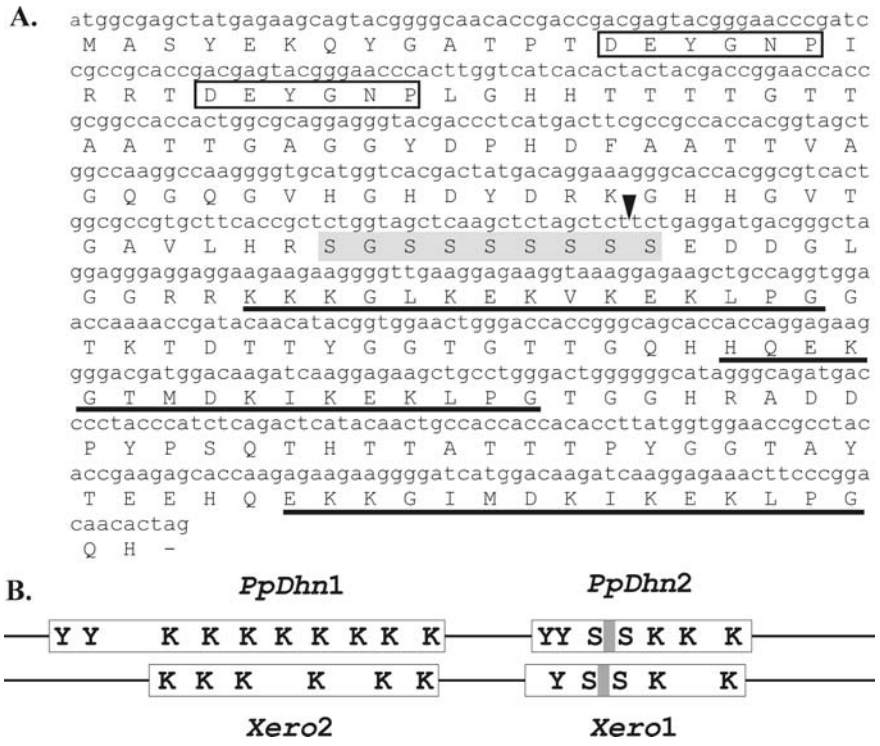

Figure 1. Sequence comparison of *PpDhn1* and *PpDhn2* and two *Arabidopsis thaliana* dehydrins. (A) Nucleotide and predicted amino acid sequence of *PpDhn2*. The Y-motifs are boxed, the S-tract is shaded in gray and the K-repeats are underlined. An arrowhead marks where an intron is present. The amino acid sequence is in one-letter code. (B) Schematic comparison of the G10a genomic subclone containing *PpDhn1* and *PpDhn2* and a portion of Chromosome 5 from *Arabidopsis thaliana*. The YSK motifs are indicated by their respective letters and the identically positioned introns of *PpDhn2* and *Xero1* are indicated by gray shaded areas.

A week of severe water deficit ($\Psi = -2.0$ MPa) resulted in the accumulation of both *PpDhn1* and *PpDhn2* transcripts in bark, although *PpDhn1* transcript was also present in prepotted plants (Figure 2B). *PpDhn2* transcripts accumulated in leaves during severe water deficit whereas *PpDhn1* transcripts did not. Transcripts from both dehydrin genes declined to background values in bark after recovery from water deficit. As expected, concentrations of ABA increased in leaves and bark in response to the water deficit (Table 2).

Seasonal patterns of transcript accumulation of both genes were examined in field-grown bark tissues. As previously reported (Artlip et al. 1997) *PpDhn1* transcript accumulation occurred on a seasonal basis, rising sharply during the autumn and winter months, declining during the spring and nearly disappearing during summer (Figure 3). In contrast, *PpDhn2* did not appear to be seasonally regulated.

PpDhn1 transcripts accumulated markedly in bark tissues and to a small degree in leaf tissues in response to low temperatures, regardless of photoperiod (Figure 4). Accumulation was evident in samples harvested at both 3 and 5 weeks. In contrast, *PpDhn2* did not appear to be consistently induced by either LT or SD in both bark or leaf tissues. The exception was at 5 weeks where at 5 °C there was a noticeable accumulation of *PpDhn2* transcript in samples exposed to LD photoperiod (Figure 4). The responsiveness of the two dehydrin genes to low temperature and photoperiod was further confirmed in samples taken from trees exposed to 4 weeks of SD at 25 °C followed by 4 weeks of SD at 5 °C (Figure 5). Four weeks of SD at 25 °C did not appear to induce *PpDhn1* in either the bark or leaves. However, after an additional 4 weeks of SD at 5 °C *PpDhn1* transcripts were clearly present in the bark (leaves were senescent and abscised from the trees by this time and did not provide usable RNA). In contrast, *PpDhn2* transcripts did not appear to accumulate under any conditions.

Analysis of the *PpDhn1* promoter was conducted using several web-based applications (e.g., Place (Higo et al. 1999) and PlantCare (Lescot et al. 2002)) available online at (http://www.arabidopsis.org/index.jsp) from The Arabidopsis Information Resource (TAIR). These analyses indicated that two dehydration-responsive-element (DRE)/C-repeats were present (Figure 6). Several ABA-responsive element (ABREs) were also present as well as two motifs (GCN4 and Skn1) associated with expression in seeds. Similar analysis of the *PpDhn2* (Figure 7) promoter for these elements failed to reveal the presence of any DRE/C-repeats; however, several ABRE or ABRE-like elements were present, as well as another water-deficit responsive motif (MYCRD22).

Discussion

Previous work established that *PpDhn1* is seasonally regulated in bark tissues, responsive to water deficit and ABA and

Table 2. Abscisic acid (ABA) concentrations in leaves and bark from control ($\Psi = -0.2$ MPa) and water-limited plants ($\Psi = -2.0$ MPa).

Treatment	ABA (pmol g_{fw}^{-1})	ABA recovery (%)
Control leaves	436	50
Stressed leaves	1945	44
Control bark	894	43
Stressed bark	1890	43

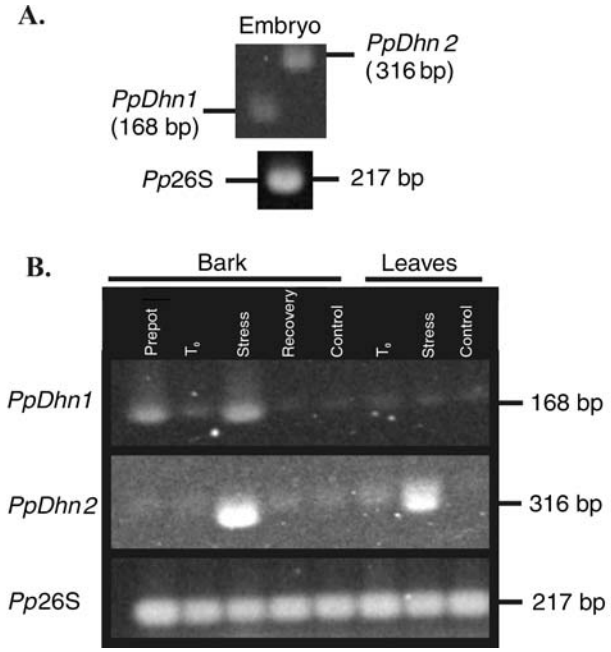

Figure 2. Analysis by reverse-transcriptase polymerase chain reaction (RT-PCR) of the expression of *Ppdhn1* and *Ppdhn2* in mature peach embryos in response to soil water deficit. (A) Embryos. (B) Water deficit. Abbreviations: Prepot = samples taken from dormant trees (overwintered and stored at 4 °C) before potting; T_0 = samples taken from trees at the onset of the experiment; Stress = samples taken from trees subjected to one week of water deficit (Ψ = –2.0 MPa); Recovery = samples taken from trees that had been re-watered for one week and had recovered from water deficit; and Control = samples taken at the end of the experiment from the control trees that had been well-watered throughout the experiment. *Pp26S* is the product of 26S ribosomal RNA subunit-specific primers, and serves as a loading control. Sizes of the PCR products are indicated on the right. Non-saturating cycles were as follows: panel A, *PpDhn1* and *PpDhn2* for 24 cycles; panel B, *PpDhn1* for 26 cycles, *PpDhn2* for 32 cycles; and *Pp26S* for 15 cycles.

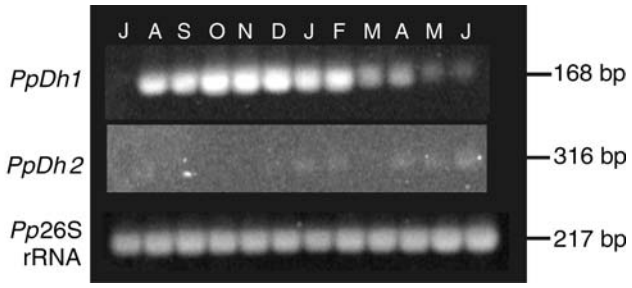

Figure 3. Analysis by reverse-transcriptase polymerase chain reaction (RT-PCR) of the expression of *Ppdhn1* and *Ppdhn2* in a seasonal collection of peach bark. Months are abbreviated as the first letter of the month, starting with July. *Pp26S* is the product of 26S ribosomal RNA subunit-specific primers and serves as a loading control. Sizes of the PCR products are indicated on the right. Non-saturating cycles were as follows: *PpDhn1* for 24 cycles, *PpDhn2* for 35 cycles; and *Pp26S* for 15 cycles.

quantitatively associated with extent of cold hardiness (Arora et al. 1992, Arora and Wisniewski 1994, Artlip and Wisniewski 1997, Artlip et al. 1997). Differential expression of this dehydrin gene (*PpDhn1*) and protein (PCA 60) in deciduous and evergreen peach, which differ in their capacity for cold acclimation and dormancy, suggested that *PpDhn1* responds to SD photoperiod and cold-acclimating temperatures (Artlip et al. 1997). However, this was not directly confirmed. The discovery of a second dehydrin, *PpDhn2*, indicates the need for a more detailed analysis of the regulation of both genes, especially as Welling et al. (2004) has recently reported the differential expression of two birch (*Betula pubescens* J. F. Ehrh.) dehydrins by photoperiod and temperature.

The predicted amino acid sequences of the products of *PpDhn1* and *PpDhn2* differed considerably as did the arrangement of their conserved motifs (Figure 1). The protein coded by *PpDhn1* exhibited a Y_2K_9 arrangement whereas the protein coded by *PpDhn2* had a Y_2SK_3 arrangement. Several studies have shown that differences in the structures of dehydrins correlate to environmental signals inducing gene expression, as well as potential biochemical properties (Close 1996, 1997, Wise 2003, Wise and Tunnacliffe 2004). The K_n-type represented by the *Xero2* (Figure 2) tends to be strongly induced by low temperatures whereas the Y_nSK_2-type represented by

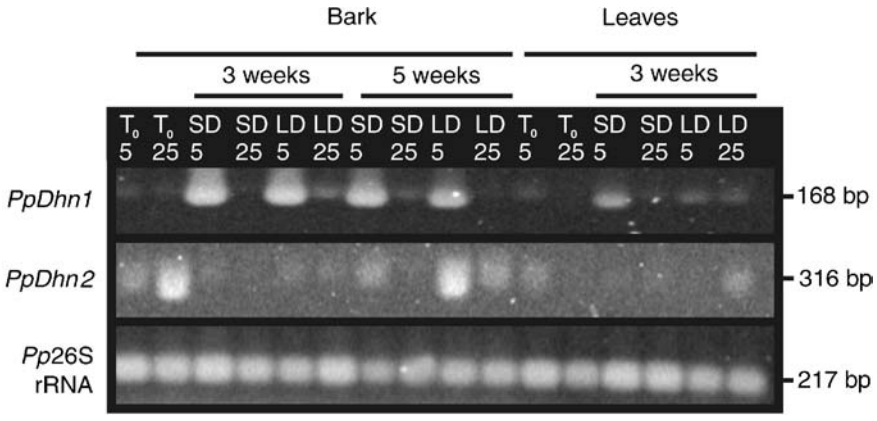

Figure 4. Analysis by reverse-transcriptase polymerase chain reaction (RT-PCR) of the expression of *Ppdhn1* and *Ppdhn2* in response to photoperiod and temperature in bark and leaf tissues. Samples were harvested after three and five weeks; leaves were senescent at five weeks and were not collected. Abbreviations: SD = short day; LD = long day; and T_0 = time zero controls. Temperatures are indicated beneath the SD, LD and T_0 descriptors. *Pp26S* is the product of 26S ribosomal RNA subunit-specific primers and serves as a loading control. Sizes of the PCR products are indicated on the right. Non-saturating cycles were as follows: *PpDhn1*, 26 cycles; *PpDhn2*, 32 cycles; and *Pp26S* for 15 cycles.

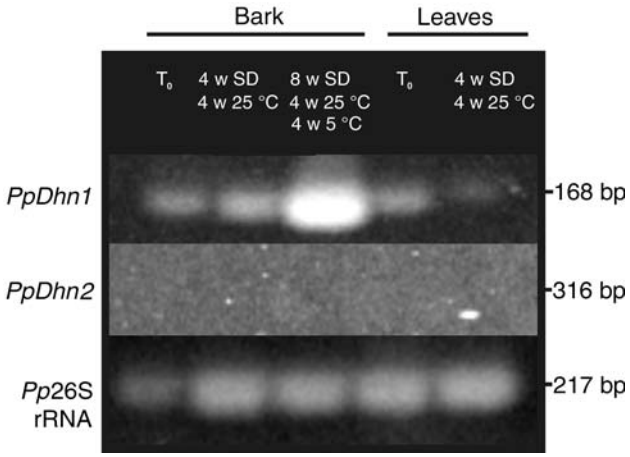

Figure 5. Analysis by RT-PCR of the expression of *Ppdhn1* and *Ppdhn2* in response to a prolonged short-day photoperiod in bark and leaf tissues. Samples were harvested after 4 and 8 weeks; leaves were senescent at 8 weeks and were not collected. Abbreviations: T_0 = time zero controls; 4 w SD = four weeks under short day photoperiod at 25 °C; and 8 w SD/4 w 4 °C = 8 weeks under short day photoperiod, with the last 4 weeks at 4 °C. *Pp26S* is the product of 26S ribosomal RNA subunit-specific primers, and serves as a loading control. Sizes of the PCR products are indicated on the right. Non-saturating cycles were as follows: *PpDhn1*, 26 cycles; *PpDhn2*, 32 cycles; and *Pp26S* for 15 cycles.

Xero1 and *PpDhn2* (Figure 1B) tends to be strongly induced by dehydration. In a more recent analysis of the family of late-embryogenesis (LEA) proteins, Wise and Tunnacliffe (2004) suggested additional possible functions beyond the original premise that dehydrins are involved in promoting the stability of membranes and proteins during dehydration.

The similar structure and arrangement of *Xero1* and *Xero2* in *Arabidopsis* and *PpDhn1* and *PpDhn2* in peach suggest some degree of synteny. This is in agreement with the findings of Georgi et al. (2003) who compared a peach BAC clone to the complete *Arabidopsis thaliana* genome and observed congruent gene order limited to 2–3 genes in sequence. Georgi et al. (2003) also reported that peach introns were nearly twice the length of *Arabidopsis* introns, a feature noted in the peach ethylene receptor gene *PpEtr1* (Bassett et al. 2002).

PpDhn2 is nearly identical to *Parab21* from *Prunus amygdalus* at the nucleotide level and at the predicted amino acid level, reflecting the close relationship between *Prunus persica* (peach) and *Prunus amygdalus* (almond) which are capable of producing fertile F_1 hybrids (Kester and Hansen 1966, Grassely and Damavandy-Kozakonane 1974). As observed for *Parab21* (Campalans et al. 2000), it is likely that *PpDhn2* migrates as a 30 kDa polypeptide in SDS-PAGE because both proteins have predicted molecular masses of about 21.4 kDa. It is also likely that almond contains an equivalent to *PpDhn1*, because an EST clone (GenBank Accession no. BQ641120) has BLASTn E-values of e–108 and 7e–99 to the genomic and mRNA sequences of *PpDhn1* (GenBank Accession nos. U62486 and U34809, respectively) and there are no other accessions with greater homology. Campalans et al. (2000) reported that *Parab21* transcript, as well as the translated dehydrin protein, accumulate in embryos during maturation. Similarly, *PpDhn2* transcript was present in mature peach embryos (Figure 2A) but we did not determine protein concentrations.

Soil water deficits as well as exogenous application of ABA are known to induce dehydrin gene expression in woody plants (Artlip et al. 1997, Rinne et al. 1998, Welling et al. 1997) and plants in general (Close 1997, Svensson et al. 2002, Wise 2003). Our finding of increased expression of *PpDhn1* in bark

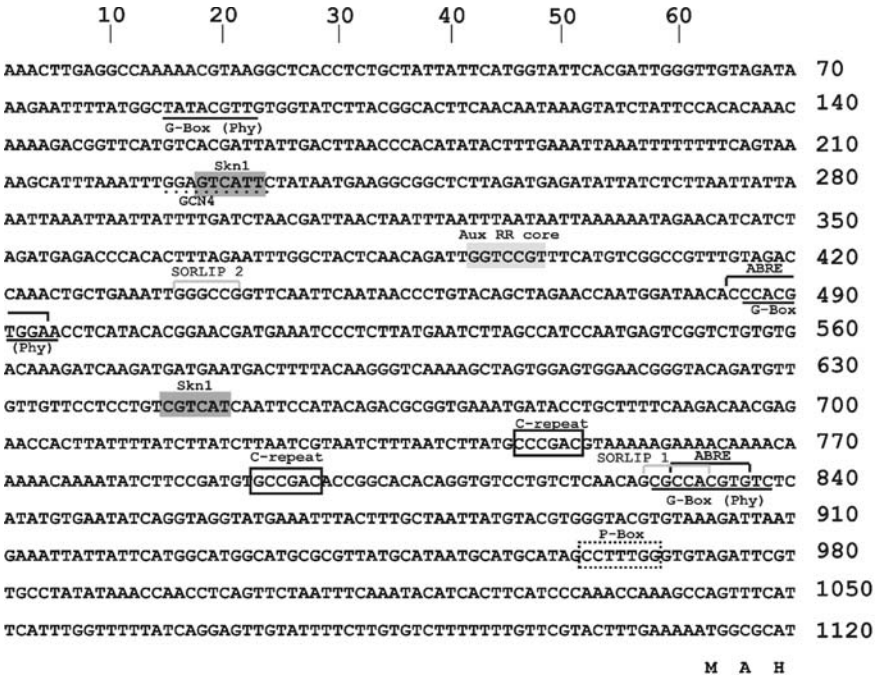

Figure 6. *PpDhn1* promoter sequence. Selected promoter elements are noted. The first few amino acids from the coding region are included. G-Box (phy) indicates elements predicted to bind transcription factors related to phytochrome, ABRE indicates putative abscisic acid response elements, C-repeat indicates the cold/dehydration (ABA-independent) DRE/C-repeat elements and SORLIP indicates sequences over-represented in light-induced promoters (PhyA related). Dark gray shaded or dotted-underlined Skn1, Prolamin and GCN4 refer to *cis*-acting elements related to seed expression whereas light gray shaded or dotted boxes refer to auxin- and GA-responsive elements, respectively. For clarity, only a few of the potential elements are noted.

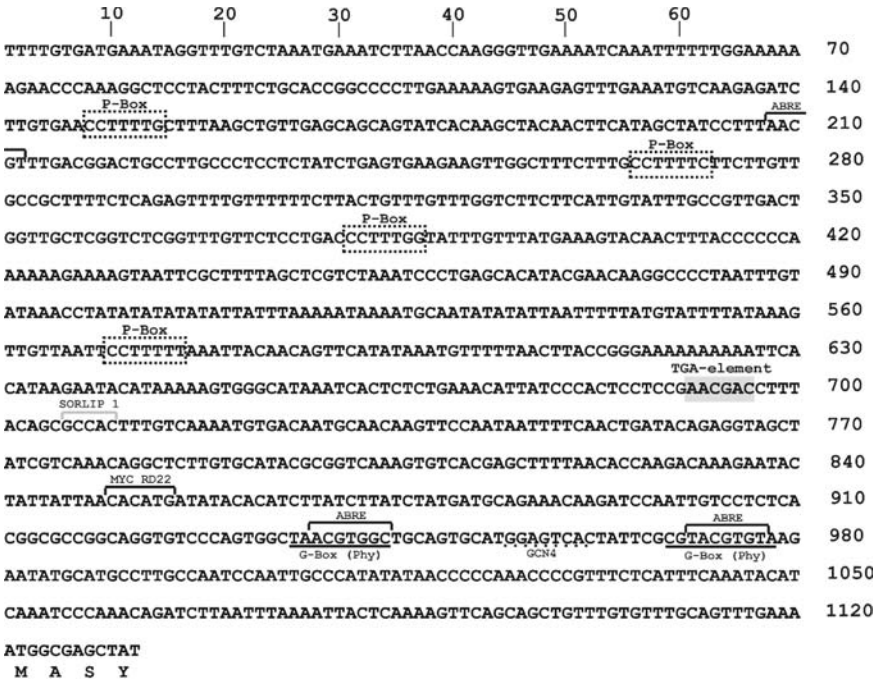

Figure 7. *PpDhn2* promoter sequence. Selected promoter elements are noted. The first few amino acids from the coding region are included. Abbreviations: G-Box (phy) indicates elements predicted to bind transcription factors related to phytochrome; ABRE indicates putative abscisic acid response elements; MYC RD22 refers to an element predicted to bind a MYC transcription factor related to dehydration; SORLIP indicates sequences over-represented in light-induced promoters (PhyA related); and GCN4 refer to a *cis*-acting element related to seed expression. Light gray shaded and dotted boxes refer to auxin- and GA-responsive elements, respectively. For clarity, only a few of the potential elements are noted.

tissues, but not in leaves, in response to water deficit confirms our earlier reports (Artlip and Wisniewski 1997, Artlip et al. 1997). Similarly, our observation that *PpDhn2* transcripts accumulated to a high degree in bark and leaves of peach in response to severe water deficit (–2.0 MIA for 1 week) and then recovered to pre-stress values (T_0) after 1 week of rehydration (Figure 2B) closely matches the expression of *Parab21* (Campalans et al. 2000). The strong regulation of *PpDhn2* by water deficit supports the premise that Y_nSK_2-type dehydrins tend to be more strongly induced by dehydration than by low temperatures (Close 1996, 1997). The presence of *PpDhn1* transcript in seedlings before they were potted for the photoperiod and temperature experiment was likely because these were dormant trees that had been stored at 4 °C before potting. The increase in ABA concentration in the drought-stressed plants compared with the control plants probably contributed to the accumulation of *PpDhn1* and *PpDhn2* transcripts in response to water deficit (Table 2). Artlip and Wisniewski (1997) showed that *PpDhn1* is responsive to exogenously applied ABA, so it is likely that *PpDhn2* is also.

Seasonal accumulations of specific transcripts and proteins have been observed in several temperate woody plant species (e.g., Arora et al. 1992, Arora and Wisniewski 1994, Wisniewski et al. 1996, Artlip et al. 1997, Sarnighausen et al. 2002, Wisniewski et al. 2003, Welling et al. 2004). The pattern shown in Figure 3 for *PpDhn1* from field-collected bark tissue matches that previously observed by Artlip and Wisniewski (1997), i.e., transcript levels increased in late summer and early autumn, reached their highest values in late autumn and early winter and then declined in spring to background values in early and midsummer. In contrast, *PpDhn2* did not display these seasonal kinetics, nor was there any discernable pattern to the limited transcript accumulation that was detected. The PCR for *PpDhn2* was run for 35 cycles compared with 24 cycles for *PpDhn1*, which further reinforces the idea that *PpDhn2* transcripts do not accumulate on a seasonal basis. This suggests that *PpDhn2* transcription is not induced by environmental cues such as photoperiod or low temperature.

To better characterize the response of *PpDhn1* and *PpDhn2* to environmental cues, trees were exposed to either SD or LD photoperiods at 25 or 5 °C for up to 5 weeks. *PpDhn1* transcripts accumulated in bark tissue in response to low temperatures but not to SD (Figure 4). In leaves, low temperature induced *PpDhn1* transcript accumulation when combined with SD, whereas LD appeared to limit the response to low temperature. In contrast, *PpDhn2* transcripts appeared to accumulate in bark tissues only after 5 weeks, and the accumulation was limited to the 5 °C + LD treatment. No significant accumulation was seen in leaf tissue under any condition. The experiment in which trees were exposed to conditions promoting dormancy and cold acclimation further confirmed that *PpDhn1* is induced by low temperature (5 °C) but not SD and that *PpDhn2* is not induced by either factor (Figure 5). Similiar differential expression of dehydrin genes in woody plants has been reported in birch (*Betula pubescens* Ehrh.) (Welling et al. 2004), where *BpuDhn1* responds to both SD and low nonfreezing temperatures whereas *BpuDhn2* is relatively SD insensitive and responds mainly to low temperature.

It is possible that the method we used to simulate LD conditions (15 min night break) was ineffective; however, this seems unlikely because the terminal bud set data indicated that trees kept under SD conditions exhibited growth cessation and set terminal buds whereas the LD plants continued to grow without setting terminal buds (data not shown). Trees kept in 5 °C

+ LD conditions ceased to grow but did not set terminal buds. Therefore, the conditions used in this study appeared to simulate the effects of temperature and daylength in the natural environmental. The prolonged dormancy + cold acclimation experiment further confirmed the validity of the results obtained when SD and LD conditions were simulated in the same chamber. Collectively, the data indicate that, unlike *PpDhn1*, *PpDhn2* is not involved in acclimation to low temperatures but plays a role in the response to water deficit and is present in the embryos of peach seeds.

The lack of induction of *PpDhn1* by SD under our experimental conditions is problematic and indicates that the regulation of SD-inducible genes may be complex. Arora and Wisniewski (1994) and Artlip et al. (1997) observed an increase in *PpDhn1* transcript and its translated protein (PCA60) in field-collected samples beginning in August at a time when mean maximum and minimum temperatures in the study area (Kearneysville, WV) are 33 and 17 °C, respectively (National Weather Service, Silver Springs, MD). This led to the hypothesis that *PpDhn1* was initially induced by SD, the effect later reinforced by low temperature. However, this interpretation is not supported by the results of our growth chamber studies (Figures 4 and 5).

Welling et al. (2004) found that birch ecotypes respond differentially to photoperiods particularly during the induction of freezing tolerance; moreover, there was a variable response of dehydrin expression in the autumn between the ecotypes and between the two dehydrins. Similarly, Artlip et al. (1997) observed differing patterns of dehydrin expression in different cultivars of peach. Although genetic diversity may account for some of the variation observed, the fact that commercial cultivars of peach have a very narrow genetic base (Scorza et al. 1985) and that prolonged SD treatments (3–5 weeks) were used in the present study do not support the premise that genetic diversity accounts for the differences between field-collected samples and those collected from the environmental chamber experiments. The dormancy induction experiment (Figure 5) was conducted with 'Early Loring' which differs from 'Loring' primarily in that it ripens 2 weeks earlier (Okie 1998) and it displayed no SD induction. This result also supports the contention that genetic diversity is not responsible for differences between seasonally collected and environmental-chamber-collected samples.

Rinne et al. (1998) compared water status, ABA concentrations and an LEA Group 2-like (dehydrin) proteins in a wild-type and an ABA-deficient genotype of birch under various combinations of photoperiod, temperature and water deficit as well as under field conditions. Three weeks of SD was sufficient to induce detectable amounts of dehydrin-like proteins in the wild-type genotypes, whereas water deficit for a similar period of time was not. They noted that an ABA peak observed in birch buds coincided with significant water loss in field-grown trees between mid-July and August which in turn increased the concentration of the dehydrin-like protein. The pattern of dehydrin-like protein accumulation observed in their study was similar to that observed for the dehydrin protein/gene (PCA60/*PpDhn1*) reported by Arora and Wisniewski (1994) and Artlip et al. (1997). Rinne et al. (1998) proposed a model in which ABA in conjunction with short days is necessary for the expression of dehydrin-like proteins. This model is consistent with reports by Karlson et al. (2003) and Marian et al. (2004) on dehydrins in red-osier dogwood (*Cornus sericea* L.) and rhododendron (*Rhododendron* cv. 'Chionides'). Both groups indicated that a reduction in water content occurred under SD conditions and that dehydrin accumulation was correlated to this reduction. Therefore, it is possible that water content of the plants was not reduced sufficiently in the SD + 25 °C treatment in our growth chamber experiments to induce the production of insufficient ABA to act synergistically with SD in promoting *PpDhn1* expression. Unfortunately, we measured neither stem Ψ nor ABA concentration in these experiments.

Low temperature induction of *PpDhn1* is consistent with the presence of DRE/ C-repeats in the promoter of *PpDhn1* (Shinozaki et al. 2003, Yamaguchi-Shinozaki and Shinozaki 2004) (Figure 6). Rinne et al. (1998) showed that other dehydrin-like proteins present in the shoot apex also behave like *PpDhn1*, in that they are readily detectable following exposure to low temperatures but are barely detectable under short day conditions. In addition, other plant growth regulators such as indole acetic acid (IAA) or GA may influence the expression of dehydrin-like genes. Li et al. (2003) examined the effect of photoperiod on growth, water content and changes in ABA and IAA concentrations in a series of latitudinal birch ecotypes and concluded that IAA may play an important role in the photoperiodic control of cold acclimation, growth and dormancy and cited other studies suggesting a similar role for GA in these same processes. In this context, it is noteworthy that several *cis*-acting elements reflecting regulation by IAA and GA are present in the upstream regions of *PpDhn1* and *PpDhn2*.

In summary, we have identified a new peach dehydrin gene (*Ppdhn2*) and examined the response of this gene and a previously identified dehydrin (*PpDhn1*) to SD, low-temperature and water deficit. We conducted an in silico analysis of the promoters of each of the genes. *PpDhn2* was strongly induced by water deficit but not by low temperature or SD. It was also present in mature embryos of peach. In contrast, *PpDhn1* was induced by water deficit (although not as strongly as *PpDhn2*) and low temperature but not SD. Analysis of the promoter region indicated that the promoter of *PpDhn1* contained two DRE/C-repeats that are responsive to low temperature and several ABREs. In contrast, *PpDhn2* contained no any low temperature elements but contained several ABREs and a MYCRD22 motif. The latter motif is associated with binding by a water-deficit-induced MYC transcription factor that is apparently involved in the induction of other dehydration-induced genes (Abe et al. 1997). Both promoter analyses are consistent with the observed expression patterns. The lack of corroboration between field-collected samples and growth chamber experiments on the expression of *PpDhn1* in response to SD is problematic and suggests that SD-induced expression of dehydrin genes is complex and may be the result of several interacting factors.

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