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Assessing Field *Prunus* Genotypes for Drought Responsive Potential by Carbon Isotope Discrimination and Promoter Analysis

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Abstract: In order to improve the effectiveness of breeding practices for *Prunus* rootstocks, it is essential to obtain new resistance resources, especially with regard to drought. In this study, a collection of field-grown *Prunus* genotypes, both wild-relative species and cultivated hybrid rootstocks, were subjected to leaf ash and carbon isotope discrimination ($\Delta^{13}C$) analyses, which are strongly correlated to water use efficiency (WUE). Almond and peach wild relative species showed the lowest $\Delta^{13}C$ ratios, and therefore, the highest WUE in comparison with hybrid genotypes. In addition, drought-related *cis*-regulatory elements (CREs) were identified in the promoter regions of the effector gene *PpDhn2*, and the transcription factor gene *DREB2B*, two genes involved in drought-response signaling pathways. The phylogenetic analysis of these regions revealed variability in the promoter region sequences of both genes. This finding provides evidence of genetic diversity between the peach- and almond-relative individuals. The results presented here can be used to select *Prunus* genotypes with the best drought resistance potential for breeding.

Keywords: almond wild-relative species; ash content; *DREB2B* transcription factor; peach wild-relative species; *PpDhn2*; rootstock; water use efficiency

1. Introduction

Drought stress is a significant challenge to agriculture, especially in arid and semi-arid climates [1] such as the Mediterranean region, where water availability is the most important factor for plant survival. In plants, water stress response is a complex combination of different factors at the biochemical, molecular and physiological levels leading to plant adaptation under drought conditions [2,3]. Late embryogenesis abundant (LEA) proteins are involved in this functional adaptation. Their accumulation plays a crucial role in protecting protein structure and binding metals under osmotic and oxidative stresses induced by drought, cold and salinity [4,5]. Dehydrins, which belong to the Group II LEA proteins, are one of the most important proteins that accumullate during water stress [6,7]. The role of dehydrins in abiotic stress tolerance has been demonstrated in different woody species [8–12]. In particular, three dehydrin genes (*Ppdhn1*, *Ppdhn2* and *Ppdhn3*) have been described in peach confirming their induction by cold and/or drought [13–15], and the presence of specific *cis*-regulatory elements (CREs) in their promoter regions is thought to contribute to their induction by several abiotic stresses [14,15]. Recently, Bielsa et al. [16] confirmed the drought-induction of two genes: a gene encoding a homologous protein to D-29 LEA protein and

the *PpDhn1* gene in three interspecific hybrids of *Prunus.* Dehydration-responsive element-binding (DREB) transcription factors (TFs), which belong to the APETALA 2/ethylene-responsive element binding factor (AP2/ERF) family [17], are important in abiotic stress responses by interaction with a specific dehydration-responsive element/C-repeat (DRE/CRT) *cis*-element (G/ACCGAC), located in the promoter regions of several stress responsive genes, including dehydrins [18–21]. DREB2B is one such gene whose expression is induced by dehydration, salinity and heat in an abscisic acid (ABA)-independent manner, thereby improving multiple stress tolerances in different plant species including model plants and crops [17,21–24].

Molecular responses to drought are reflected in physiological-adaptive mechanisms such as stomatal closure, reduction of cellular growth and photosynthesis deprivation [20]. Due to drought tolerance being a sophisticated and complicated process, phenotyping this physiological trait can be highly challenging. Several parameters have been established to assess drought tolerance, including water status [25], leaf hydraulic conductivity [26], stomata features [27] and water use efficiency (WUE) [28]. WUE is a physiological assessment extensively used in comparative studies due to WUE being tightly associated with plant drought adaptation [29–31]. WUE can be determined via comparison of different physiological assessments, namely via determining the; (i) ratio between net CO_2 assimilation rate and stomatal conductance (this is defined as intrinsic WUE) and (ii) ratio between net CO_2 assimilation and transpiration rates (this is defined as instantaneous WUE) [28]. These measurements provide information about plant responses to short-term drought conditions. Improving WUE is a key in ensuring future production in rain deficit environments. In breeding programs, it is also crucial to understand changes that are induced by exposure of the plant to long-term drought conditions. Carbon isotope discrimination ($\Delta^{13}C$) analysis is suggested as an appropriate indicator of long-term WUE at the leaf level [28,32]. The basis of this indirect method has been extensively studied [32–34] and suggests a negative correlation between WUE and $\Delta^{13}C$. Furthermore, the relationship between $\Delta^{13}C$ and ash content has been studied in cereals [35,36], apple [37] and peach [38] in order to improve phenotyping and breeding for WUE. The association among these three parameters (WUE, $\Delta^{13}C$ and leaf ash content) is based on the passive transport of minerals via the xylem and their accumulation in growing and transpiring tissues. Therefore, the rate of transpiration, the higher the rate of mineral transport is to those tissues leading to an increase in ash content [39]. The correlation of high WUE with low leaf ash content and low $\Delta^{13}C$ is well documented [37,40,41].

Rootstocks are responsible for water and nutrient uptake, resistance to soil-borne pathogens, and tolerance to environmental stresses [42]. In *Prunus*, several species such as *P. amygdalus* (L.) Batsch, *P. persica* (L.) Batsch, *P. cerasifera* Ehrh., *P. davidiana* (Carr.) Franch, *P. mira* (Koehne) Kov. et Kost., *P. domestica* L., and *P. insititia* L. are used as rootstocks. In addition, interspecific hybrids have been developed from almond × peach and peach × *P. davidiana* [43–46]. Currently, the aim of several stone fruit rootstock-breeding programs is to create more interspecific hybrids to obtain desirable and useful traits from different *Prunus* species. Wild-relative species have also been utilized both for direct rootstock development, such as, *P. bucharica* (Korsh.) Fetdsch., *P. kuramica* (Korsh.), *P. webbii* (Spach) Vieh. or *P. kotschii* (*A. kotschii* Boiss.), and to create interspecific hybrids, e.g., *P. webbii* × almond, to introgress genes encoding for their natural abiotic and biotic resistances into cultivated *Prunus* rootstocks [47,48].

The objective of this study was to identify plant lines that displayed drought resistance potential among field *Prunus* collection, including a number of peach and almond wild-relative species and cultivated rootstocks. The identification of such lines was based on (i) their phenotype by leaf ash content and $\Delta^{13}C$ analyses to estimate their long-term WUE, and; (ii) their genetic distances obtained from the promoter analysis of *PpDnh2* and *DREB2* TF, two genes involved in WUE.

2. Materials and Methods

2.1. Plant Material and Growth Conditions

A total of 48 individuals, listed in Table S1, were used in this study. The genotypes were located at the CITA (Centro de Investigación y Tecnología Aroalimentaria de Aragón) facilities in Zaragoza, Spain (41°43′26″ N, 0°48′31″ W) belonging to a rootstock and wild relatives collections, respectively. Conventional orchard practices were used in tree training and weed control. Water requirements were supplied by surface irrigation for the hybrids and their parentals, and drip irrigation for the almond wild-relative species.

2.2. Leaf Ash Content Analysis and Carbon Isotope Discrimination Analysis

WUE was estimated from two analyses: (i) leaf ash content and (ii) leaf carbon isotope discrimination ($\Delta^{13}C$). Approximately 15 leaves per tree were collected, washed with deionized water, and air dried at 60 °C for 48 hours (h). The tissue was dried further at 70 °C for 72 h, ground to a degree that would allow passage through a 40-mesh screen, and analyzed for ^{13}C content (University of California, Davis Stable Isotope Facility, Department of Plant Sciences, Davis, CA, USA). Carbon isotope discrimination ($\Delta^{13}C$) was calculated according to [49]. The carbon dioxide isotope composition in air was assumed to be −7.8 parts per thousand [50]. The same sample leaf tissue weight (0.5 g) was placed in a preheated porcelain crucible and burnt in a muffle furnace at 550 °C for 24 h to determine ash content using a thermogravimetric analyzer (Leco, Inc., St. Joseph, MO, USA, model TGA701). Correlation analysis was performed to relate leaf ash content with $\Delta^{13}C$ using IBM SPSS Statistics v21.0 (SPSS Inc./IBM Corp., Chicago, IL, USA).

2.3. DNA Isolation

Leaves were collected and stored at −20 °C. Total DNA was extracted from 50 mg of frozen leaves as described by Doyle and Doyle [51]. In brief, each sample was ground in a mortar with liquid N_2. The ground material was lysed with 700 µL of CTAB (100 mM Tris-HCl $C_4H_{11}NO_3$, 20 mM EDTA, 2% CTAB, 1.4 M NaCl, pH 8, 1% PVP-40, 0.1% $NaHSO_3$) and 0.4 µL of 2-mercaptoethanol and transferred to a 1.5 mL Eppendorf tube. Cellular lysis was further assisted via incubation at, at 65 °C for 25 min. Next, 700 µL of chloroform-isoamyl alcohol (24:1, v/v) was added. Samples were then homogenized, it was centrifuged at 5590× *g* for 15 min, at room temperature. After centrifugation, 450 µL from the upper phase were transferred to a new 1.5 mL Eppendorf tube and an equal volume (450 µL) of cold isopropanol was added and samples thoroughly mixed. The precipitated nucleic acid was recovered by centrifugation at 10,956× *g* at room temperature for 5 min, washed in 800 µL of 10 mM ammonium acetate in 76% ethanol for 45 min. After the washing step, the sample was centrifuged again at 10,956× *g* at room temperature for 5 min. Finally, the supernatant was removed and the pellet dried at room temperature. DNA was re-suspended in 100 µL of TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0) and stored at 4 °C overnight. The following day, the samples were quantified using a NanoDrop® ND-1000 UV-vis spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

2.4. PCR Amplification

In order to obtain the approximate 1000 bp upstream sequence of the translation start codon to represent the promoter region, primers were designed based on the nucleotide sequences of the *PpDhn2* (*ppa011637m.g*) and *DREB2B* (*ppa022996m.g*) according to the sequences of the assembled and annotated peach genome (*P. persica* genome v1.0; http://www.rosaceae.org/). Approximately 150 ng of genomic DNA were amplified using a Platinum Taq DNA Polymerase High Fidelity kit according to the manufacturer's instructions (Invitrogen, Life Technologies, Carlsbad, CA, USA) and the *PpDhn2*-specific primers, forward 5′-TTGAGCAGCAGTATCACAAGC-3′, and reverse: 5′-GGTGGTTCCGGTCGTAGTAG-3′; and the *DREB2B*-specific primers, forward 5′-ACGTGGGACAAAACAGGGTA-3′, and reverse: 5′-TACCAAGCCAAAGACGACTG-3′. The PCR

conditions used were 1 min at 94 °C, followed by 35 cycles of 30 s at 94 °C, 1 min at 60 °C and 2 min at 68 °C, followed by a final extension of 10 min at 72 °C. After agarose gel electrophoresis, the PCR products were purified using a DNA Clean and concentrator™-5 kit (Zymo Research, Orange, CA, USA) following the manufacturer's recommendations.

2.5. Cloning and Sequencing

The gDNA fragments of 1074 bp and 1003 pb obtained for the putative promoter regions of the *PpDhn2* and *DREB2B* genes, respectively, were subsequently cloned into the pCR™2.1-TOPO® vector (Invitrogen, Life Technologies, Carlsbad, CA, USA) following the manufacturer's instructions. The plasmid DNA of the positive transformants was isolated using GeneJET™ Plasmid Miniprep kit (Thermo Fisher Scientific, Waltham, MA, USA). After digestion with *Eco*R1 using EcoR1-HF™ RE-Mix® (New England, BioLabs Inc., Ipswich, MA, USA) for checking the quality and the integrity of the gDNA insert within the vector, positive clones were sent to Beckman Coulter Genomics (Danvers, MA, USA) and Secugen S.L. (Madrid, Spain) for sequencing using the universal M13 forward and reverse primers.

2.6. In Silico Analysis of PpDhn2 and DREB2B Promoter Regions

Chromatograms from the sequencing of the studied fragments were edited by BioEdit software version 7.2.5 [52], vector sequences were removed using VecScreen software from NCBI (http://www.ncbi.nlm.nih.gov/tools/vecscreen/). Next the resulting sequences were aligned using MUSCLE software from EMBL-EBI (http://www.ebi.ac.uk/Tools/msa/muscle/) [53] and assembled by the Contig Assembly Program CAP3 (http://mobyle.pasteur.fr/cgi-bin/portal.py?#forms::cap3) [54].

The phylogenetic trees for each promoter region of the *PpDhn2* and *DREB2B* gene were constructed to classify our individual plant lines on the basis of their respective promoter sequences. This analysis was done using MEGA 6.0 [55] with the neighbour-joining (NJ) method [56], and a bootstrap analysis was conducted using 1000 replicates [57]. The evolutionary distances were determined using the Kimura 2-parameter method [58].

Two databases of *cis*-acting regulatory elements (CREs) motifs: PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) [59] and PlantPAN 2.0 (http://plantpan2.itps.ncku.edu.tw/promoter.php) [60] were used to identify CREs involved in drought response.

3. Results

3.1. Relationship between Leaf Ash Content and $\Delta^{13}C$

Mean $\Delta^{13}C$ ratios varied among genotypes and ranged from 17.71‰ to 23.17‰ and mean ash content varied from 5.96 to 17.97% (Table 1). There was a significant ($p < 0.05$) positive relationship between $\Delta^{13}C$ and leaf ash content (Figure 1) *P. davidiana* individuals had the lowest value for both $\Delta^{13}C$ ratio and leaf ash content (Table 1 and Figure 1). The $\Delta^{13}C$ values of almond-related wild species were close to the average (20.99‰) with ratios between 19.96‰ to 20.87‰ (Table 1). Genotypes with highest $\Delta^{13}C$ ratios were 'Nemared' (23.17‰), 'Monegro' (23.11‰) and 'Mira × Pecher' (22.95‰). $\Delta^{13}C$ ratios of the individuals belonging to G × N series, except for the genotype 'GN-8', were above average (Table 1). Genotype 'GF-677' had the highest leaf ash content and the fourth highest $\Delta^{13}C$ value (Table 1). Variability of $\Delta^{13}C$ values was low with an overall standard deviation value of 1.31 and coefficient of variation (CV%) of 6.40, while the overall standard deviation of ash content values was 3.10 with a CV% of 34.99 (Table 1).

Table 1. Carbon isotope discrimination [$\Delta^{13}C$ (‰)] and leaf ash content (%) of 21 *Prunus* genotypes. (SD: Standard Deviation; nd: no data; CV: Coefficient of variation).

Genotypes	$\Delta^{13}C$ (‰)	Ash (%)
P. davidiana T1	17.798	6.190
P. davidiana T2	18.157	5.850
P. davidiana T3	17.186	5.830
P. mira T1	19.601	8.070
P. mira T2	18.944	7.210
P. vavilovi T1	20.011	7.180
P. vavilovi T2	20.785	6.790
P. vavilovi T3	18.712	7.170
P. vavilovi T4	20.349	6.830
P. webbii F17 T1	19.202	11.760
P. webbii F17 T2	19.785	12.840
P. webbii F17 T3	20.662	12.680
P. webbii F3 T1	20.321	12.950
P. webbii F3 T2	20.970	8.000
P. gorki T1	20.484	6.210
P. gorki T2	21.139	8.240
P. gorki T3	21.077	8.050
P. gorki T4	18.441	8.260
P. zabulica F1 T1	21.056	5.750
P. zabulica F1 T2	21.241	6.030
P. zabulica F18 T1	20.383	7.630
P. zabulica F18 T3	19.104	7.850
P. zabulica F18 T4	20.257	8.120
P. bucharica F2 T1	19.053	9.060
P. bucharica F7 T1	19.710	7.990
P. bucharica F7 T2	21.200	8.370
P. bucharica F7 T3	20.286	8.030
P. bucharica F7 T4	22.344	6.930
'GN-8'	20.548	9.890
P. persica var. *nucipersica*	20.627	11.570
P. orientalis T1	20.926	7.810
P. orientalis T2	19.714	8.450
P. orientalis T3	21.789	8.990
P. orientalis T4	20.216	7.350
P. kotschii T1	20.112	7.010
P. kotschii T2	21.596	6.690
P. kotschii T3	20.554	6.520
P. kotschii T4	21.210	6.030
Cadaman'	20.880	9.660
Garfi' almond	20.994	7.380
'GN-10'	21.154	11.540
Barrier'	21.292	10.860
'Garnem'	21.979	14.470
'Felinem'	22.146	14.450
'GF-677'	22.235	17.970
'Nemared' peach	23.169	14.600
'Mira × Pecher'	22.948	17.600
'Monegro'	23.105	13.240
Mean	20.530	9.124
Standard deviation	1.314	3.102
CV (%)	6.401	33.998

Comparing both $\Delta^{13}C$ and ash content values, *P. davidiana* individuals had the lowest values (Figure 1), indicating higher WUE than the other genotypes. Conversely, 'GF-677' and 'Mira × Pecher' hybrids had the highest values for ash content and $\Delta^{13}C$, indicating the lowest WUE (Table 1 and Figure 1). The almond wild-relative species had similar low ash content values to *P. davidiana* except

for *P. webbii* individuals F3 and F17, and low $\Delta^{13}C$ values compared to the peach and the peach hybrid values. Overall, these peach relatives had higher $\Delta^{13}C$ and ash content values than almond wild-relative species. Among the G × N series, 'GN-8' and 'GN-10' had lower ash and $\Delta^{13}C$ values than 'Felinem', 'Garnem', 'Monegro' and 'Nemared' (Table 1).

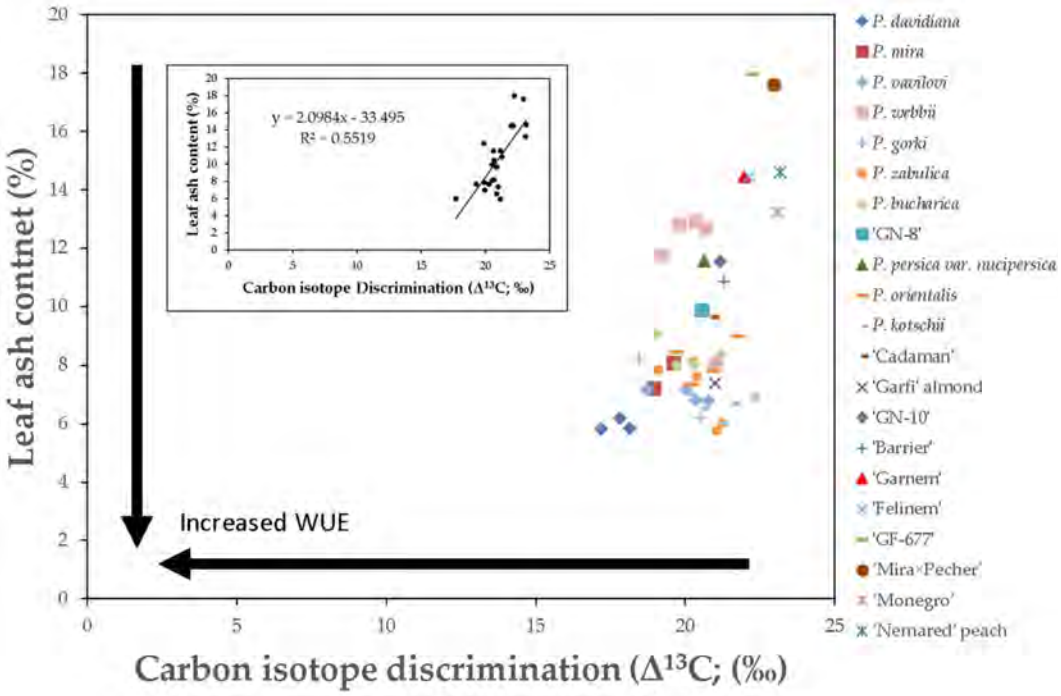

Figure 1. Relationship between carbon isotope discrimination [$\Delta^{13}C$ (‰)] and leaf ash content (%) for *Prunus* genotypes. Arrows indicate the negative relation between these two parameters and WUE.

3.2. Phylogenetic Analysis Based on Promoter Regions of PpDhn2 and DREB2B Genes

The 5′ regulatory region of the *PpDhn2* gene amplified from the 47 genotypes and species assessed were classified into six clusters (Figure 2) based on the dendrogram tree obtained by the NJ method. Cluster I contained 28 individuals, including all the hybrids and their parentals, except one individual belonging to *P. mira* genotype, which was grouped in cluster II, as well as 12 individuals belonging to 6 different wild-relative species (Figure 2). Cluster II included one *P. mira* individual, *P. mira* T1, as above-mentioned, and another six plant lines from four different wild-relative species (Figure 2). Clusters III, IV and V were the only clusters containing just one individual from 2 wild-relative species, *P. gorki* for Cluster III and the *P. webbii* F3T2 and F3T1 lines for clusters IV and V, respectively. (Figure 2). Finally, cluster VI was formed by nine wild-relative almond species (Figure 2). These results revealed the diversity in the promoter region of the *PpDhn2* gene.

The promoter region of the *DREB2B* TF encoding gene from 48 plant lines was grouped in four clusters (Figure 3). The largest cluster, cluster I, contained 29 individuals including the 'Garfi' almond and individuals belonging to the almond wild-relative species *P. vavilovi*, *P gorki*, *P. webbii*, *P. bucharica*, *P. orientalis*, *P. zabulica*, and *P. kotschii*, as well as the 'GF-667' hybrid (Figure 3). Cluster II contained four wild-relative almond individuals belonging to *P. zabulica* and *P. kotschii* species (Figure 3). The smallest group was cluster III and was formed by the single genotype *P. mira* T2 (Figure 3). All hybrid individuals and most of the parentals were found in cluster IV (Figure 3). This dendrogram showed evolutionary distances close to zero, indicated a high level of conservation in the 5′ regulatory region of the *DREB2B* gene for each individual analyzed.

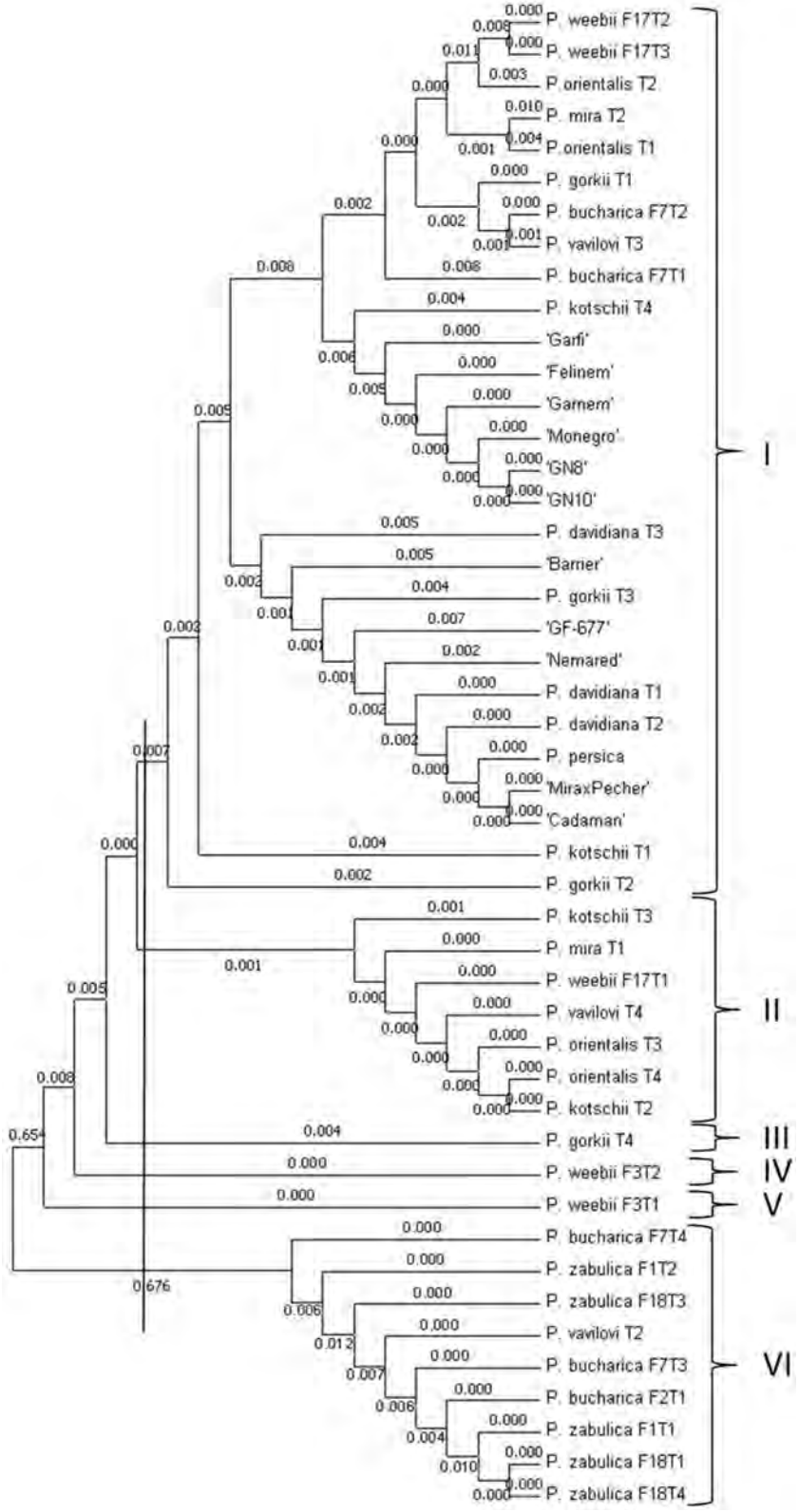

Figure 2. Dendrogram representing the phylogenetic differences in *PpDhn2* promoter gene regions. The tree was constructed using the neighbor-joining method with 1000 bootstrap replicates.

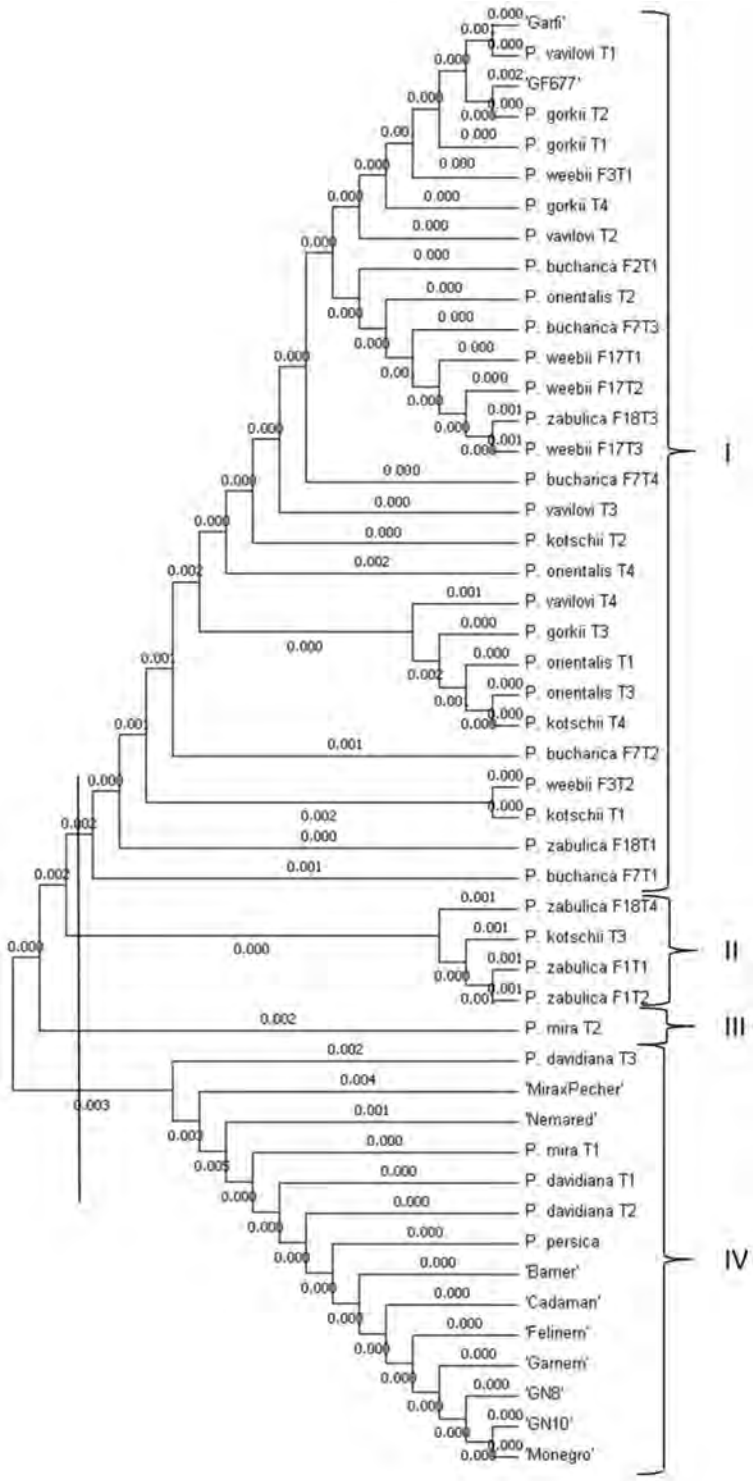

Figure 3. Dendrogram representing the phylogenetic differences in *DREB2B* promoter gene regions. The tree was constructed using the neighbor-joining method with 1000 bootstrap replicates.

3.3. Drought-Related cis-Regulatory Elements Found in PpDhn2 and DREB2 Promoters

In order to construct a more detailed understanding of the expression regulation of the *PpDhn2* and *DREB2B* genes in response to drought, we next searched for CREs in the putative promoter regions of each analyzed plant line. Based on the dendrograms resulting from the phylogenetic analysis, the nucleic acid sequences of selected individuals from each cluster were aligned. Individuals of each

group of the alignment were selected again depending on the nucleic acid differences found in the alignment analysis. Finally, CREs were found not only responsive to drought stress, but also to other processes and stresses such as light, development, hormone, biotic and abiotic stress responses in both promoter regions

For the *PpDhn2* gene, we analyzed the promoter regions of *P. mira* T2 and *P. webbii* F17T2 from cluster I, *P. gorki* T4 from cluster III, and *P. zabulica* F1T2 from cluster VI as representatives of each cluster. For CREs analysis, clusters II, IV and V were represented by *P. gorki* T4 from cluster III because the promoter regions of all these grouped plant lines harbored the same complement of CREs in their respective promoter regions. Different families of CREs associated with drought stress and ABA signaling response were predicted in both sense and antisense positions. Four CRE classes were found in all genotypes: different ABA- and dehydration-responsive elements; several (basic leucine zipper) bZIP TFs also related to ABA signaling; an element regulated by calcium signals; several myeloblastosis (MYB) motifs, as well as a myelocytomatosis (MYC) and the SEF4 TF (Figure 4a, and Table 2 and Table S2). Among the CREs, EBOXBNNAPA was the most abundant element with a repetition range of 18 to 4 in the promoter region of each genotype, followed by ACGTATERD1 with a range of 8 to 6 repetitions (Table S2). Clear differences between individuals from cluster VI and individuals from the rest of the clusters (I, II, III, IV and V) were identified (Figure 4a and Table S2). Three different CREs families were only represented in the promoter region of genotypes from cluster VI: a heat shock promoter element (HSE); a low-temperature-responsive element (LTRE-1); and three MYB elements (Figure 4a, and Table 2 and Table S2). Six CREs were found in individuals from clusters I, II, III, IV and V, but not in individuals from cluster VI: the ABA-responsive element (ABRE) motif ABREDISTBBNNAPA; a T-box ACGTTBOX; the dehydration-responsive (DRE) element DRE1COREZMRAB17; the MYC elements MYCATERD1 and MYCATRD22, and the MYC recognition site G-box. Furthermore, individuals from clusters I, II, III, IV and V contained a GT3 box in their promoters (Figure 4a, and Table 2 and Table S2). The ethylene-responsive element (ERE), ERELEE4, was only identified in cluster I, but not in clusters II, III, IV and V (Figure 4a, and Table 2 and Table S2). Finally, five CREs were found only in individuals from clusters II, III, IV and V, but not in clusters I and VI: four different dehydration-responsive (DRE) elements CBFHV, DRE, DRE1COREZMRAB17 and DRECRTCOREAT; and a LTRE element LTRECOREATCOR15 (Figure 4a, and Table 2 and Table S2).

The study of the *DREB2B* TF gene promoter region was conducted in 'Garfi', 'GF-677', *P. orientalis* T4, *P. vavilovi* T4, *P. bucharica* F7T2, *P. kotschii* T1 and *P. bucharica* F7T1 from cluster I; *P. kotschii* T3 from cluster II; *P. mira* T2 from cluster III; and *P. davidiana* T3, 'Mira × Pecher', *P. persica* and 'Garnem' from cluster IV. CREs were located in both the sense and antisense orientation, presenting a more conserved sequence than the *PpDhn2* gene promoter region. We identified in all individuals several ABA-, and dehydration-responsive elements also identified in the *PpDhn2* promoter region however, we also identified an additional ABRE-element, namely the ABARE-element HEXMOTIFTAH3H4. Other CRE families were also identified in this analysis, including ERELEE4 motif; HSE element; the motif LTRE1HVBLT49; several MYB elements; the calmodulin-binding motif CAMTA3; SR1, and the MYC element EBOXBNNAPA (Figure 4b, and Table 2 and Table S3). The motif most frequently identified was the ACGTATERD1 being identified on 8 occasions. Interestingly, the *cis*-element MYB2CONSENSUSAT was only identified among cluster I members, but not in individuals belonging to clusters II, III, IV and V. (Figure 4b and Table S3). The bZIP TF DPBFCOREDCDC3 element was found in clusters I, II, III and IV, but not in cluster V (Figure 4b and Table S3). The motif SEF3MOTIFGM was presented in individuals from cluster II, III and IV (Figure 4b, and Table 2 and Table S3). Finally, the SEF4 element was only found in clusters I and V and interestingly, the position of this TF binding site was identified at different positions within the promoter regions of members of each cluster (Figure 4b, and Table 2 and Table S3).

Table 2. Description of drought-related cis-regulatory elements (CREs) revealed in the promoter region of *PpDhn2* and *DREB2B*.

Family	Element Name	Element Sequence (5→3′)	Description
		***PpDhn2* promoter sequence**	
bZIP	ABRELATERD1	AACGT	Abscisic acid (ABA)-responsive element
(Motif sequence only)	ACGTATERD1	ACGT	ABA-responsive element
(Motif sequence only)	ABRERATCAL	MACGYGB	ABA-responsive element
(Motif sequence only)	ACGTABREMOTIFA2OSEM	ACGTGKC	ABA-responsive element
bZIP	ASF1MOTIFCAMV	TGACG	ASF-1 binding site related to ABA signaling
bZIP	DPBFCOREDCDC3	ACACNNG	Basic leucine zipper (bZIP) encoded by ABI5
(Motif sequence only)	SEF4MOTIFGM7S	RTTTTTR	SEF4 binding site; ABA-responsive element
(Motif sequence only)	ABREDISTBBNNAPA	GCCACTTGTC	dist B (distal portion of B-box) shown similarity to ABRE/dist B ABRE mediated transactivation by ABI3 and ABI3-dependent response to ABA
(Motif sequence only)	ACGTTBOX	AACGTT	T-box according to the nomenclature of ACGT elements
CG-1; CAMTA	CAMTA3; SR1	[ACG]CGCG[GTC]	Calmodulin-binding transcription activator 3
(Motif sequence only)	CBFHV	RYCGAC	Binding site of CBF1
(Motif sequence only)	DRE1COREZMRAB17	ACCGAGA	DRE1 core
AP2; ERF	Dehydration-responsive element (DRE)	[AG]CCGAC	Mediates cold or dehydration-inducible transcription
(Motif sequence only)	DRE2COREZMRAB17	ACCGAC	DRE2 core
(Motif sequence only)	DRECRTCOREAT	RCCGAC	Core motif of DRE/CRT *cis*-acting element
Dehydrin	LTRECOREATCOR15	CCGAC	Core of low temperature responsive element (LTRE)
(Motif sequence only)	ERELEE4	AWTTCAAA	Ethylene responsive element
HSF	Heat shock promoter element (HSE)	AGAAnnTTCT	Heat shock element
(Motif sequence only)	LTRE1HVBLT49	CCGAAA	Low-temperature-responsive element (LTRE-1)
(Motif sequence only)	MYB2CONSENSUSAT	YAACKG	Myeloblastosis (MYB) recognition site
Myb	MYBCORE	CNGTTR	*MYB2* TF
(Motif sequence only)	MYBCOREATCYCB1	AACGG	MYB recognition site
(Motif sequence only)	MYBST1	GGATA	MYB recognition site
(Motif sequence only)	MYB1AT	WAACCA	MYB recognition site
Myb/SANT; MYB	MYBGAHV	TAACAAA	Myb-like DNA-binding domain

Table 2. *Cont.*

Family	Element Name	Element Sequence (5→3′)	Description
		***PpDhn2* promoter sequence**	
(Motif sequence only)	MYBPLANT	MACCWAMC	MYB binding site
bHLH	EBOXBNNAPA	CANNTG	Myelocytomatosis (MYC) recognition site
NAC; NAM	MYCATERD1	RCCGAC	MYC recognition sequence
bHLH	MYCATRD22	CACATG	Binding site for MYC
bHLH	G-box	CACNTG	*MYC2* gene
Trihelix	GT3 box	GGTAAA	Negative regulator of water use efficiency
		***DREB2B* promoter sequence**	
bZIP	(ABARE) HEXMOTIFTAH3H4	ACGTCA	Abscisic acid response element (ABARE)
bZIP	ABRELATERD1	AACGT	ABA-responsive element
LEA_5	ABREMOTIFAOSOSEM /LEA5	TACGTGTC	Motif A ABRE-like sequence
(Motif sequence only)	ABRERATCAL	MACGYGB	ABA-responsive element
(Motif sequence only)	ACGTABREMOTIFA2OSEM	ACGTGKC	ABA-responsive element
(Motif sequence only)	ACGTATERD1	ACGT	ABA-responsive element
bZIP	ASF1MOTIFCAMV	TGACG	ASF-1 binding site related to ABA signaling
(Others)	DPBFCOREDCDC3	ACACNNG	Novel bZIP encoded by ABI5
CG-1; CAMTA	CAMTA3; SR1	[ACG]CGCG[GTC]	Calmodulin-binding transcription activator 3
(Motif sequence only)	ERELEE4	AWTTCAAA	Ethylene responsive element
HSF	Heat shock promoter element (HSE)	AGAAnnTTCT	Heat shock element
(Motif sequence only)	LTRE1HVBLT49	CCGAAA	Low-temperature-responsive element (LTRE-1)
(Motif sequence only)	MYB1AT	WAACCA	MYB recognition site
Myb	MYBCORE	CNGTTR	*MYB2* TF
(Motif sequence only)	MYBCOREATCYCB1	AACGG	Myb core
Myb/SANT; MYB	MYBGAHV	TAACAAA	Myb-like DNA-binding domain
(Motif sequence only)	MYBPLANT	MACCWAMC	MYB binding site
(Motif sequence only)	MYBST1	GGATA	MYB recognition site
bHLH	EBOXBNNAPA	CANNTG	MYC recognition site
(Motif sequence only)	MYB2CONSENSUSAT	YAACKG	MYB recognition site
(Motif sequence only)	SEF3MOTIFGM	AACCCA	SEF3 binding site
(Motif sequence only)	SEF4MOTIFGM7S	RTTTTTR	SEF4 binding site

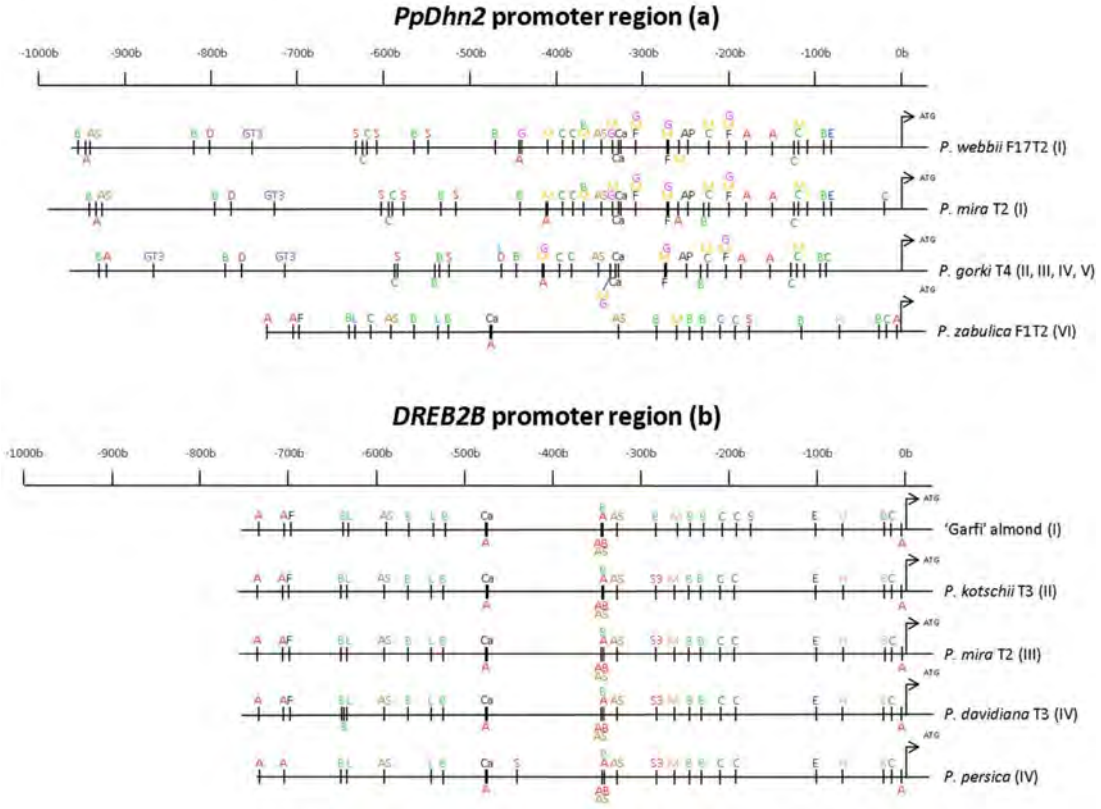

Figure 4. Schematic representation of the 1000 pb region upstream of the *PpDhn2* (**a**) and *DREB2B* (**b**) promoters in each of the cluster-representing individuals. Promoter regions were defined as the first 1000 pb 5′ of the translation start site. A: Abscisic acid responsive (ABRE)-elements, in red; AB: ABARE-element, in red; AP: APETALA 2/ethylene-responsive element binding factor (AP2/ERF), in black; AS: ASF-1 binding site, in brown; B: myeloblastosis (MYB)-elements, in light green; C: CAAT-box, in dark green; Ca: Calmodulin-binding motif, in black; D: dehydration-responsive (DRE)-elements, in pink; E: ethylene-responsive element (ERE), in dark blue; F: basic leucine zipper (bZIP) TF, in black; G: G-box, in pink; H: heat shock promoter element (HSE), in salmon; L: low-temperature-responsive element (LTRE), in blue; M: myelocytomatosis (MYC)-elements, in orange; S: SEF4 TF, in red; S3: SEF3 TF, in red; T: TATA-box, in yellow; GT3: GT3-box, in purple.

4. Discussion

Drought tolerance must be one of the primary criteria when selecting a rootstock that we be cultivated in areas where water availability is limited. The drought response in plants is controlled by complex prototypical and physiological components. The relationship between genotype and phenotype is crucial in order to understand this response for enhancing the expression of desired traits related to drought tolerance, such as WUE. Therefore, increasing WUE in rootstocks is important to ensure future economical fruit tree production in less water-friendly environments [61]. Here, leaf ash content and carbon isotopic composition were carried out to estimate the long-term WUE [37,62] in peach and almond wild-relative species, in a number of interspecific *Prunus* hybrids and their parental genotypes. Furthermore, in the same plant material, a molecular genetics approach was used to assess the promoter region landscapes of two drought-responsive genes involved in key responsive pathways, the effector gene, *PpDhn2* and the gene encoding the *DRE2B* TF was performed. Our data revealed the genotypes with the highest WUE (Figure 1); as well as documenting the variability between the *PpDhn2* and *DREB2B* genes for each assessed plant line. (Figures 2–4).

Leaf ash content and $\Delta^{13}C$ were returned a positive correlation, and further; the ratios were similar to ratios obtained in previous reports in apple [37] and peach [38]. Also, it is known that ash

content and $\Delta^{13}C$ can be used to evaluate long-term WUE in fruit trees [37]. Based on these criteria, all *P. davidiana*, *P. mira* and the almond wild-relative species presented higher WUE than the hybrid genotypes and their parentals. This improved WUE could be due to the natural adaptation of these species to severe conditions, which represent a different growing strategy than the hybrid rootstocks studied. These wild-relative species originate from the arid steppes, deserts, and mountainous areas [63–66] in which the lack of water is a common factor. In these species stomata closure, a proven adaptation to water restrictive environments, results in decreased $\Delta^{13}C$, and therefore; $\Delta^{13}C$ would be a reliable phenotypic measure of long-term drought survival [30,40,67]. Both leaf ash content and $\Delta^{13}C$ appear to be suitable phenotypic parameters for assessing drought stress in *Prunus*. Further, Brendel et al. [61] were able to identify different quantitative trait loci (QTLs) for WUE as estimated by leaf $\Delta^{13}C$ in *Quercus robur* L.

Promoter analysis of the *PpDhn2* and *DREB2B* genes revealed the presence of CREs associated with ABA- and dehydration-response. We found that all individuals shared ABREs in both gene promoter regions, although the number of ABREs varied depending on the genotype and the gene promoter sequence. ABRE is the most abundant CRE in ABA-responsive gene expression, and at least two copies of an ABRE are necessary for ABA-responsive induction of transcription [3]. Different MYB motifs and a MYC element were also distributed throughout the promoter regions of both genes in each assessed genotype. Both MYB and MYC TF binding sites have been associated with drought responsiveness and are fundamental to ABA- and drought-responsive expression [68–70]. Further, specific CREs for *PpDhn2* gene from each cluster were identified. This finding indicates additional expression regulation opportunities for the *PpDhn2* locus compared to the *DREB2B* gene among the plant lines analyzed, which indicate more diversity of that promoter region compared to DREB2B promoter along the studied genotypes. The nine almond individuals from this cluster, that belong to *P. bucharica*, *P. zabulica* and *P. vavilovi* also had in common a HSE, a LTRE, as well as three MYB motifs that were more abundant elements than in the other individuals. However, we identified in the promoters of individuals from clusters I, II, III, IV and V an ABRE motif, one DRE element, three MYC recognition sites, and a GT3 box, the CRE to which the negative regulator of WUE Trihelix TF binds to, to regulate the expression of the *SDD1* locus [71]. Other specific CREs were found in the almond wild-relative species and in a *P. mira* T1 belonging to clusters II, III, IV and V. Their *PpDnh2* promoter regions harboured 3 DREs, one C-repeat binding factor and one LTRE. In previous reports, the promoters of the *PpDhn2* gene in peach was studied and founding ABRE and MYC CREs, but not MYB binding sites, nor DRE/CRT or LTRE elements on the sense strand [14,15]. In our work, the DRE motifs and LTRE were located in the negative strand, but not in the sense strand. The influence of this *cis*-element and its orientation in gene promoter regions remains an area of debate with both dependent and independent orientation motifs [72,73]. Recent research did not find evidence of the influence of motif orientation in regulatory gene expression in a number of *cis*-elements studied in *A. thaliana* [74]. Similar to our findings, Bassett et al. [14] and Wisniewski et al. [15] observed that no DRE elements were found in positive sense in the *PpDhn2* promoter and suggested that the absence of this *cis*-element was related to the lack of expression in response to cold. However, other reports have confirmed the presence of one DRE/CRT element in the promoter region of the Y_nSK_n dehydrin class, which includes *PpDhn2* [75]. In spite of this observation, it is known that Y_nSK_n dehydrins are not expressed in response to cold. García-Bañuelos et al. [76] concluded that *MdDhn*, which shows great similarity with *PpDhn2*, was accumulated after a period of acclimation in apple trees. Based on that, Zolotarov and Strömvik [75] affirmed that cold-induced expression of Y_nSK_n-type dehydrins would not be detected in some cases because of a limited time of exposure to low temperature. So that, the presence of the DRE and LTRE elements found in the anti-sense position in our individuals could have some effect in the expression of *PpDhn2* in a possible response to cold.

All species shared essentially the same CREs in their *DREB2B* promoter region, evidencing a lower variability among the promoter sequences of every studied genotype. Beside the elements described before, we identified in sense orientation a HSE element, which binds to heat shock factors responsible

for heat stress tolerance [77]. Moreover, although several reports demonstrated that *DREB2B* is not induced by low temperatures [1,17,67], a LTRE element, an important motif for the induction of cold regulated genes [78], was located upstream of the transcription start codon. The presence of ABREs motifs in the promoter region of *DREB2B* denoted the implication of this TF in ABA-dependent signal transduction pathway [79]. In the literature, the relation between dehydrin expression and an increase of WUE in cereals has been demonstrated. Sivamani et al. [80] confirmed an improvement of biomass and WUE in transgenic barley plants expressing *HVA1* gene under drought conditions. Furthermore, Melišová et al. [33] suggested that elevated expression of the *HvDhn4* gene, which is also a Y_nSK_n-type dehydrin and similar to *PpDhn2*, was associated with the high WUE observed in a drought-tolerant variety of barley at 12 h after ABA treatment. Moreover, *DREB* TFs improved tolerance to abiotic stress in transgenic plants by regulation of genes involved in abiotic stress responses, so *DREB* TFs could increase WUE under water deficit conditions [81]. Furthermore, tobacco transgenic lines with overexpression of *SbDREB2A*, homolog to DREB2B, showed higher WUE and also, a higher expression of different dehydrins including *ERD10B*, *ERD10D* and *LEA5*, conferring drought tolerance [82].

The promoter regions of both genes also contained multiple *cis*-elements related to other plant responses. For example, SORLIP or I-box motifs which are usually upstream elements are regulated by light and the circadian clock; other elements are associated with to development responses, including the O_2-site involved in zein metabolism regulation and a CAT-box linked to meristem expression. Some motifs are related to hormone responses including an ARR1AT motif (cytokinin response regulator), several CGTCA-motifs involved in methyljasmonate-responsiveness, and the GARE-motif associated with gibberellin-responsiveness, as well as others linked to additional stress. The presence of these CREs could reflect the role of *DREB2B* and *PpDhn2* in other processes in addition to cold and drought [79,83–85].

Based on our data, the presence of DRE elements in *PpDhn2* promoter belonging to the genotypes from clusters I, II, III, IV and V, suggest that *PpDhn2*, in addition to the ABA-dependent pathway, is also induced in an ABA-independent manner by the binding of DREB2B TF to these *DRE* elements under drought conditions [18–21]. From our promoter analysis of the *PpDhn2* gene, we are unable to find a definitive association between *PpDhn2* and different WUE and Ash measurements in a variety of *Prunus* genotypes.

In conclusion, our phylogenetic classification of the *Prunus* collection based on the CREs identified in the promoters of both genes showed a clear distinction between peach relatives and almond relatives. Nevertheless, in both *PpDhn2* and *DREB2B* phylogenetic trees, it was demonstrated that *P. davidiana* (the highest WUE), a peach wild-relative specie [86], was closer to the other parental plant lines and their hybrids (lowest WUE). Our results show that phenotyping data is useful as an early selection criteria and that there are relatively few differences in promoter regions of the genes examined here, which suggests that improving drought survival could be accomplished by introgressing one or more of these genes/promoters into standard *Prunus* rootstock germplasm. According to our results, almond wild-relative species would be the genotypes with the best drought resistance potential for incorporation into future breeding programs aimed at generating new cultivars with drought tolerance potential.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4395/8/4/42/s1, Table S1: List of the 48 individuals used in this study, Table S2: *Cis*-regulatory elements of *PpDhn2* promoter gene region in each of the cluster-representative individual. Cells in grey color, CREs outside of the first 1000 bp 5′ of the translation start site. In red color, CREs in negative strand. In black color, CRES in positive strand, Table S3: *Cis*-regulatory elements of *DREB2B* promoter gene region in each of the cluster-representative individual. Cells in grey color, CREs outside of the first 1000 bp 5′ of the translation start site. In red color, CREs in negative strand. In black color, CRES in positive strand.

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