Dehydrins in maritime pine (*Pinus pinaster*) and their expression related to drought stress response

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Abstract Maritime pine (*Pinus pinaster*) is an important commercial species throughout its Atlantic distribution. With the anticipated increase in desiccation of its habitat as a result of climate change, the selection of genotypes with increased survival and growth capability under these conditions for breeding programs is of great interest for this species. We aimed to study the response to a realistic drought stress under controlled conditions, looked for a method to measure dehydration resistance, and analyzed dehydrin expression in drought-resistant and drought-sensitive clones from different ecotypes. We report here the sequence characteristics and the expression patterns of five dehydrins from *P. pinaster*, along with the physiological characterization of drought stress

responses in different genotypes (clonally replicated plants), originating from a broad geographical distribution across France and Spain (provenances). In total, we distinguished five different dehydrin genes in silico, grouped into two types—K₂ and SK_n. Three of the dehydrin genes had several sequence variants, differing by multiple or single amino acid substitutions. Only two of the dehydrins (*PpinDhn3* and *PpinDhn4*) showed an increase in transcription with increased drought stress which was dependent on provenance and genotype, suggesting their involvement in drought resistance. The other dehydrins showed decreased expression trends with increased severity of the drought stress. The lack of close association between the drought stress and expression patterns of these dehydrin genes suggest that they could have other functions and not be involved in drought resistance. Our results suggest large differences in function between different dehydrin genes.

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Introduction

Maritime pine (*Pinus pinaster* Ait.) is one of the major and most widely distributed forest species of the occidental Mediterranean basin and the Atlantic coastal region of southern Europe (Miguel Pérez et al. 2002; González-Martínez et al. 2004, 2005; Wahid et al. 2006), extensively used in plantations and highly relevant for Iberian forestry (Alía et al. 1995; Alía and Martín 2003). Currently, there is considerable interest in selecting genotypes which maintain growth performance under drought conditions (Nguyen-Queyrens and Bouchet-Lannat 2003).

Global warming and the associated likely increase in the incidence of drought in southern regions is one of the most

severe environmental stresses that affects plant survival and productivity (Rampino et al. 2006). Drought resistance is a physiological concept that includes both “dehydration avoidance” and/or “dehydration tolerance”. When plants are exposed to drought, they initially maintain high water status or cellular hydration level (desiccation postponement; see Taiz and Zeiger 2006) and then develop dehydration tolerance, defined as the relative capacity to sustain or conserve plant function in a dehydrated state (Blum 2005).

Some studies have concluded that *P. pinaster* may be among those species that postpone desiccation, because it has adopted several mechanisms to reduce loss of water (e.g., adjustment of water content; osmotic adjustment, with accumulation of osmolites such as proline, glutamate, and sugars; stomatal closure; alterations in root development, etc.), thus enabling growth to be maintained under drought conditions. Because *P. pinaster* has high intraspecies variability and also shows geographic variation in response to drought (Nguyen-Queyrens and Bouchet-Lannat 2003), researchers have found it difficult to develop pertinent criteria for the selection of drought tolerant or sensitive genotypes.

Several physiological parameters have been used to study the response to drought. One of the most informative is the measurement of water potential, which provides information on both soil and plant state (Ritchie and Hinckley 1975). In addition, during water deficit, the expression of a large number of genes is modified, giving rise to strict regulation of the physiological stress responses which is controlled by many factors (Rampino et al. 2006). Among the genes involved in stress responses identified, dehydrins are, so far, those most often considered relevant to dehydration stress. They are desiccation-induced proteins (Artlip and Wisniewski 1997) produced in response to dehydration during environmental stress such as drought (Campbell and Close 1997), low temperatures or freezing (Rinne et al. 1999; Welling et al. 2004; Yakovlev et al. 2008), and increased salinity (Close 1997; Rorat et al. 2006). Dehydrins are also known as the late embryogenesis abundant (LEA) proteins, belonging to the LEA DII family, and they accumulate in plant tissues in response to cellular dehydration resulting from developmental events (Tompá and Kovács 2010) such as during the late stage of seed maturation, but their exact function remains unclear. In spite of this, different studies suggest that dehydrins may participate in establishing the stress tolerance of plants by taking on a protector function thereby mitigating the damage caused by the stress (Close et al. 1993; Battaglia et al. 2008; Bies-Ethève et al. 2008; Hara 2010; Tompá and Kovács 2010).

The dehydrins are characterized by the presence of different amino acid motifs, Y-, S-, and K-motifs. They are divided into five subclasses based on their number and order: YnSKn, SKn, Kn, YnKn, and KnS (Close et al.

1993; Campbell and Close 1997). The Y-segment (T/VDEYGNP) is found near the N-terminal region (there can be between one and three copies), and they might work in the same way as the nucleotide binding site of chaperones (Close 1997). The S-motif, a phosphorylatable serine tract containing five to eight residues, participates in nuclear targeting of the dehydrins (Close 1997). The K segment is a conserved lysine-rich, 15-amino-acid sequence (EKK-GIMDKIKEKLPG) that is found in all dehydrin genes, generally near the C-terminal region (Kosová et al. 2007). There may be one or several copies of this region (Rorat et al. 2006) which act as an interface between hydrophobic membrane phospholipids and the hydrophilic cytosol in plant cells, forming an amphipathic helix (Close 1997; Campbell and Close 1997; Hara et al. 2009). Additionally, there are usually polar amino acids, called Φ -segments, which are interspersed between the conserved segments.

In the last 20 years, different, but structurally related dehydrins have been isolated from crops (Close et al. 1989; Ismail et al. 1999; Zhu et al. 2000; Borovskii et al. 2002; Lee et al. 2005; Bhattarai and Fetting 2005; Hinniger et al. 2006), herbaceous plants (Ouvrard et al. 1996; Volaire 2002; Olave-Concha et al. 2004; Yakubov et al. 2005; Pulla et al. 2008; Bies-Ethève et al. 2008), and woody plants (Arora and Wisniewski 1994; Muthalif and Rowland 1994; Artlip et al. 1997; Rinne et al. 1999; Wisniewski et al. 1999, 2006; Richard et al. 2000; Kontunen-Soppela et al. 2000; Caruso et al. 2002; Welling et al. 2004; Porat et al. 2004; Deng et al. 2006; Yakovlev et al. 2008; Bassett et al. 2009; Hara et al. 2009; Vornam et al. 2011). An accumulation in leaves and stems has been demonstrated, which appears to change during the yearly seasonal cycle.

In this study, we analyzed the response of drought-resistant and drought-sensitive genotypes of *P. pinaster* originating from the latitudinal and altitudinal range of distribution (Fernández et al. 1999; Eveno et al. 2008; Aranda et al. 2010; Sánchez-Gómez et al. 2010). They were exposed to repetitive drought under controlled conditions, and their state was assessed using several physiological methods. We made an in silico search and analysis of dehydrins among available *Pinus* expressed sequence tags (ESTs) and genomic sequences. Furthermore, we studied the expression of five dehydrin genes (*PpinDhn1–5*) found in needles in relation to the pine’s ability to tolerate long drought periods. The expression was quantified by real-time quantitative RT-PCR (qRT-PCR).

Several of the dehydrins analyzed in this study have been described previously, although they may have been given different names (see Table 3 and Electronic supplementary material 1, for more specific information). This work extends the information of the dehydrins of *P. pinaster* and knowledge of their structure and possible functions in relation to drought resistance.

Material and methods

Plant material

Clonal material of maritime pine (*P. pinaster* Aiton) from three provenances, Oria (Spain), Coca (Spain), and Mimizán (France), were chosen based on differences in climatic characteristics at the sites of seed origin (Mediterranean, Continental, and Atlantic, respectively), to represent the likely range in drought tolerance (Fernández et al. 1999; Eveno et al. 2008; Aranda et al. 2010; Sánchez-Gómez et al. 2010). Within each of the three provenances, two different genotypes, one drought-tolerant and one drought-sensitive, were used. The clones were preselected based on a previous assessment in a greenhouse experiment of survival; 12 seedlings from 223 genotypes were kept in a hydroponic culture and subjected to progressive increases in water stress mediated by the additions of an osmoticum (polyethylene glycol 8000) to the nutrient solution until reaching -2 MPa after 8 months. Early characterization to drought tolerance was expressed as the number of days to reach 50% mortality/survival (T_{50} value, unpublished data) (Table 1).

The clones were propagated in 2005 as rooted cuttings from stock being grown in a greenhouse. After several months, they were transplanted to 2-l containers filled with peat/perlite (70:30 v/v) and were placed in a greenhouse in “La Mata” Asturias, Spain ($43^{\circ} 32' N$ $7^{\circ} 00' W$, 65 m). At the end of 2008, the trees were moved into a climatically controlled chamber with a day/night temperature of $25 \pm 2^{\circ}C$, $60 \pm 10\%$ relative humidity, and 16/8 h of daylight/night. They were acclimated for 1 month in the new conditions, watered three times a week, and supplemented with mineral elements once a week (1:0.42:1.47, N/P₂O₅/K₂O). The drought treatment was then implemented for 1 month. Three ramets for each genotype, i.e., 18 3-year-old cuttings in total were used.

Drought treatment, harvests, and measurements

The plants were fully watered (field capacity) prior to the start of the experiment. In the first two weeks of the trial, the plants were left to dry out to the point of 5% soil water content (water reduction treatment, until 50% of initial pot weight was reached), followed by a 1-week acclimation treatment under this condition. Subsequently, they were subjected to a 1-week period without watering, showing values near to 0 (drought treatment, until 37% of initial pot weight was reached) (Aranda et al. 2010). Finally, the plants were re-watered to field capacity for 2 weeks (recovery period).

The water content of the pots was measured by gravimetric measurements every day and monitored with time domain reflectometry (TDR; Topp et al. 1980; Topp and Davis 1985) sensors throughout the experimental period. Water

was added in order to maintain the level of stress according to TDR and gravimetric measurements (Fig. 1).

Several needles were collected every week for needle pre-dawn water potential measurements (Ψ_{nwpd}), using the Scholander chamber (PMS Instruments, Corvallis, OR, USA) as described by Scholander et al. (1965) and Turner (1988).

Three harvests were made during the experiment: the first, at the beginning of the trial prior to the water reduction treatment, the second, at the end of the 1-week acclimation period following the water reduction treatment, and the third at the end of the drought treatment. For each of these harvests, approximately 1 g of needles was collected from each tree and immediately frozen in liquid nitrogen for dehydrin analysis. To measure the osmotic potential (Ψ_{π}), three further needles per tree were collected at each harvest and frozen in liquid nitrogen. Sap osmolality was measured with a thermocouple psychrometer (C52 chamber, Wescor, USA) using 10 μ l sap samples. To obtain the needle relative water content (RWC), two further needles were weighted immediately after each harvest (fresh weight (FW)), then placed in water to obtain their fresh weight at full hydration (FW₁₀₀), and finally dried at $70^{\circ}C$ for 48 h to obtain their dry weight (DW). RWC was calculated using the following equation:

$$RWC = (FW - DW) / (FW_{100} - DW)$$

To compare plants' physiological responses to the treatments, the Δ of different parameters (RWC, Ψ_{π} , and Ψ_{nwpd}) were calculated as the difference between treatments (Table 2): with a being the difference between second harvest (water reduction treatment) and first harvest (field capacity), and b the difference between third harvest (drought treatment) and second harvest (water reduction treatment). In this way, the rise in accumulated solutes ($\Delta\Psi_{\pi}$) was quantified by comparing the difference between the initial and final Ψ_{π} between treatments.

$$\Delta\Psi_{\pi}^a = \Psi_{\pi 2} - \Psi_{\pi 1} \text{ and } \Delta\Psi_{\pi}^b = \Psi_{\pi 3} - \Psi_{\pi 2}$$

Loss of water was calculated as increase/decrease in RWC:

$$\Delta RWC (\%)^a = RWC_2 - RWC_1 \text{ and } \Delta RWC (\%)^b = RWC_3 - RWC_2$$

And the increase in Ψ_{nwpd} as:

$$\Delta\Psi_{nwpd}^a = \Psi_{nwpd 2} - \Psi_{nwpd 1} \text{ and } \Delta\Psi_{nwpd}^b = \Psi_{nwpd 3} - \Psi_{nwpd 2}$$

Gene searching and in silico sequence analysis

An initial search of dehydrin genes was performed with BLAST (tblastn) using Y-segment (T/VDEYGNP), K-segment (KIKEKLPG), and S-motif amino acid virtual probes,

Table 1 Climatic and physiological characteristics of the *P. pinaster* genotypes in the different provenances

Provenance origin	Seed origin	Latitude/longitude	Altitude	Precipitation, mm	Mean temperature, °C	Genotype	Behavior	T ₅₀ , days ^a
Coca Continental	Segovia/Spain	41°22'N/4°05'W	788	461	11.2	F3P5 F2P4	Sensitive Resistant	102 204
Oria Mediterranean	Almería/Spain	37°29'N/2°17'W	1232	348	12.5	F1P3 F4P4	Sensitive Resistant	107 141
Mimizán Atlantic	Mimizán/France	44°08'N/1°18'W	37	995	13.8	F2P1 F2P2	Sensitive Resistant	102 211

^a T₅₀, value of drought tolerance expressed as days to reach 50% mortality/survival

comparing the results against those of the National Centre for Biotechnology Information (NCBI) database restrained by *Pinus* taxa. The obtained sequences were used for a subsequent BLAST (blastn) search against NCBI nucleotide and EST databases, again confined to the *Pinus* taxa. Collected genomic fragments and ESTs were aligned and combined into contigs for in silico full-length gene construction. Contig clusters were considered as separate dehydrin genes. Multiple sequence alignment was made using WWW resources: Multiple Sequence Alignment (MultAlin) Interface (<http://bioinfo.genotoul.fr/multalin/multalin.htm>) (Corpet 1988) and T-Coffee (<http://tcoffee.vital-it.ch/cgi-bin/Tcoffee/tcoffee.cgi/index.cgi>) (Notredame et al. 2000). To obtain the exon–intron structure of the defined genes, the fragments of cDNA and gDNA were aligned using the Spidey mRNA program for genomic alignment at the NCBI (<http://www.ncbi.nlm.nih.gov/IEB/Research/Ostell/Spidey/spideywebeg.html>).

Translated amino acid sequence alignments and phylogenetic tree construction for the selected dehydrin gene models were conducted using the MEGA software version 4.1 (Tamura et al. 2007) using the neighbor-joining method with default parameters.

RNA isolation

Total RNA was extracted using Spectrum™ Plant Total RNA kit (Sigma, St. Louis, MS, USA, #STRN50) with a minor change from the manufacturer's instructions to improve the extraction: instead of using 500 µl of binding solution and 500 µl of lysis solution/2ME-mixture, we used 750 and 700 µl, respectively (page 5 and protocol A, page 7 of instruction manual). Contaminating DNA was removed from total RNA samples using the DNA-Free™ kit (Ambion, Austin, TX, USA, #1906) following the manufacturer's protocol.

Fig. 1 Correlation between available water, measured with TDR and the weight of pots at three different times during the experiment: **a** well-watered plants; **b** water reduction treatment; **c** drought treatment

Table 2 Increase in osmotic potential (Ψ_{π}), relative water content (RWC), and predawn water potential (Ψ_{nwpd}) of 3-year-old ramets of *P. pinaster* dependent on water deficit treatments

Genotype	Ψ_{π}	$\Delta\Psi_{\pi}^a$	$\Delta\Psi_{\pi}^b$	RWC _i (%)	Δ RWC (%) ^a	Δ RWC (%) ^b	Ψ_{nwpd}	$\Delta\Psi_{nwpd}^a$	$\Delta\Psi_{nwpd}^b$
COCA_F3P5	-1.987ab	-0.385a	0.744b	100a	-4.8a	-0.2a	-0.45b	0.00b	-0.97a
COCA_F2P4	-1.903a	0.117a	-0.143a	97.8a	-2.2a	-3.8a	-0.62b	0.22b	-1.62a
MIMI_F2P1	-1.950a	0.121a	-0.138a	97.8b	-1.1b	-5.2a	-0.70b	0.07b	-0.89a
MIMI_F2P2	-1.764a	0.432a	-0.571a	99.1a	-1.0a	-1.3a	-0.48b	-0.19b	-0.98a
ORIA_F1P3	-1.797b	-0.426a	0.612b	97.0a	-1.9a	-5.2a	-0.48b	0.05b	-1.62a
ORIA_F4P4	-2.100a	0.253a	0.015a	99.4b	-4.3ab	-3.0a	-0.50b	-0.07b	-1.03a

Parameters with the same letter are not significantly different at $P=0.05$ (Tukey's test)

_i values for control plant

^a Increase between harvests 1 and 2

^b Increase between harvests 2 and 3

RNA was quantified using GeneQuant spectrophotometric OD₂₆₀ measurements, and quality was assessed by OD₂₆₀/OD₂₈₀ ratios, followed by electrophoresis on 1% agarose gels stained with ethidium bromide. All RNA was stored at -80°C .

cDNA synthesis

cDNA was synthesized from 1 μg of total RNA using the Taqman Reverse Transcription Reagents (Applied Biosystems, Foster City, CA, USA, #N8080234) according to the manufacturer's procedure. Reverse transcription was carried out in a 50- μl reaction volume containing 500 μM of dNTPs mixture (2.5 mM each), 2.5 μM of oligo d(T)₁₆, 1 $\times$ TaqMan RT buffer, 5.5 mM of MgCl₂ solution, 0.4 U/ μl of RNase Inhibitor, and 1.25 U/ μl of Multiscribe Reverse Transcriptase. Reactions were incubated at 25°C for 10 min, then at 48°C for 30 min, and 95°C for 5 min to inactivate the reverse transcriptase in a GeneAmp[®] PCR System 9700 (Applied Biosystems). After heat-deactivation of the enzyme, ss cDNA obtained was diluted twice and stored at -80°C .

Quantitative real-time PCR (qRT-PCR)

Specific primers for reference genes and dehydrins were designed using Primer 3 software (http://biotools.umassmed.edu/bioapps/primer3_www.cgi) (Rozen and Skaletsky 2000). Primers were designed to have a size of 19–27 base pairs (bp), with GC content of 44–69% and T_m of 67–71 $^{\circ}\text{C}$. Other aspects were also taken into account such as likelihood of primer self-annealing and formation of dimers. The predicted fragment size ranged from 80 to 100 bp (Electronic supplementary material 1). Oligonucleotides were synthesized by Invitrogen (Paisley, UK) and Eurofins MWG Operon (Ebersberg, Germany).

Real-time PCR reactions were performed in a 25- μl volume containing 250 nM of each primer, 2 μl of cDNA, and 1 $\times$ SYBR Green PCR master mix (Applied Biosystems #4309155). Real-time PCR was performed on the 7500 Real-Time PCR System (Applied Biosystems) using the parameters for absolute quantification experiment recommended by the manufacturer (2 min at 50°C , 10 min at 95°C , and 40 cycles of 95°C for 15 s and 60°C for 1 min). Each PCR reaction was repeated twice, giving three experimental replications. We used the geometric mean of the three reference genes *PpinAct1*, *PpinAct2*, and *PpinAct3* as the internal control. The transcripts of these genes were all more homogeneous (unaffected by the treatments) than other tested genes such as the Ubiquitin gene (*PpinUbq*) and RNA Polymerase II (*PpinRpolII*) (Brunner et al. 2004; Yakovlev et al. 2006) (Electronic supplement 2).

The specificity of the amplifications was verified at the end of the PCR run using 7500-system SDS Dissociation Curve Analysis Software, ensuring that only one distinct peak was present. Results were then averaged from different replicates, since the expression patterns were almost equal.

Statistical analysis

Data acquisition and analysis was carried out using the 7500 real-time PCR system SDS software for absolute quantification and then MS Excel software. The relative amounts of transcript were subjected to statistical analysis as described previously by Johnsen et al. (2005a, b). The quantitative data used in the analysis of the transcripts was delta Ct (threshold cycle) of cDNA obtained from mRNA of three 3-year-old ramets of three different provenances and two different tolerance levels (drought-tolerant and drought-sensitive). The analysis corresponds to a randomized complete block design with three replicates,

using SAS/STAT® Software (US and Canada), following a linear model for the expected value of general response y_{ijkl} :

$$Y_{ijkl} = \mu + P_i + C_j + T_k + PC_{ij} + PT_{ik} + CT_{jk} + PCT_{ijk} + e_{ijkl}$$

where μ is the general mean; P , C , and T represent the additive fixed main effects of provenance (P), category (C) (sensitive or tolerant), and treatment (T) (well-watered, 5% available water, and withholding water), respectively. PC is the interaction between provenance and category; PT is the interaction between provenance and treatment; CT is the interaction between category and treatment, and PCT is the interaction between all possible effects: provenance, category, and treatment. e_{ijkl} is the residual value of the individual values of i , j , k , l .

Analysis of variance (one-way ANOVA), using an SPSS 15.0 Inc.® Win™ statistics package (SPSS, Richmond, CA), was applied to the data in order to discriminate between genotypes and drought treatments. Comparisons of means were made using post hoc comparisons with the Tukey's test ($P < 0.05$). No significant interactions between main effects were obtained.

Relationships between variables and different graphics were made using SigmaPlot® 10.0 procedures (2006 Systat Software, Inc.).

Results

Plant material and drought treatment

Throughout the experimental period, soil and leaf water status were monitored by measuring the gravimetric soil water content, the volumetric content of water (θ , %, measured with TDR technology), and the needle predawn water potential (Ψ_{nwpd}). A negative linear relationship ($r^2 = 0.90$) was obtained between gravimetric values (pot weight) and θ over the time exposed to drought, indicating available water for plants throughout the treatment period (Fig. 1). Part A presents the water status in the well-watered plants (maintained at field capacity) prior to drought. Continental and Mediterranean genotypes maintained relatively higher θ values than Atlantic genotypes. During the water reduction treatment (part B), water availability was drastically reduced to 28.29%, and the mean weight of pots decreased to 56.78%. Both genotypes of the Continental and Atlantic provenances consumed a similar quantity of water ($\approx 55\%$ and $\approx 63\%$, respectively). In the Mediterranean provenance, the sensitive genotype consumed a higher amount of water compared with the tolerant genotype (56.48% and 48.62, respectively). For the Atlantic provenance, the average use of water was lower than in the other provenances (between 8% and 11%). In part C, all plants exposed to severe drought, i.e., no available water, no differences between genotypes were found.

Predawn leaf (needle) water potential (Ψ_{nwpd}) is an indicator of water availability in the soil near the root system, reflecting plant water status (Nguyen-Queyrens et al. 2002). Ψ_{nwpd} (megapascals) values measured during the experiment are shown in Fig. 2. Part A shows the condition of plants before the water reduction treatment, where quite similar values in Ψ_{nwpd} (approximately -0.5 MPa) were obtained in each provenance and genotype. Part B shows the values from the successive measurements taken during the water reduction treatment during which soil water content was reduced and maintained at approximately 5%. During this period, Ψ_{nwpd} initially decreased in both genotypes of the Continental and Atlantic provenances as well as the Mediterranean sensitive genotype and then returned to levels nearly as high as in the well-watered period. The Ψ_{nwpd} of the tolerant Mediterranean genotype, however, remained the same as in part A. Following the water reduction period, the drought regime was established (part C) and Ψ_{nwpd} values further decrease. This was most pronounced in the Mediterranean and the Continental provenances, where quite clear opposite trends between tolerant and sensitive genotypes were observed. For the Atlantic provenance, we did not find any consistent difference between the tolerant and sensitive genotypes, suggesting an interaction between provenance and clone category. During the re-watering period (part D), no general pattern of recovery from drought stress to higher water potentials was found. However, 66% of the plants of each genotype from the Continental provenance, and 33% of the sensitive genotype from the Atlantic provenance died. There was zero mortality among the plants from the Mediterranean provenance and the tolerant Atlantic genotype. In the sensitive genotypes, only that from the Mediterranean provenance recovered to -1.0 MPa, when re-watered.

Plant water relations

Table 2 shows the osmotic potential (Ψ_π), the RWC, and predawn water potential (Ψ_{nwpd}). The delta (Δ) parameters were estimated as the differences between periods (a and b) during the drought treatment.

For the Continental provenance, only the sensitive genotype showed a passive osmotic adjustment with a reduction in RWC (4.8% for Δ^a), and significant differences between treatments were observed (Table 2) at $P < 0.05$ (one-way ANOVA, post hoc Tukey's test). In period a, this was the genotype which showed the largest differences, though it was closely followed by the sensitive genotype of the Mediterranean provenance, although this latter did not show any changes in RWC. The tolerant genotype of Atlantic provenance showed a non-significant active osmotic adjustment during period b ($\Delta\Psi_\pi -0.571$ MPa) without significant differences in RWC. The remaining genotypes did not show any significantly different osmotic adjustment.

Fig. 2 Needle predawn potential (Ψ_{nwpd} , megapascals) measured during the experiment: **a** well-watered plants (point 1); **b** water reduction treatment (points 2, 3, and 4); **c** drought treatment (point 5); **d** recovery period, after re-watering of plants (points 6 and 7)

With regards $\Delta\Psi_{nwpd}$, all genotypes showed significant differences between treatments ($P < 0.05$): the highest being obtained in the drought treatment for all genotypes. The lowest values for Ψ_{nwpd} were observed in the sensitive genotype of Mediterranean provenance (-2.05 MPa) and in the tolerant genotype of Continental provenance (-2.02 MPa), at the end of the experiment.

Sequences and structure of the dehydrins

The search of the GenBank EST database, confined to *Pinus* genus, revealed a large set of ESTs and genomic sequences containing dehydrin-specific S- and K- amino acid motifs. However, we did not find any sequences containing a dehydrin-specific Y-motif. All ESTs were aligned and combined in contigs based on nucleotide sequences. The contigs obtained allowed, in most cases, the construction of full-length cDNA with complete open reading frames (ORFs). They were also aligned with dehydrin genomic sequences allowing us to obtain the intron–exon structures of putative dehydrin (*Dhn*) gene models. In total, we defined and characterized five different *Dhn* genes in *P. pinaster*, three of which (*PpinDhn1*, *PpinDhn2*, and *PpinDhn5*) had several (at least two) sequence variants, differing by single or several amino acid substitutions (Fig. 3). Based on the presence of the dehydrin-specific amino acid S- and K-motifs (Close 1996; Campbell and Close 1997), we grouped the five *Dhn* models into two types, as shown in Table 3.

The first type of *P. pinaster* dehydrin was the most abundant and included six ESTs containing S- and K-motifs, which we defined as a SK_n-type dehydrins. The first of these, *PpinDhn1.1*, encoded a putative protein of 238 amino acids (aa), had one described intron between position 248 and 355 bp, and contained four K- segments (K(I/S)KEK(L/I)PG) and an S-motif of eight-serine residues upstream of the first K-motif. *PpinDhn1.2* encoded a putative protein of 238 aa with four different K-motifs (K(I/N)KEK(I/L)(P/H)(G/A)) and

an S-motif of six serine residues and two aa substitutions. Its genomic sequence contains one intron between 333 and 486 bp. The protein sequences of *PpinDhn2.1* and *PpinDhn2.2* differed only in three aa. Each had an ORF of 582 bp encoding a protein of 193 aa; their structure was formed by two K-motifs, K(I/T)KEK(I/L)PG followed by an S-motif with eight-serine residues. *PpinDhn3* had one ORF of 516 bp, encoding a putative protein of 171 aa, two K-motifs (KIK(E/D)KLPG), and nine-serine residues. *PpinDhn4* had a full length of 1,755 bp with an ORF of 981 bp, contained one intron of 572–905 bp in the S-motif, and had a structure of five K-motifs (K(I/T/F)K(E/K)K(L/V)P(G/A/V)) and seven-serine residues.

The second dehydrin type was categorized as a Kn-type. It had no S-motif, and the amino acid sequences had only two K-motifs. *PpinDhn5.1* with an ORF of 309 bp encoded a protein of 102 aa and contained two K-motifs (KIK(E/Q)KLPG), and *PpinDhn5.2* had one ORF of 270 bp, encoded a protein of 89 aa, and also contained two K-motifs (KIK(E/Q)KLPG).

Phylogenetic relationships of *P. pinaster* dehydrins

The full-length dehydrin coding regions of various other woody plants, among which were other *Pinus* species, *Picea abies*, *Cupressus sempervirens*, *Ginkgo biloba*, *Rhododendron catawbiense*, *Betula pubescens*, *Tamarix hispida*, *Pyrus pyrifolia*, *Malus domestica*, *Populus* species, *Prunus* species, and *Citrus* species, were aligned using the ClustalW program (Larkin et al. 2007) and their phylogenetic tree (constructed using MEGA software version 4.1, Tamura et al. 2007).

This tree (Fig. 4) was then used to compare the translated aa sequences of the *P. pinaster* with the other woody plant dehydrins. Fourteen groups combining dehydrins from different species and structure types (Y_nSK_n, Y_nKn, K_nS, S, SK_n, K_n) were found. The Genbank accession numbers of the analyzed sequences are given in the Electronic supplementary material 3.

Fig. 3 Alignment of the deduced amino acid sequences of *P. pinaster* dehydrins: **a** type 1 (SK_n) and **b** type 2 (K_n). The boxes indicate the S-motif and the K-motifs

The *P. pinaster* *dhn* genes were arranged in two different groups within two *dhn* gene classes. *PpinDhn1.1* and

PpinDhn1.2 (SK₄ type) were present in group 1, very close to *Pinus ponderosa*, *Pinus sylvestris*, and *Pinus conorta*

Table 3 Description of *P. pinaster* dehydrin genes

Type	Gene, name-class	Description	<i>P. pinaster</i> EST database ID	GeneBank acc. no.	Published ID <i>P. pinaster</i> <i>Dhn</i> (references)
Type 1-SK _n	<i>PpinDhn</i> , 1.1-SK ₄	Contains one S-segment with eight serine residues and four K-segments—KSEKEKLP, KIKEKIPG, KIREKIPG, and KIKEKLP	>CAM58808; >DR388209 full-length ORF containing one intron	AM502292.1 + DR388209	<i>Dhn1 P. pinaster</i> (Eveno et al. 2008)
	<i>PpinDhn</i> , 1.2-SK ₄	Contains one S-segment with six serine residues and four K-segments—two KIKEKIPG, KNKEKLHG, and KIKEKIPA	>ACL27837; >ACL27839 full-length ORF containing one intron	FJ415451.1 + DR011056 + FJ415453.1	<i>Dhn9 P. pinaster</i> (Palme et al. 2009)
	<i>PpinDhn</i> , 2.1-SK ₃	Contains one S-segment with eight serine residues and three K-segments—KTKEKLP, KIKEKIPG, and KIKEKLP	>CT583101; >CT577827; >BX250279; >BX253143; >BX251756; >CT582728; >CT576916; >CT582573; >BX251211; >BX251710 full-length ORF	FJ415436.1;	<i>Dhn2 P. pinaster</i> (Palme et al. 2009)
	<i>PpinDhn</i> , 2.2-SK ₃	Contains one S-segment with eight serine residues and three K-segments—KTKEKLP, KIKEKIPG, and KIKEKLP	>CT583101 full-length ORF	FJ415436.1;	<i>Dhn2 P. pinaster</i> (Palme et al. 2009)
	<i>PpinDhn</i> , 3-SK ₂	Contains one S-segment with nine serine residues and two K-segments—KIKEKLP and KIKDKLP	>DT626947; >BX250897; >BX677751; >ABU93876.1 full-length ORF containing one intron	EUI20235.1	<i>Dhn</i> -like protein <i>P. pinaster</i> (Pyhajarvi et al. 2007)
Type 2-K _n	<i>PpinDhn</i> , 4-SK ₅	Contains one S-segment with seven serine residues and five K-segments—two KIKEKLP, KTKKKVPG, KFKELPA, and KIKEKLPV	>BX253097; >CT575006; >CT580714; >ACL27818 full-length ORF containing one intron	CT582038 + FJ415432	<i>P. pinaster</i> cDNA + <i>Dhn1 P. pinaster</i> (Palme et al. 2009)
	<i>PpinDhn</i> , 5.1-K ₂	Contains two K-segments—two KIKEKLP but lacks S-segments	>CT582715; >CT576152; >CT575239; >CT579312.1; >CT574757; >CT582598; >CT582734; >CT577498; full-length ORF	FJ415446.1	<i>Dhn7 P. pinaster</i> (Palme et al. 2009)
	<i>PpinDhn</i> , 5.2-K ₂	Contains two K-segments—KIKEKIPG and KIKQKLP but lacks S-segments	>ACL27826 full-length ORF	FJ415440.1	<i>Dhn3 P. pinaster</i> (Palme et al. 2009)

Fig. 4 Phylogenetic tree showing sequence relations between *P. pinaster* dehydrins and selected woody plant dehydrins. Species codes: *Ppin*: *P. pinaster*; *Bp*: *B. pubescens*; *Cits*: *Citrus sinensis*; *CuS*: *C. sempervirens*; *Prp*: *Prunus persica*; *Th*: *Tamarix hispida*; *Pop*: *Populus nigra*, *Populus maximowiczii*, *Populus alba*, *Populus tremula* var *glandulosa*, *Populus tremula* var *Davidiana*, *Populus canadensis*, *Populus x canadensis*, *Populus trichocarpa*; *Rc*: *R. catawbiense*; *Md*: *M. x domestica*; *Prd*: *Prunus dulcis*; *Pyp*: *P. pyrifolia*; *Gb*: *G. biloba*; *Pa*: *P. abies*; *Cit*: *Citrus unshiu*; *Pt*: *Pinus taeda*; *Ps*: *P. sylvestris*; *Ppo*: *P. ponderosa*; *Pc*: *P. conorta*. For detailed references, see Electronic supplementary material 3. *P. pinaster* dehydrins highlighted with red boxes. Cluster numbers consist of indication of dehydrin types included into distinct cluster

(SK₄). *PpinDhn4* (SK₅ type) was present in the same group, although more associated with *P. sylvestris* (*PsDhn1.2*). *PpinDhn3* was placed in group 1 together with *dehydrin2.1* of *Pinus taeda* (*PtDhn2.1*), and *PpinDhn2.1* and 2.2 were found in the same group, very close to dehydrins belonging to *P. abies*, *C. sempervirens*, and *P. sylvestris*. Finally, group 7 was formed by sequences with K₂ motifs, which included *PpinDhn5.1* and 5.2 from *P. pinaster* (two proteins that differ in seven gaps and eight aa) together with dehydrins of *P. sylvestris* and *C. sempervirens*.

The remaining groups included dehydrins from different Magnoliophyta orders (Rosales, Caryophyllales, Sapindales, Fagales, Malpighiales, Ericales) together with some *Cupressus sempervirens* dehydrins, with different dehydrin motifs (S, SK₂, K₂, K₃, K₆, Y₃SK, Y₂SK₃, Y₂K₉, YK₃). Magnoliophyta orders were present in three groups: group 3 with K₂S-motif, group 5 with a dehydrin structure that included the Y-motif, and group 10 with SK and SK₂ motifs. Group 4 included the *G. biloba* dehydrin with an S-motif structure.

Different dehydrin structures of the K_n type, belonging to *P. abies*, *C. sempervirens*, and *Pinus* and *Populus* species, were placed into groups 6, 7, and 8.

Quantitative real-time PCR (qRT-PCR)

The expression of dehydrin genes was measured using quantitative real-time PCR of RNA samples isolated from three ramets per genotype of the three *P. pinaster* provenances. The initial sample copy numbers were calculated from the difference between the mean *Ct* and specific *Ct*s for each genotype and treatment (Fig. 5). The statistical model applied to test differences in the expression of dehydrins showed significant differences for all dehydrins ($P < 0.05$). Significant differences in transcription were observed between treatments at $P < 0.001$ or $P < 0.05$, in the dehydrins studied. Significant differences between provenances were obtained only for *PpinDhn1*, *PpinDhn2*, and *PpinDhn3* ($P < 0.05$).

PpinDhn1, *PpinDhn2*, and *PpinDhn5* showed similar trends, which indicated decreasing transcript levels as water deficit became more severe. The highest transcript levels were found at the beginning of the experimental period. We found no differences in *PpinDhn5* transcript levels between

drought-sensitive and drought-tolerant genotypes or between treatments. In the tolerant genotype of Mediterranean provenance, however, the *PpinDhn2* transcript levels increased at the beginning of the drought phase, though they

Fig. 5 Expression profiles of dehydrins in drought-sensitive and drought-resistant genotypes of three provenances of maritime pine (*P. pinaster* Aiton): Oria (Spain–Mediterranean), Coca (Spain–Continental), and Mimizán (France–Atlantic). Transcript levels are presented as the difference between geometric mean of three preselected reference

genes (*PpinAct1* and 2 and *PpinTub*) and the chosen transcripts relative to the mean value for the genes targets (Delta dCt). Asterisks depict significant differences in the interaction among provenance, categories, and treatment ($P < 0.05$). 1: well-watered plants, 2: water reduction treatment and 3: drought treatment

later dropped significantly under severe drought conditions (third sampling point).

Expression patterns of *PpinDhn3* and *PpinDhn4* differed significantly between different provenances and between drought-sensitive and drought-tolerant genotypes. *PpinDhn3* transcript levels increased significantly with increasing drought stress for Mediterranean provenance, more so for the sensitive than the tolerant genotype. The same overall effect was seen in the Continental tolerant genotype where a more than 128-fold rise in transcript levels was found, showing significant differences ($P < 0.0001$) between the beginning and end of the drought stress. However, for the Continental sensitive genotype, onset of drought stress was accompanied by a drastic decrease in dehydrin transcripts, followed by a

slight recovery when the drought conditions became very severe at the end of the drought treatment. The Atlantic provenance scarcely changed *PpinDhn3* expression in relation to the treatments.

PpinDhn4 showed a stable increase in its transcript levels in all tolerant genotypes and also in Continental sensitive genotype. However, the influence of drought stress on Atlantic and Mediterranean sensitive genotypes resulted in a slight though non-significant response in transcript levels. In general, there was a more than three- to fourfold difference between tolerant and sensitive genotype of Continental and Mediterranean provenances and a less than twofold difference was obtained for Atlantic genotypes.

Accumulation of dehydrin transcripts in relation to the physiological response

Figure 6 shows the relationship between transcription of the dehydrins and predawn water potential (Ψ_{nwpd}). For *PpinDhn3* and *PpinDhn4*, an increase in the accumulation of transcript was found when Ψ_{nwpd} decreased. For the other dehydrins, a decrease in Ψ_{nwpd} was associated with a decrease in accumulation of transcript, and the overall pattern was similar for resistant and sensitive genotypes. The treatment response pattern for *PpinDhn3* and *PpinDhn4*, however, differed depending on provenance and clone type within provenance. This was particularly clear for *PpinDhn3*, where we found a strong upregulation under severe drought stress for the sensitive clone of Mediterranean provenance, but this was not so for the sensitive genotypes from the two other provenances (sub-figure A for *PpinDhn3*). The Atlantic tolerant genotype showed no changes in transcription with increasing drought stress (sub-figure B for *PpinDhn3*), while the transcription level for two other provenances clearly increased with rising drought stress intensity. An increase in *PpinDhn4* transcript levels during severe drought stress was found for the sensitive genotypes of the Continental provenance (sub-figure A for *PpinDhn4*) but not for the sensitive genotypes from the two other provenances. This increase in transcription of *PpinDhn4* during severe drought stress was consistent among all the resistant clones but more pronounced in those from the

Continental and Mediterranean provenances (sub-figure B for *PpinDhn4*).

For the rest of the dehydrins (*PpinDhn1*, *PpinDhn2*, and *PpinDhn5*), the downregulation of transcription during increased drought stress severity was consistent within provenances and within provenance clone types though some minor deviations from this general trend were observed for *PpinDhn1* and *PpinDhn5*.

Discussion

The main aim of this experiment was to analyze the gene expression response to a drought period in *P. pinaster* by exposing the plants to a realistic level of drought, i.e., of comparable strength to that which may naturally occur under field conditions. Both drought-resistant and drought-sensitive genotypes from across the species' latitudinal range of distribution (three provenances) (Fernández et al. 1999, 2000; Eveno et al. 2008; Aranda et al. 2010; Sánchez-Gómez et al. 2010) were included in the study and were selected according to the results of a previous experiment (unpublished data). Under drought stress conditions, plants produce an array of proteins as part of a global stress response, which leads to the protection of cell metabolism. From the various hydrophilic proteins produced (Caruso et al. 2002; Wisniewski et al. 2006; Khurana et al. 2008), dehydrins were chosen for analysis here.

Fig. 6 Relationship between needle predawn water potential (Ψ_{nwpd} , megapascals) and accumulation of *PpinDhn* transcripts (cycle difference), expressed as mean values for the genotypes in the different treatments: 1

well-watered plants; 2 water reduction treatment; 3 drought treatment. Plot A and plot B correspond with sensitive and tolerant plants, respectively. Significant differences were obtained at $P < 0.05$

We report here the sequence characteristics and the patterns of expression of five dehydrins from maritime pine, as well as a physiological characterization of samples from drought-exposed plants and their controls. Dehydrin genes were identified based on the pine EST collection at the NCBI. We found relatively high intragenic diversity with several nucleotide (and amino acid) substitutions among the analyzed ESTs, which allowed us to distinguish between several variants of the same gene. We suspect these to be alleles, but further work needs to be carried out to verify. In total, we identified eight dehydrin gene models, combined in five genes. After analyzing the amino acid sequences, we distinguished two different types of dehydrin: SK_n and K_n. Most dehydrins found in this study (*PpinDhn1*–*PpinDhn4*) belong to the SK_n type and have one S-segment of six to eight serine residues and 2–5 K-segments. Only one of the dehydrins found (*PpinDhn5*) was of the K-type and had two K-segments. All the dehydrins in this study were shown to have a similar structure, i.e., they lack a Y-segment, as do the dehydrins from other conifers like *P. abies* (Yakovlev et al. 2008), *P. sylvestris*, *P. conorta*, *P. ponderosa*, or *C. sempervirens* (Fig. 4, Electronic supplementary material 3). Y-segments have in fact only been found in fruit tree dehydrins, such as *M. domestica*, *P. pyrifolia*, and *Prunus* sp. (Artlip et al. 1997; Wisniewski et al. 2006) which all contain one Y-segment, and *B. pubescens* (Welling et al. 2004) which has three Y-segments.

Cluster and phylogenetic tree analysis indicated that available dehydrin genes in the woody plants analyzed can be divided into three large groups (Fig. 4). The first includes cluster 1, formed by the order Pinales with an SK_n structure. The second by deciduous trees belonging to the order Magnoliophyta (clusters 5 and 10) and possessing various different dehydrin structures (SK_n, K_nS, K_n, Y_nK, and Y_nSK_n types). The third group is represented mainly by different conifers from the *Pinus*, *Picea*, *Populus*, and *Cupressus* taxa and is composed of K_n dehydrin.

P. pinaster dehydrins, although dispersed across two clusters, tended to be very close to other *Pinus* and *C. sempervirens* dehydrins but not so closely associated with *P. abies* dehydrins.

Currently, various parameters are commonly used to define the dehydration resistance of a plant. Of these, we decided to apply water deficit to *P. pinaster* ramets for a month, monitoring the soil water content with TDR technology and measuring the gravimetric soil water content (Fig. 1). Throughout the experiment, different levels of water consumption between plants of the same genotype (ramets of a clone) were observed, possibly because of the significant differences in size among ramets of the same clone (Electronic supplementary material 4). However, as water reduction reached the point of 5% available water in pots, water consumption of different ramets became more similar, showing sensitive genotypes to generally consume more water than the resistant clones, except for the

Continental provenance, where similar consumption levels were seen irrespective of water availability.

Throughout the experiment, Ψ_{npd} values decreased and finally reached values of almost –2.5 MPa. Drought tolerance can clearly be influenced by the origin of the plants (Fernández et al. 1999). During the recovery period, we recorded 66% and 33% mortality for Continental and sensitive Atlantic plants, respectively. However, none of the plants of Mediterranean provenance (from Oria in southern Spain) died, thus demonstrating considerably more drought tolerance. This has been demonstrated previously in other studies, e.g., Fernández et al. (2000), which showed that Oria tended to be more efficient in water use than other provenances and Fernández et al. (1999), where it was suggested that plants originating from the region might save more water. The tolerant Continental genotype exhibited lower values of Ψ_{npd} than the sensitive genotype. Although whole plant leaf area, heights, or diameters were not measured, ramets of this genotype were observed to be larger than sensitive ramets. Larger plants are likely to have a higher transpiring leaf area and therefore consume more water. Thus, when these plants are subjected to water limitation in a restricted root volume, the ramets show rapidly decreasing values of water potential (Nguyen-Queyrens et al. 2002). The sensitive genotype of the Mediterranean provenance showed lower Ψ_{npd} values than the tolerant genotype. In this case, ramets of both genotypes were similar in sizes, but in spite of this, the sensitive genotype had a greater transpiring leaf area, and this feature could explain the lower values observed (Electronic supplementary material 4).

qRT-PCR analysis indicated that three dehydrin genes (*PpinDhn1*, *PpinDhn2*, and *PpinDhn5*) were most highly expressed in the well-watered plants. Their transcript levels decreased with increasing drought intensity. Therefore, their regulatory role in drought resistance in maritime pines is quite doubtful, although they could be involved in response to other types of stresses, such as low temperatures or osmotic, salt, or other biotic stresses. The results obtained in this study suggest that, within the dehydrin group of genes, large difference in function may exist.

Though the exact functions of dehydrins in plants are still not clear, they have been shown to be involved in a wide range of adaptive processes (Hara 2010). In trees, levels of dehydrins have been associated with cold hardiness/dormancy and content/state of water in the tissue (Rowland and Arora 1997; Erez et al. 1998; Kalberer et al. 2006). It seems that accumulation of dehydrins is a common feature of cold acclimation and onset of dormancy, and levels of dehydrin transcripts stay very high during the winter (Wisniewski et al. 1996; Yakovlev et al. 2008). Many studies report a positive correlation between the accumulation of dehydrin transcripts or proteins and tolerance to freezing, drought, or salinity (Karlsón et al. 2003; Kosová et al. 2007; Mehta et al. 2009; Rampino et al. 2006; Rorat et al. 2006; Tommasini et al. 2008; Wisniewski et al.

2008; Hara 2010). Joosen et al. (2006) have demonstrated a positive correlation between cold acclimation and upregulation of K₂ type dehydrins. However, the same authors point out that the expression of different classes of dehydrins (such as SK₄) was unrelated to physiological parameters. In our study, K₂-type dehydrins, together with SK₃ and SK₄, showed downregulated expression under water stress and were inversely correlated to drought tolerance, whereas the dehydrins type SK₂ and SK₅ were upregulated. These results suggest that there is no clear correlation between expression and dehydrin types in conifer species.

The data from in vitro dehydrin studies indicate that dehydrin proteins are involved in a variety of functions during plant growth and in response to dehydration stress, such as the binding of macro- or small molecules, the prevention of protein denaturation, or by performing as antioxidant molecules (Rorat et al. 2006; Hara 2010; Tompa and Kovacs 2010).

Expression patterns for the two genes *PpinDhn3* and *PpinDhn4* showed a rather close relationship with the intensity of the drought stress, but revealed different patterns in response to it, depending on provenance and genotype. In the water reduction treatment stage, there was a decrease in transcript levels of *PpinDhn3* for all provenances, but this was more marked for the sensitive genotypes than the resistant. Furthermore, the drought treatment phase resulted in an increase in *PpinDhn3* transcript levels compared with the level of well-watered plants in the Atlantic provenance, as well as to levels above those of the well-watered plants for the Continental tolerant and both Mediterranean genotypes.

In summary, we found clear opposite trends in *PpinDhn4* expression between sensitive and tolerant genotypes of Mediterranean provenance. Increasing drought conditions were accompanied by an increase in transcript levels in the drought-tolerant genotype but a decrease in the sensitive genotype. In the Continental provenance, we found a trend of increasing *PpinDhn4* expression for both the sensitive and tolerant genotypes as drought intensity increased, but the initial *PpinDhn4* expression level in well-watered sensitive plants was nearly twice as low as for the tolerant genotype. For Atlantic provenance, no pattern in gene expression was noted. As we did not find any clear relationship between *P. pinaster* *PpinDhn3* and *PpinDhn4* transcript levels and drought intensity which was common to all genotypes, we tentatively suggest that different provenances use different strategies for survival under drought condition. For example, in the case of the Mediterranean provenance, the difference in *PpinDhn3* expression between genotypes could be related to the decrease in Ψ_{nwpd} at the end of the stress period, possibly explained by water loss and osmotic adjustment (Table 2). There was high expression of *PpinDhn3* in the sensitive genotype of this provenance which showed a low Ψ_{nwpd} and early osmotic adjustment, whereas the tolerant genotype showed a high Ψ_{nwpd} , with no osmotic adjustment and low water loss. This

suggests that dehydrins may be regulated osmotically. On the other hand, the tolerant genotype of Continental provenance showed a higher *PpinDhn4* expression than the sensitive one: the sensitive genotype showing osmotic adjustment in the first phase of the drought treatment caused by loss of water, while the tolerant genotype expressed osmotic adjustment and decreasing Ψ_{nwpd} in the final phase. Thus, we may conclude that the early response of the sensitive genotype led to the accumulation of fewer transcripts than in the tolerant genotype, which had a later response to the drought. These results suggest a clear relationship between dehydrin expression, in our case, *PpinDhn3* and *PpinDhn4*, and drought stress (Fig. 6) using parameters such as water loss or Ψ_{nwpd} , as described by other authors, e.g., Cellier et al. (1998) and Volaire (2003).

Variation in gene expression was found between genotypes and provenances for the dehydrin genes studied (Figs. 5 and 6) and has also been observed in other species (Tschaplinski et al. 1998; Vaseva et al. 2010; Wilkins et al. 2009). Caruso et al. (2002) demonstrated a constitutive expression of *PeuDhn1* transcripts in leaves of *Populus euramericana*, which increased during drought conditions, but with transcript levels reaching their highest level following 3 days of re-watering rather than in the maximum drought condition. In other studies, it has been necessary to apply severe drought conditions to see large differences in transcript expression (e.g., Cellier et al. 2000).

Our results may also be attributable to other causes: Richard et al. (2000) observed differences in expression between needles, buds, stems, and roots of spruce, and it is also important to note that night/day fluctuations have been found to be significant in *Helianthus annuus* L (Cellier et al. 2000), with higher transcript levels in the early morning under mild drought and at midday under severe drought. Moreover, in studies of cocksfoot (*Dactylis glomerata*) (Voltaire et al. 2005), dehydrin expression differed between cultivars and seasons. It is also known that dehydrins might show different expression patterns when the stress period is increased, and between seasons and cultivars, as well as between drought-tolerant and drought-sensitive genotypes (Voltaire 2002; Cellier et al. 1998).

The complexity of the situation in relation to barley (*Hordeum spontaneum*) has been described by Suprunova et al. (2004), where the authors hypothesized that higher drought stress resistance could be attributed to the combination of different factors such as the high expression level of dehydrin genes, the presence of more efficient transcription factors, and/or more efficient signal transduction and early cellular perception of the stress. And Vaseva et al. (2010) observed that some wheat cultivars, defined as resistant to water deficit by means abscisic acid results, were found to be drought-sensitive when dehydrin expression profiles were used. This complexity of the drought-tolerance trait consequently implies that it cannot be genetically analyzed as a monogenetic character. It is obvious that in vitro evidence combined with dehydrins expression

profiles suggests that their function indeed correlates with the stress-survival of the plants.

Further investigations focusing on other physiological parameters as well as the analysis of expression of dehydrins and other water-stress-related genes are needed to provide further insight into the molecular aspects of drought tolerance in plants and also to clarify the roles of single members of the dehydrin gene family in the general pattern of stress response.

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