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Research article

Differential expression of heat shock protein genes in preconditioning for photosynthetic acclimation in water-stressed loblolly pine

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ABSTRACT

Heat shock proteins (HSPs) are induced not only under heat stress conditions but also under other environmental stresses such as water stress. In plants, HSPs families are larger than those of other eukaryotes. In order to elucidate a possible connection between HSP expression and photosynthetic acclimation or conditioning, we conducted a water stress experiment in loblolly pine (*Pinus taeda* L.) seedlings involving progressive treatment consisting of one cycle of mild stress (−1 MPa) followed by two cycles of severe stress (−1.7 MPa). Net photosynthesis was measured at each stress level. Photosynthetic acclimation occurred in the progressive treatment after the first cycle, but not in the severe treatment, suggesting that a cycle of mild stress conditioned the trees to adapt to a more severe stress. Real time results indicated specific patterns in needles in the expression of HSP70, HSP90 and sHSP genes for each treatment, both at maximum stress and at recovery. We identified a pine homolog to GRP94 (ER resident HSP90) that was induced after rehydration coincident with acclimation. Further analysis of the promoter region of the pine GRP94 showed putative cis-elements associated with water stress and rehydration, corresponding to the expression pattern observed in our experiment.

1. Introduction

Drought stress affects a large range of species, causing substantial losses in many crops. Even though the knowledge of mechanisms of tolerance to drought stress has increased lately, the functions of genes related to the events after the perception of the stress and the recovery response are not fully understood. Furthermore, in most cases, the drought stress has been imposed as a "shock" treatment, while dehydration in the field is gradual and prolonged. Under prolonged drying, plants have the opportunity to adapt their water relation status and photosynthetic capacity to the adverse conditions by stress-mediated gene expression and modification of physiological function and morphology. There have been many physiological studies of prolonged dehydration i.e. [1]. However, the effects of prolonged dehydration on gene expression have only been analyzed in a few cases: Arabidopsis (*Arabidopsis thaliana* L.) [2], tobacco (*Nicotiana tabacum* L.) [3] and barley (*Hordeum vulgare* L.) [4]. Moreover, these studies focus on the changes of gene expression under water stress for one cycle of water stress and do not investigate the possible conditioning effects of a first stress cycle on plant responses during consecutive cycles of water stress, which is what occurs in nature.

The genes that are expressed under water stress can be classified into two groups. The first group consists of genes that are related to drought stress-mediated signaling, such as transcription factors or kinases. The second group are downstream genes related to tolerance to water stresses, such as LEA proteins [5], enzymes for metabolism of osmolytes [6], antioxidant proteins [7] and chaperones or HSPs [2,3].

Our work focused on loblolly pine (*Pinus taeda* L.), which covers approximately 134,000 km^2 in the US [8]. Drought stress is the most common cause of pine mortality in the US [9]. Relatively few studies of water stress responses have been conducted in forest trees and fewer yet in conifers [10,11]. A recent study, has identified water stress responsive genes in loblolly pine roots using EST libraries of roots of plants that were exposed to a severe level of water stress (−1.75 MPa) [12].

Abbreviations: HSP, Heat shock protein; BIP, Luminal binding protein; PDI, Protein disulfide isomerase; UPRE, Unfolded protein response element; STRE, Stress response element.

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Consecutive cycles of water stress can result in water stress "acclimation", in the form of higher rates of photosynthesis during later cycles, as we observed in loblolly pine seedlings under mild stress conditions [13]. We showed that expression patterns of genes encoding specific heat shock proteins (HSPs) are correlated with photosynthetic acclimation under mild water stress.

HSPs are classified according to their molecular size and some are expressed constitutively, while others are induced by stress. There is some knowledge of the possible protective role of these proteins during stress responses, but little is known about their role and mode of action during successive cycles of water stress responses and in gymnosperms. Overexpression of an ER resident HSP70 (BIP) in tobacco [14] conferred water-stress tolerance. The regulated expression during seed development of an Arabidopsis sHSP, sHSP17.4, suggested a protective role in desiccation tolerance [15] and overexpression of a tomato mitochondrial sHSP in tobacco increased thermotolerance [16].

In this study, we investigated the behavior of heat shock proteins during photosynthetic acclimation and different levels of water stress in loblolly pine seedlings. Real time PCR was used to determine the expression of HSPs that belong to the HSP70, HSP90 and sHSPs families. We identified a pine homolog to GRP94 (ER localized HSP90) associated with photosynthetic acclimation. We also found that the expression of homologs of the cytosolic HSP70 gene family is differentially regulated, depending on the level of water stress.

2. Materials and methods

2.1. Plant material and water stress application

Rooted cuttings of loblolly pine from the Atlantic Coastal Plain were propagated clonally by Dr. Barry Goldfarb at North Carolina State University (NCSU). Trees were grown in pots in a greenhouse with supplemental lighting to maintain 16 h day-length and with the temperature controlled at 24 °C during the day and 18 °C at night. Plants were watered as needed and fertilized once a week with half strength Hoagland's solution. Trees were subjected to three cycles of either mild or severe water stress, or one cycle of mild stress, followed by two cycles of severe stress (progressive treatment). Each treatment (severe, mild and progressive) had its own set of well-watered (control) trees. Mild stress was defined by a pre-dawn water potential of −1.0 MPa and severe stress by a water potential of −1.7 MPa. Water potential was measured using a Plant Water Status Console (Soilmoisture, Santa Barbara, CA) on at least two seedlings. Once stressed plants had reached the desired water potential, net photosynthesis measurements were made on at least two seedlings and the plants were re-watered. Photosynthesis was measured at light saturation on a Li-Cor 6400 (LICOR Biosciences, Lincoln, NE). Needles were harvested at different points throughout the drying cycle (−0.4 and −0.7 MPa for mild and −0.6 and −1.2 in severe), at the point of maximum stress (−1.0 for mild or −1.7 MPa for severe) and 24 h after re-watering from both treated and well-watered, control seedlings (Fig. 1). At each sampling time, needles were taken from four different seedlings (two treated and two control), flash frozen with liquid nitrogen and stored at −80 °C until RNA extraction. In total 36 seedlings were used for each treatment (18 treated and 18 control).

2.2. RNA extraction and real time PCR

RNA was extracted from needles according to Watkinson et al. (2003). Two step real time PCR was performed to measure the level of expression. Total RNA was DNAse treated with the DNAfree kit (Ambion, Austin, TX). Then 2 μg of RNA were reverse transcribed using Superscript II (Invitrogen, Carlsbad, CA).

Real time PCR was performed with 5 μL of cDNA (from a 20 ng/μL dilution) using SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA) in a 25 μL reaction volume on an ABI Prism 7700 Sequence Detection System (Applied Biosystems), with 0.5 μM primer final concentration and the following cycling steps: initial denaturation for 10 min at 94 °C, followed by 34 cycles with 15 s at 94 °C, 30 s at 56 °C and 30 s at 72 °C and a 20 min gradient from 60 to 90 °C to obtain a melting curve. The data were collected at the extension step (72 °C). Absolute quantification was carried out using a 10 fold dilution of the plasmids containing the *P. taeda* sequences in concentration between 10 pg and 0.1 fg.

The adenosine kinase gene (AK), the expression of which was shown previously to remain unchanged during water stress in loblolly pine [13] was used for normalization (Table 1). At least three technical repeats per biological repeat were analyzed. Deviations from threshold values were less than 0.5 cycle for technical replicates and less than 1 cycle for biological replicates. The following genes were amplified: GRP94, BIP3, PDI5, HSC70-1, HSC7 70-3, HSP70-10, DnaJ, sHSP17.6, sHSP25.3 and sHSP23.6 (Table 1). The names assigned to the genes are the names assigned to the Arabidopsis homologs in TAIR. All primers pairs were tested for dimer formation before using them with the actual samples.

2.3. Cloning of pine GRP94 cDNA

Primers were designed using the loblolly contig for the GRP94 homolog in the Gene Index Project Database: (http://compbio.dfci.harvard.edu/tgi/plant.html) (TC73014, Table 2). A PCR reaction using cDNA as template was performed using 5 μL of cDNA (from a 20 ng/μL dilution) and GoTaq green master mix (Promega, Madison, WI) in a 20 μL reaction volume with 0.5 μM final primer concentration. The cycle steps were: denaturation for 3 min at 94 °C, followed by 34 cycles of 30 s at 94 °C, 30 s at 56 °C and 1 min 30 s at 72 °C. The PCR product obtained was sequenced in the Virginia Bioinformatics Institute (VBI) core laboratory facilities (Blacksburg, VA), using the ABI BigDye Terminator kit (Applied Biosystems) and analyzed on an Applied Biosystems 3730. The 5′UTR region was obtained using the 5′-full RACE Core Set (Takara, Madison, WI) using a 5′ phosphorylated primer located within 200 bp of the cDNA already obtained (RACE_1, Table 2) following the manufacturer protocol. The PCR product obtained was then cloned into the pGEM T-easy vector (Promega) and sequenced at VBI.

2.4. Cloning of promoter region of pine GRP94

DNA was isolated using the same protocol as that used for RNA isolation [13] but using isopropanol to precipitate the DNA. A modified version of TAIL-PCR [17] was then used to amplify the 5′ flanking region of GRP94. Five specific primers were designed close to the 5′ end of the coding region, two in the first exon: GRP94_A, GRP94_B and three in the 5′UTR region: GRP94_C1, GRP94_C2 and GRP94_C3 (Table 2). GRP94_A was paired with one of 5 random primers previously used for RAPDs in pine: 203, 210, 297, 244 and 278 [18] and used for the primary PCR amplification (unspecific). The primary PCR reaction was performed with 100 ng of genomic DNA in a 20 μL reaction with 0.4 μM final GRP94_A primer concentration, 2 μM of final random primer concentration, GoTaq master mix (Promega) and the cycling steps for primary PCR from Terauchi and Kahl [16]. The product of this reaction was diluted 1:50. The secondary PCR reaction (20 μL) was performed with 5 μL of a 1:50 dilution from the primary PCR reaction, with 0.4 μM final GRP94_B primer concentration, 2 μM of final random primer concentration, GoTaq master mix (Promega) and the cycling

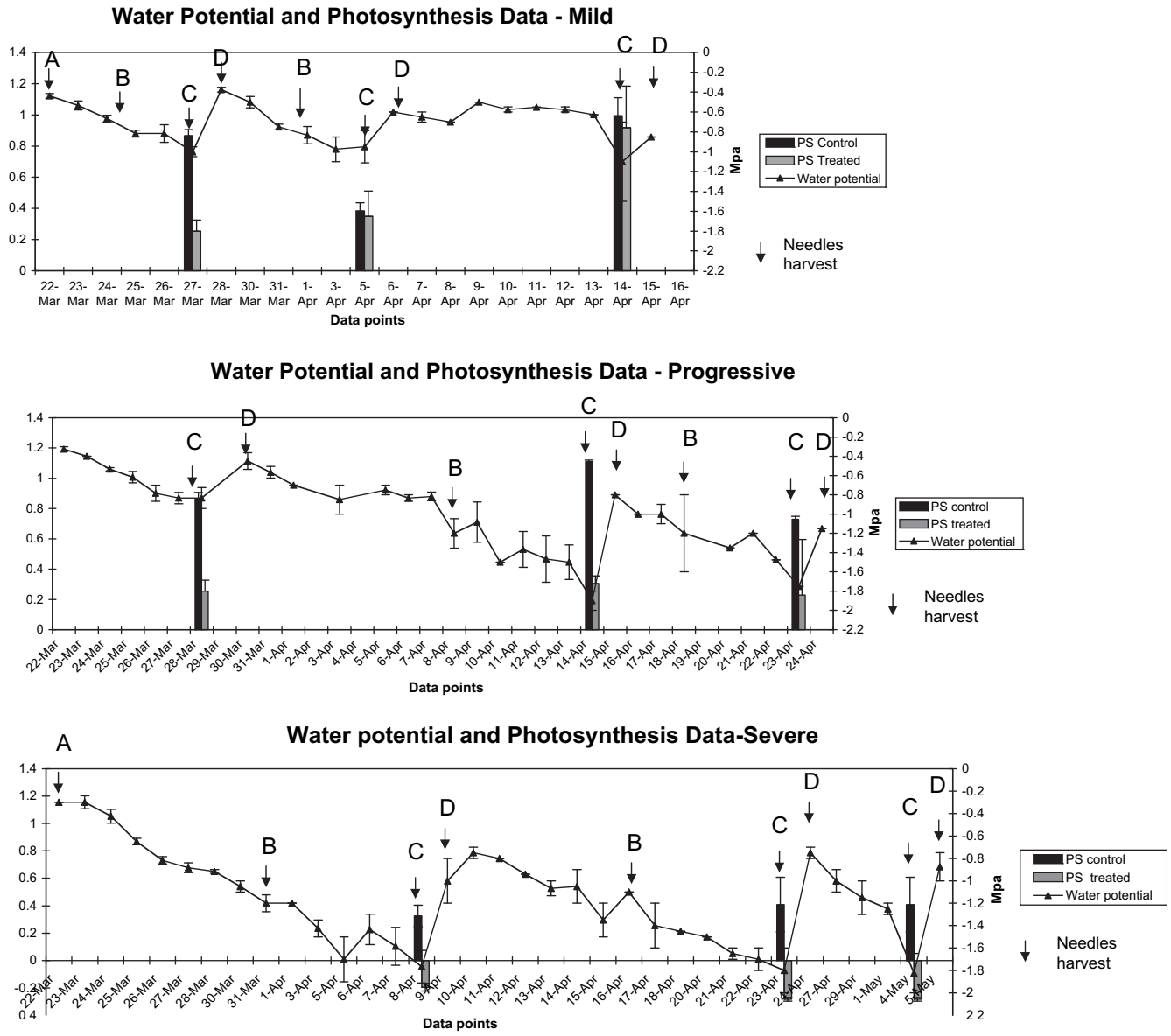

Fig. 1. Effects of water stress on water potential and photosynthesis. Physiological responses to water stress in the mild treatment (a) and the progressive treatment (b). Net photosynthesis data (PS) are represented as columns and the water potential of treated plants as lines. Point A: Beginning of the stress, B: 50% of initial water potential, C: maximum stress (−0.8 to −1.0 MPa for mild stress and −1.6 to −1.8 MPa for severe stress), D: 24 h after re-watering (recovery). Arrows show the time of harvest of needles. Error bars represent standard error, at least two replicates were measured. The water potential of control plants was around −0.3 MPa (not shown).

Table 1
List of genes and primers used for real time PCR amplification.

Gene name	Pine EST	Arabidopsis homolog	Subcellular location	Forward primer	Reverse primer
GRP94	NXNV_149_E10	At4g24190	ER	5′-TGGAGGGTTGTTGGTTAGAG-3′	5′-AGGGACATCTAGCACACACC-3′
BIP3	NXNV_158_D06	At1g09080	ER	5′-TGGGTGTTTTTCTAATTG-3′	5′TCATCCTACACTATTTGC-3′
PDI5	NXCI_042_G05	At1g21750	ER	5′-TTTACCTTCTTTCAAGTGGC-3′	5′-CTAATGGCAAAAGCGTTG-3′
DnaJ	NXNV_132_E06	At3g44110	Cyt	5′-GGCACGAGAGACAACTTTG-3′	5′-TGTTGCTGCTTCCTTCTTAG-3′
HSC70-3	NXSI_117_C08	At3g12580	Cyt	5′-GTCCCAAGATCGAGGAAGTG-3′	5′-CACGCAGGAGGTGATGAAG-3′
HSC70-1	ST17D07	At5g02500	Cyt	5′-TATTCCTGCGTTGGGGTGTGGC-3′	5′-CTCCAATGAGTCGCTCAGTATC-3′
HSP70-10	NXNV_123_C06	At5g09590	Mit	5′-GTTATGACTGTTGACTCTGG-3′	5′-TGGACCATTTGAATAAGG-3′
sHSP25.3	NXLV_066_C03	At4g27670	Chl	5′-ATGTGAAGGTCTCTGTGGTGGAGG-3′	5′-GTATGGTATCTGATGTGCGAAGGC-3′
sHSP23.6	NXSI_067_H12	At4g25200	Mit	5′-AACAGAAATACAGTAGCCGCATCG-3′	5′-AGCACGCCATTCTTCATCTG-3′
sHSP17.6	ST40F04	At1g53540	Cyt	5′-CGAAGCATTCACACATCTGG-3′	5′-AGTATTGGCTACGGCAGAAG-3′
AK	NXSI_116_A09	At3g09820	Cyt	5′-GTGAGTTCCAGTTGCCTTTG-3′	5′-GGGTGGGAGACTGACAATG-3′

ER: Endoplasmic Reticulum, Cyt: Cytosol, Mit: Mitochondria, Chl: Chloroplast.
Names assigned to the pine genes are according to the names assigned to the Arabidopsis homologs found in The Arabidopsis Information Resource database (TAIR).

Table 2
List of primers used for GRP94 full length and promoter cloning.

Primer name	Sequence
TC73014_FP	5′-GGACCGATACAATGTGCC-3′
TC73014_RP	5′-AACCCTCCAAAATTCCTG-3′
RACE_1	5′-/5Phos/CAAAGCATCAGAGGC-3′
GRP94_A	5′-CGCAACATCTGCGTCCGTAGAGAGT-3′
GRP94_B	5′-GGACTGGCATTCTCATCGGCAGG-3′
GRP94_B_in	5′-GGACCTGCACAAAACCCCAATC-3′
GRP94_C1	5′-GGCACTTGAACTCGCACACTCC-3′
GRP94_C2	5′-ACCTTACTACAACCCCCTGGCCCT-3′
GRP94_C3	5′-GCTTTTATAGGCACATTGTATCGGTCC-3′
203	5′-CACGGCGAGT-3′
210	5′-GCACCGAGAG-3′
297	5′-GCGCATTAGA-3′
244	5′-CAGCCAACCG-3′
278	5′-GGTTCCAGCT-3′
NeedleGRP94_F	5′-CCAAGTCACGGGTGACAAAA-3′
RootGRP94_F	5′-CGCGAAAATGCAACTGCTAGAATATC-3′

steps from secondary PCR from Terauchi and Kahl [16]. The tertiary PCR reaction (20 μL) was performed with 5 μL of a 1:25 dilution from the secondary PCR reaction, with 0.4 μM final GRP94_C1 or GRP94_C2 or GRP4_C3 primer concentration, 0.5 μM of final random primer concentration, GoTaq master mix (Promega) and the following cycling steps: 10 s at 94 °C, 1 min at 64 °C, 2.5 min at 72 °C, 10 s at 94 °C, 1 min at 29 °C and 2.5 min at 72 °C for 15 cycles and a final extension of 5 min at 72 °C.

3. Results

3.1. Some preconditioning for severe stress occurs after exposure to a mild stress

Fig. 1 shows the effect of different levels of water stress on photosynthesis. After one cycle of mild water stress (−0.8 MPa aprox.) the trees "acclimated", by the criterion of higher photosynthetic rates under stress after the second cycle, when rates in treated trees was similar to control trees, as we observed previously [13]. Water potential of the control plants was between −0.35 and −0.3 MPa during the whole experiment.

In the second cycle of the progressive experiment, in which the trees experienced a level of stress similar to the second cycle of the severe treatment, photosynthesis was reduced compared to the control treatment (treated: 0.3 ± 0.05 μmol $CO_2/m^2/s$, control: 1.1 ± 0.01 μmol $CO_2/m^2/s$) but it was not completely inhibited as we observed for plants subjected to severe stress with no prior mild (preconditioning) water stress cycle (−0.20 ± 0.03 μmol $CO_2/m^2/s$, −0.28 ± 0.012 μmol $CO_2/m^2/s$ and −0.28 ± 0.013 μmol $CO_2/m^2/s$ for cycles 1, 2 and 3 respectively). Therefore, even though photosynthesis did not recover completely, exposure to a mild level of water resulted in some photosynthetic acclimation.

3.2. Some HSPs are associated with photosynthesis preconditioning

In our previous experiment [13] we observed a differential expression of HSPs between mild and severe treatments. Therefore, in order to see if there were HSP genes whose expression correlates with preconditioning to water stress, the expression of HSP genes that showed induction during photosynthetic acclimation or that have been shown to be induced during water stress in other systems was analyzed through real time PCR.

From those results, the expression patterns of two chaperone/protection and repair genes were found to be correlated with acclimation, GRP94, ER localized HSP90, and PDI5, ER localized PDI (Fig. 2a and b). GRP94 and PDI5 were induced during the first cycle of recovery under both treatments, mild and progressive (both mild stresses). GRP94 expression was also induced at the recovery point of the second and third cycle of both progressive and mild treatments. HSC70-1, on the other hand, was induced at maximum stress under the two severe cycles of the progressive treatment and not responsive under the mild stress, while HSC70-3 was induced under the mild treatment and not responsive throughout the progressive treatment.

In contrast, GRP94 and HSC70-1 were induced only during the first cycle of severe stress (drying down to −1.7 MPa), when no "pre-conditioning" mild stress (drying only down to −0.8 MPa aprox.) had been administered (Fig. 3a). After that, during the subsequent two cycles of severe stress, their expression levels were not affected, in contrast to the results obtained in the plants that had received the progressive stress treatment (Fig. 3a). PDI5 and HSC70-3 fold change did not show any pattern with values between 0.6 and 1.0 for the 3 cycles with the exception of a value of 2.0 of PDI5 during recovery in the 3rd cycle (point S3D).

3.3. Other members of HSP families increase their expression after more than one cycle of severe stress

Real time PCR results obtained with cDNA from severely stressed samples (−1.7 MPa) showed an increased expression of HSP70-10 and sHSP23.6, both mitochondrial proteins, and ER-localized BIP3 at each point of maximum stress (Fig. 3b).

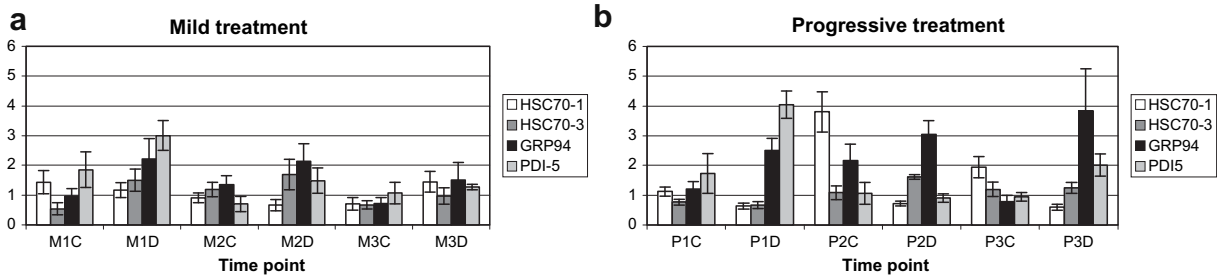

Fig. 2. Relative expression of genes associated with photosynthetic acclimation. Real time PCR results from needle cDNA in cases where acclimation occurred: 2nd and 3rd cycle of mild treatment (a) and 2nd and 3rd cycle of progressive treatment (b). Each bar represents the fold change of treated vs control plants (2 samples with 3 replications). a) M1C, M2C, and M3C are from samples taken at the maximum stress point of the 1st, 2nd and 3rd cycle of mild stress respectively. M1D, M2D and M3D are from samples taken at the "recovery" point (24 h after watering) of the 1st, 2nd and 3rd cycle respectively. b) P1C, P2C and P3C are from samples taken at the maximum stress point at the 1st, 2nd and 3rd cycle of progressive stress, respectively. P1D, P2D and P3D are from samples taken 24 h after watering at the 1st, 2nd and 3rd cycles, respectively.

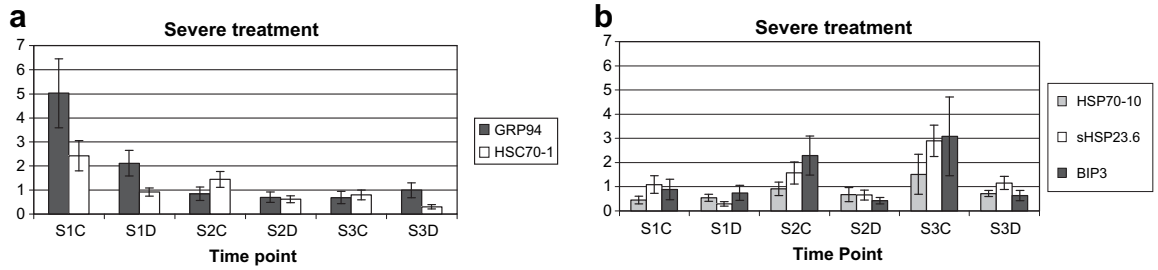

Fig. 3. Relative expression of protection and repair genes during severe stress. Real time PCR results obtained with cDNA from needles subjected to the severe treatment that are highly induced after exposure to one severe drought stress cycle but unresponsive thereafter (a) and genes whose expression is increased after each cycle of stress (b). Each bar represents the fold change of treated vs control plants (2 samples with 3 replications). S1C, S2C and S3C are from samples taken at the maximum stress point of the 1st, 2nd, and 3rd cycle of severe stress, respectively. S1D, S2D and S3D are from samples taken 24 h after watering of the 1st, 2nd and 3rd cycles, respectively. HSP70-10 and sHSP23.6 gene products localize to the mitochondrion and BIP3 to the ER.

3.4. Pine GRP94 is closely related to GRP94s from Xerophyta viscosa baker and from monocots

GRP94 showed induction during the recovery of plants that showed photosynthetic acclimation. It was of interest, therefore, to obtain the full length cDNA sequence for this gene. This was achieved by doing PCR on pine needle cDNA with primers based on the ESTs in the Pine Gene Index Project database. The translated sequence was compared with other plant ERHSP90 proteins using Clustal W (Fig. 4). The pine protein is very similar to the other plant proteins: 76.3% similarity to the Arabidopsis ERHSP90, 77.7% to barrel clover (*Medicago trunculata* Gaertn.), 78.7% to tomato (*Lycopersicon esculentum* Mill.), 78.9% to cottonwood (*Populus trichocharpa* L.), 79.2% to maize (*Zea mays* L.), 79.2% to rice and 80.6% to *X. viscosa*. The residues that are known to be involved in ATP binding and hydrolysis [19] are identical among the 8 plant proteins (Fig. 4) and human GRP94 (data not shown), which suggest that pine ERHSP90 is an ATPase. Pine ERHSP90 is closely related to monocot GRP94s: *X. viscosa*, rice and maize (Fig. 5).

3.5. The promoter region of pine GRP94 contains cis-elements responsive to water stress and the unfolded protein response element (UPR)

Several specific primers (Table 2) were used for the isolation of the promoter region from genomic DNA, but after isolation of the 5′UTR it appeared that the promoter region obtained did not overlap with the 5′UTR region obtained for the full length cDNA. This suggested the possible existence of more than one GRP94 gene in pine and that, consequently, the 5′UTR region obtained belonged to the gene that is expressed in needles, whereas the promoter region obtained is from another member of the same gene family. In order to obtain the promoter region of the specific GRP94 gene that is expressed in needles, TAIL-PCR was used, with primers designed specifically in the 5′UTR of the full length needle cDNA. All the PCR cycles were done by pairing each specific primer with each random primer. The primer used for the secondary PCR was a primer (GRP94_B_in, Table 2) which falls in a junction between the first exon and first intron of the GRP94 expressed in needles (data not shown). The promoter region, which included part of the first exon, isolated previously did not have an intron at that location. The tertiary PCR yielded bands with GRP94_C1 using the 203, 244 and 210 primers. Each band was cloned and sequenced. Only the band from the combination of 244 and GRP94_C1 primers matched the 5′UTR sequence obtained from the GRP94 that is expressed in needles. After that an specific gene was designed (NeedleGRP94_F, Table 2) and together with the secondary primer (GRP94_B_in, Table 2) and a band of the same size was obtained. This band was then sequenced. The sequence obtained was indeed part of the promoter of the GRP94 expressed in needles. The corresponding promoter region was analyzed using the cis-element finding part of the program XcisClique [20], comparing it with the promoter of the Arabidopsis GRP94 homolog. Fig. 6 shows the motifs found by XcisClique.

Cis-elements related to water stress, such as the MYB2CONSENSUSAT and MYCCONSENSUSAT elements, where AtMYB2 and AtMYC2, dehydration and ABA responsive transcription factors, respectively, bind [21] are present in the sequence of the upstream region in pine GRP94. Mammalian GRP94 [22] and plant GRP94 [23] have been shown to be responsive to ER stress or the Unfolded Protein Response (UPR). The UPRMOTIFIAT element, which is identical to one of the two versions of the mammalian UPRE: CCACGTCA [23], is also found in the promoter region of the GRP94 gene that is expressed in needles (Fig. 6).

Other motifs found in the needle promoter region are two stress responsive elements (STRE). The cytosolic HSP90 from Arabidopsis (HSP90-1, At5g52640), is responsive to metal stress [24] and STREs are involved in that response [24].

While mining the loblolly ESTs in the TIGR database, it appeared that the putative second GRP94 loblolly gene is expressed in roots, since an assembly of ESTs expressed only in roots produces a sequence that exactly matches part of the promoter region isolated earlier. Fig. 7 shows the promoter region of the putative root GRP94 gene. This sequence was also confirmed with the following gene specific primers: Root GRP94_F and GRP94_B (Table 2). Among the motifs found was a heat shock element (HSE), but with only one mutation in the middle motif (TAC instead of TTC). It also has two water stress elements not found in the needle promoter: RAV1AAT and RAV1BAT [25]. Another cis-element found is PRE (Pro or hyposmolarity responsive element), which is also found in the upstream region of the rehydration responsive ProDH (proline dehydrogenase) gene [6]. There is no UPR element in the ~1000 bp of sequence obtained, while all the other plant GRP94s, including the pine needle expressed GRP94 have at least one UPR element within ~400 bp from the first codon.

4. Discussion

4.1. Possible role of heat shock proteins in preconditioning to water stress

Exposure to a mild water stress stress enabled the plant to become “conditioned”, or perform better when exposed to a second cycle of more severe stress. We have observed that certain HSPs are induced during recovery when photosynthetic acclimation occurs e.g. HSC70-1 and HSC70-3 (Fig. 2a and b). Overexpression of

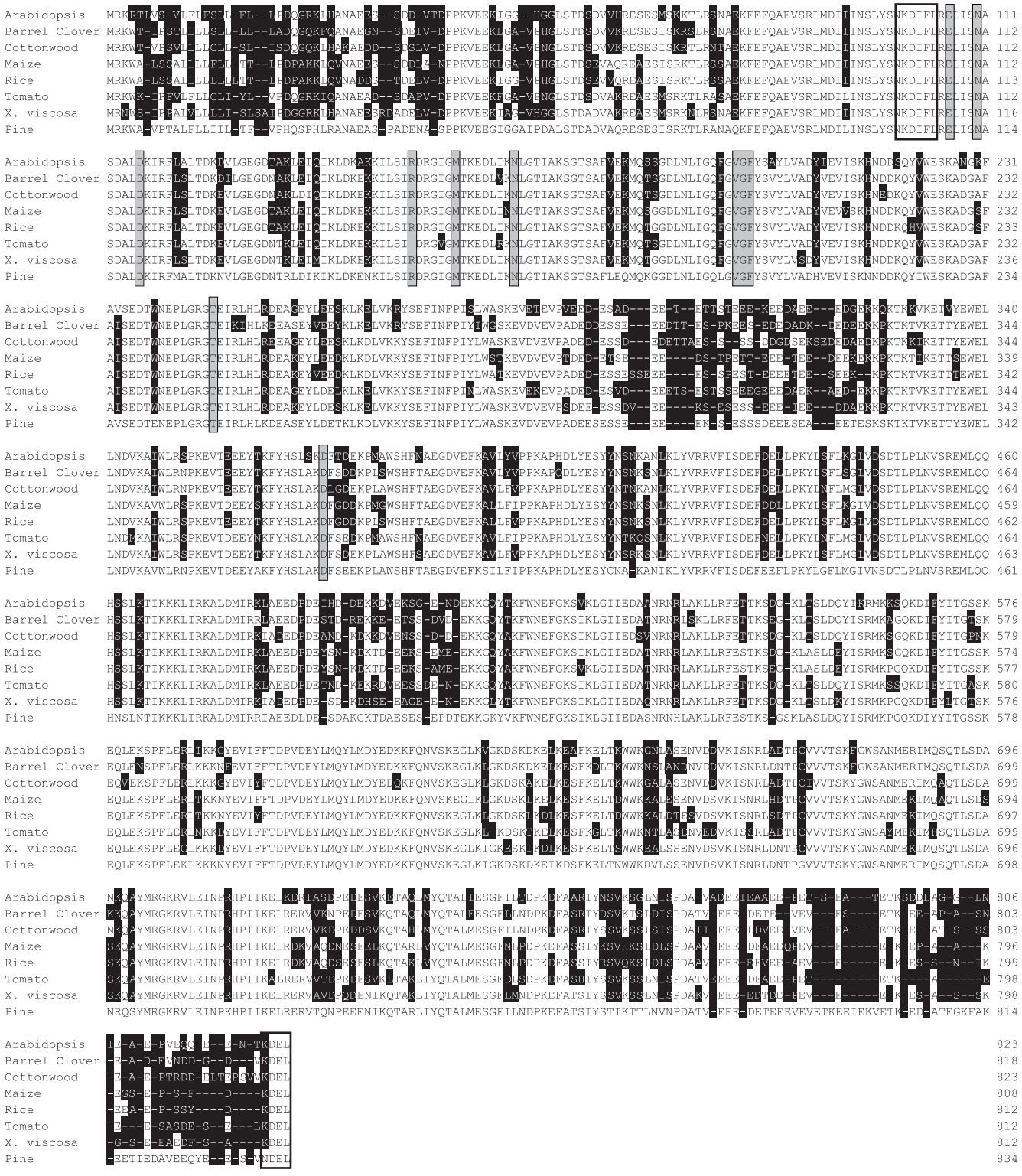

Fig. 4. Protein alignment of GRP94 from pine compared with related plant GRP94s. Individual residues that are not conserved are shaded in black. The black boxes indicate the HSP90 family signature sequence (NKDIL) and the ER retention signal (KDEL). The gray shaded boxes indicate residues that are required for ATP binding and hydrolysis: E109, N113, D116, D157, M162, N170, V200, G201, G202, T249 and K371 in the pine sequence. The GenBank accession numbers for the sequences are: Q8SB39 for rice and AAN34791 for *X. viscosa*. For maize the translation of the GenBank sequence AY103537 was used. The cottonwood sequence was obtained from the Poplar genome web page (http://genome.jgi-psf.org/Poptr1/Poptr1.home.html): ID, ESTEXT_FGENESH1_PM_V1.C_LG_V0560. The Arabidopsis sequence, At4g24190, was obtained from the Arabidopsis Genome web page (www.arabidopsis.org). The Barrel Clover sequence was obtained from the Gene Index Project Database (http://compbio.dfci.harvard.edu/tgi/plant.html) and the ID is TC94521_framefinder ORF. The pine needle expressed cDNA sequence is EU140579 in GenBank.

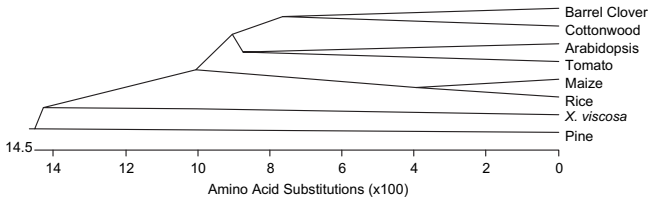

Fig. 5. Phenogram of plant GRP94s. The alignment used to generate the phenogram is the same as is shown in Fig. 4.

HSC70-1 in Arabidopsis plants resulted in increased thermotolerance [26]. Silencing of the gene did not produce viable plants, illustrating the importance of HSC70-1 under stress and normal conditions [26]. The Arabidopsis homologs of HSC70-1 and HSC70-3 are induced after virus infection and/or overexpression of proteins in the cytosol. This suggested that these genes respond to the accumulation of unfolded proteins in the cytosol, an event similar to the unfolded protein response in the ER [27]. Water stress could also cause protein accumulation in the cytosol [28] and these genes might be induced in pine for this reason.

A recent study in *N. tabacum* showed that overexpression of an homolog to HSC70-3 (NtHSP70-1) confers water-stress tolerance in 3 week old tobacco seedlings [29]. Moreover, plants overexpressing the gene were able to maintain a lower water potential compared to the empty vector and antisense plants. The overexpressing plants experienced less stress, with a strong correlation between water content and the amount of NtHSP70-1, suggesting a role in regulating water flux. More studies will be needed to find the specific mechanism by which these genes confer water-stress tolerance. In our experiment with pine, HSC70-1 showed induction at the maximum stress point in the first, second and third cycle of progressive treatment (Fig. 2b), and at the first cycle of the severe treatment (Fig. 3a). The behavior of HSP70-1 during progressive stress suggests a role in acclimation or response to severe water stress conditions, perhaps in regulating water flux.

The other genes that were induced when photosynthetic acclimation occurred were pine homologs to GRP94 and PDI, which are part of the unfolded protein response and have been shown to be induced after tunicamycin treatment [23]. The presence of the UPR element in the promoter region of GRP94 is further evidence for a role for pine GRP94 in this response to stress. It has been shown that homologs to these genes in mammalian systems are part of a protein complex [30] that binds to nascent proteins like immunoglobulin (Ig) heavy chains. The pine homologs of GRP94 and PDI might be part of a complex involved in the protection/repair/folding of proteins during mild stress, which could help the plant cope with subsequent stress cycles.

4.2. Possible role of heat shock proteins under severe water stress conditions

Pine mitochondrial HSPs such as mitochondrial HSP70 and mitochondrial sHSP (Fig. 3b) may have a functional role during high levels of stress, since their expression increased at each succeeding cycle of severe stress. During severe stress, when the levels of ROS are high [31], the increased expression of mitochondrial HSP70 might be due to an increase in refolding/transport of antioxidant proteins to the mitochondrion. The mitochondrial sHSP is homologous to the mitochondrial tomato sHSP23.6 that, upon overexpression, confers thermotolerance to tobacco plants [16]. Moreover, it has been shown that mitochondrial sHSP protects the NADH:ubiquinone oxidoreductase complex during heat stress in plants [32]. Since heat stress also causes ROS production in the mitochondrion [33] the increased expression at each cycle of this gene might be due to an increased need of protection of this complex. Therefore, these genes may help to protect the mitochondrion reduce, repair, or protect against oxidation damage and, under natural conditions, heat might also be experienced when water stress occurs [3].

The pine BIP3 that showed gradual induction after each cycle of severe stress is closely related to a soybean BIP3 that, when overexpressed in tobacco, conferred tolerance against severe levels of water stress [14]. Since BIP3 is responsive to tunicamycin, which induces accumulation of proteins and activates the unfolded protein response [34], the increasing expression of this gene might be related to an increased accumulation of unfolded protein due to the severe water stress applied.

4.3. Importance of pine GRP94 during water stress responses

Pine GRP94 was one of the genes that showed induction in both treatments where photosynthetic acclimation occurred. This protein is part of the unfolded protein response in mammalian and plant cells. Like the other members of the HSP90 family it has a "maturation" role for important proteins. In mammalian cells, among the target proteins that GRP94 regulates are proteins that are transported to the cell surface: Toll-like receptors, which activate immune cell responses, or proteins that are secreted such as IgGs (Immunoglobulin chains) [35].

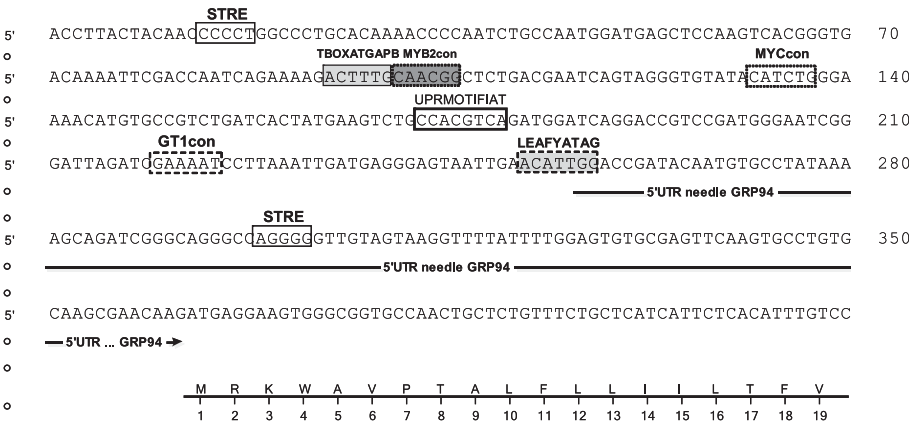

Fig. 6. Cis-elements found in the promoter region of the pine GRP94 expressed in needles. Cis-element names are according to PLACE (Higo et al., 1999), with the exception of STRE: Stress Responsive Element (Haralampidis et al., 2001), MYB2con (abbreviation of MYB2CONSENSUSAT from Higo et al., 1999) and MYCcon (abbreviation of MYCCONSENSUSAT from Higo et al., 1999). The GenBank accession number of the pine *GRP94* promoter that is expressed in needles is EU182218.

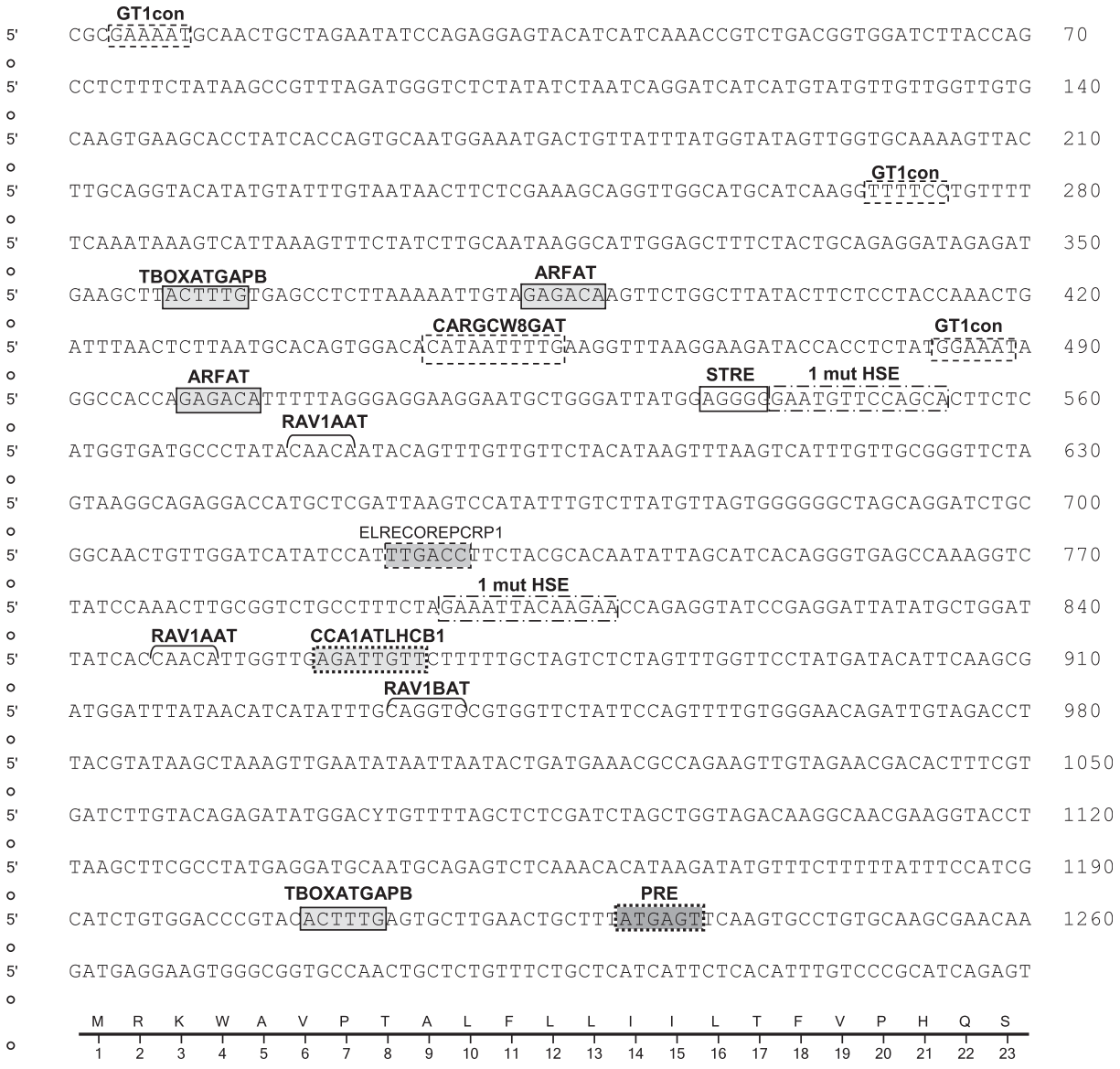

Fig. 7. Cis-elements found in the promoter region of the putative pine GRP94 expressed in roots. Cis-element names are according to PLACE (Higo et al., 1999), STRE: Stress responsive element (Haralampidis et al., 2001), 1mut HSE: Heat shock element with one mutation (Pati et al., 2006), GT1con (Abbreviation of GT1CONSENSUS from Higo et al., 1999) and PRE: Pro or hyposmolarity responsive element (Abe et al., 2003). The GenBank accession number for this promoter sequence is EU140580.

The region that is most dissimilar in pine with respect to the other known GRP94 proteins is the C-terminus which is important for protein dimerization and seems to be involved in co-chaperone interaction [36]. Cytosolic HSP90s contain the MEEVD domain in their C-terminal region for interaction with co-chaperones that contain the tetratricopeptide repeat domain (TPR) such as cyclophilins [36]. However, ER resident HSP90s do not contain that domain [37] and the pine GRP94 homolog does not contain that domain either. Nevertheless, Meunier et al. (2002) found that mammalian GRP94 forms a large multi-chaperone complex that includes a luminal cyclophilin B that does not have a TPR domain, suggesting that GRP94s may interact with co-chaperones, perhaps through an interacting domain that has not been identified yet.

The promoter region of the pine GRP94 expressed in needles contains the UPRE suggesting activation of this response during water stress. Since water stress could also cause unfolding of proteins when it is severe and we observed high induction of this gene during the first cycle of severe stress (Fig. 3), it is possible that the severe response includes the UPRE at the subcellular level. The presence of the water stress related elements together with the UPRE could partially explain the response of pine GRP94 induction during recovery in mild and progressive stress. However more research needs to be done, since the response depends on the water stress level and preconditioning cycles.

The finding of the promoter region of a putative root specific GRP94 in pine, suggests the presence of two GRP94 genes in pine. However in the plants whose genome has been already sequenced: Arabidopsis, rice and poplar (cottonwood) only one GRP94 have been found.

In Arabidopsis one of the protein targets that have been identified is a CLAVATA protein, which regulates meristem development [38]. Pine GRP94 is more similar to the *X. viscosa* GRP94 protein than to the Arabidopsis protein. The *X. viscosa* GRP94 is responsive to water stress, gradually induced during the transition from 74% to 7% RWC and also during rehydration (from 34% to 93% RWC). It is

also induced by heat and salt stress but it is not induced by cold stress and high light [39]. *X. viscosa* GRP94 was induced only by certain stresses, which might suggest that GRP94 is induced to activate/fold specific targets, since HSP90s target specific proteins. The needle expressed pine GRP94 may have a similar role under water stress and may repair specific target proteins that help to protect the photosynthetic machinery. Protein interaction experiments could help find the target proteins of this gene product.

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