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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.
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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

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