

Relationships Between Expression of Heat Shock Protein Genes and Photosynthetic Behavior During Drought Stress in Plants

Cecilia Vasquez-Robinet

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Ruth Grene, Ph.D., Chair
Eric Beers, Ph.D.
Glenda Gillaspay, Ph.D.
Lenwood S. Heath, Ph.D.

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ABSTRACT

Heat shock proteins (HSPs) are expressed in response to environmental stresses. Compared to other kingdoms, plant HSP families are larger, presumably the result of adaptation to a wide range of stresses. Following on an analysis of drought stress characteristics in loblolly pine (Watkinson et al., 2003), expression patterns of HSP gene expression during photosynthetic acclimation were examined. One cycle of mild (-1Mpa) followed by two cycles of severe stress (-1.7Mpa) were probed for conditioning effects. Photosynthetic acclimation occurred after the first cycle. No acclimation occurred without the first mild cycle. Microarray/RT-PCR analyses showed that a pine homolog to GRP94 (ER-resident HSP90) was up-regulated after rehydration coincident with acclimation. This GRP94 is closely related to GRP94 from the desiccation tolerant plant *X. viscosa*, supporting the importance of this gene during acclimation to water deficit. HSP genes whose products localized to the mitochondrion showed gradual up-regulation after consecutive cycles of severe drought.

The Arabidopsis pine GRP94 homolog, (AtHSP90-7) was then analyzed, using bioinformatics (Pati et al., 2006) and laboratory tools. Genes encoding putative candidate co-chaperones for GRP94 and other HSP90s were discovered, which contained water stress-related cis-elements. Arabidopsis (Col-0) wild type and two T-DNA insertion mutants in HSP90-7 were used to study the importance of this gene for photosynthetic acclimation. Only the mutants were able to acclimate to drought stress, with the level of AtHSP90-7 expression in the mutants being reduced compared to the wild type. AtHSP90-7 may have a different role in Arabidopsis, and its reduced expression activated other protective genes (Klein et al., 2006).

Responses to extreme drought in resistant (Sullu) and susceptible (Negra Ojosa) lines of Andean potatoes were also compared in order to identify relationships between HSPs gene expression, and tolerance, defined as the ability to maintain photosynthesis at

50% after 25 days of drought and to recover from the stress. Tolerance was correlated with up-regulation of HSPs (mostly chaperonins) and antioxidant genes all of whose gene products are located in the chloroplast.

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Chapter 1 Introduction

1.1. Drought stress effects and physiology

Low water availability is the main environmental factor that limits plant growth and yield in crops worldwide (Flexas et al., 2006; Valliyodan and Nguyen, 2006). Global climate change will likely reduce water availability still further in a larger amount of land (Hamdy et al., 2003) increasing the need for drought tolerant crops. For example, high yield varieties of rice (HYR) are very sensitive to drought stress even under short time exposures (Lafitte et al., 2007). The most consumed variety of potato (*S. tuberosum* subsp *tuberosum*) is highly susceptible to drought stress (Weisz et al., 1994; Shock et al., 1998) reducing yield up to 53% (Lahlou et al., 2003). Gymnosperms, which are not crops and therefore more exposed to environmental changes, are also affected by drought stress. Drought stress is the most common cause for pine mortality in the US according to the Southern Industrial Forestry Research Council (Report, 1986).

Drought stress greatly reduces net photosynthesis rates in plants (Flexas and Medrano, 2002; Chaves et al., 2003). This decrease in photosynthesis is initially due to the closing of stomates to avoid water loss, which results in a reduction of CO₂ availability for the chloroplasts. This phenomenon occurs in response to a water deficit with a leaf relative water content of 70-75% (Chaves et al., 2003). Stomatal closing is signaled by ABA (Schroeder et al., 2001; Israelsson et al., 2006) and experiments with dehydrated and ABA-treated leaves have shown that photochemical efficiency could be almost completely reversed after a quick transfer of the leaves to a CO₂ enriched atmosphere (Meyer and Genty, 1998), indicating that photosynthetic capacity remained high during dehydration and CO₂ limitation was responsible for reduction in net photosynthesis.

Exposure to a mild level of drought stress can “acclimate” the plant for a future exposure to drought as has been shown in wheat (Selote et al., 2004; Selote and Khanna-Chopra, 2006). Selote et al. (2004) found that exposure to a mild stress can reduce the

amount of reactive oxygen species (ROS) and lipid peroxidation compared to a plant that had not been previously exposed to stress. Previous exposure to mild drought stress induced expression of H₂O₂ metabolizing enzymes, specially ascorbate peroxidase. At the metabolite level acclimated plants maintained a reduced ascorbate-glutathione redox pool (Selote and Khanna-Chopra, 2006). We have shown that loblolly pine seedlings exposed to a mild drought stress (1-MPa water potential) acclimate in the 2nd cycle of exposure to a similar stress, keeping a photosynthesis rate similar to control plants (~3.0 μmolCO₂/m/s) even though they were at a much lower water potential(-1MPa) than control plants (Watkinson et al., 2003). Gene expression studies in these plants showed a specific up-regulation of heat shock protein genes (HSPs), late embryogenesis abundant proteins (LEAs) and aromatic amino acid flavonoid synthesis related genes, when photosynthetic acclimation occurred.

1.2. Early responses to drought stress

Early drought stress experiments have shown that ABA accumulates under water deficits (Mizrahi et al., 1971; Boussiba et al., 1975; Radin, 1981; Robertson et al., 1985). Many genes that are expressed after ABA application are also expressed under drought stress (Bray, 1997; Bray, 2002; Chaves et al., 2003; Yamaguchi-Shinozaki and Shinozaki, 2006) suggesting that ABA acts as a signaling molecule under drought stress conditions. However, microarray expression analysis has shown that there are also many genes that are expressed under drought stress but not after ABA application (Ishitani et al., 1997; Shinozaki and Yamaguchi-Shinozaki, 1997; Zhu et al., 2002) implying the existence of alternative and/or parallel signaling pathways.

Many transcription factors have been identified as drought stress responsive such as DREB1A/CBF (Stockinger et al., 1997; Liu et al., 1998), DREB2A and B (Nakashima et al., 2000), AREB/ABF (Choi et al., 2000; Uno et al., 2000) and various MYBs (Abe et al., 2003). However, a receptor or sensor for drought stress has not been identified yet, since there is no chemical ligand that participates in water stress responses that could be used for the identification of a receptor. It is believed that turgor, or membrane pressure,

can be considered a stimulus during drought stress (Morgan, 1984; Wood, 1999). Studies in an osmolarity sensitive yeast mutant *sln1* (Synthetic Lethal of N End Rule 1) showed that a two-component histidine kinase senses cellular turgor pressure by an unknown mechanism (Reiser et al., 2003). SHO1 (SH3 Domain Osmosensor) is another osmosensing protein found in yeast (Tamas et al., 2000). A screening of histidine kinases, showed that two Arabidopsis histidine kinases AtHK1 and CRE1 (Cytokinin Response 1) can complement the *sln1* deletion mutant in yeast (Reiser et al., 2003). However, as of yet, there is no proof that these proteins can work as osmosensors in plants.

Most of the drought stress experiments that have allowed the identification of transcription factors or signaling molecules during drought stress have been “shock” experiments, where the plant is exposed to a quick dehydration (Urao et al., 1993; Kiyosue et al., 1994; Seki et al., 2001). A recent paper has compared gene expression after a “shock” drought stress treatment with results obtained after slow drying (7 and 11 days) in barley (Talame et al., 2007). Only ~10% of the genes that were induced under the “shock” treatment responded in the slow drying plants. Moreover, the magnitude of expression change in slow drying plants was low, compared to the fast drying plants. Therefore Talame et al.(2007) concluded that fast drying experiments could be helpful to find genes related to signaling and recognition of the stress, but not to find genes related to acclimation that might provide long – term protection for the plant.

1.3. Protection and repair molecules

Plants can defend themselves from water stress by avoiding it (not growing during the dry season), developing structures that allow for the conservation of water (i.e. succulents), or increasing the efficiency of water use (i.e. crassulacean acid metabolism plants). All these are drought adaptation strategies. Plants that are tolerant to drought stress (i. e. by previous exposure to a mild stress) have developed mechanisms that help to avoid water loss (osmotic molecules such as proline and sugars), stabilize/repair damaged proteins due to the absence of water (HSPs, LEAs) and/or have a higher availability of antioxidant molecules or enzymes.

1.3.1. Osmotic molecules

Osmotic adjustment molecules include proteins and amino acids such as proline (Pro), aspartic acid and glutamic acid. These molecules decrease the cell osmotic potential, which allows the maintenance of water absorption and cell turgor under water deficit. Proline is the osmolyte that has been most frequently observed to accumulate under water stress. Expression of enzymes for proline synthesis are induced under drought stress and enzymes for Pro metabolism/degradation are induced during rehydration (Yoshida et al., 1997). Recent studies on Arabidopsis plants exposed to low water potential have shown that ABA accumulation is necessary for Pro accumulation, however, ABA alone is not sufficient to increase Pro accumulation. Using ABA insensitive mutants, it was observed that severity of water stress and ABA must increase in tandem to increase Pro accumulation (Verslues and Bray, 2006).

1.3.2. LEA proteins

Late embryogenesis abundant (LEA) proteins were initially characterized during the later stages of seed maturation, but they have since been found in other vegetative tissues. In vegetative tissues, they are expressed developmentally and are induced by environmental stress (dehydration, osmotic and low temperature stress) or by ABA application.

Although LEA proteins were first identified in seeds, they appear to be widespread in prokaryotes and eukaryotes (Garay-Arroyo et al., 2000; Battista et al., 2001; Browne et al., 2002; Wise and Tunnacliffe, 2004). Several roles have been proposed for LEAs based on the predicted amino acid sequences, *in vitro* studies, or ectopic expression. These roles include: water binding proteins, chaperones with the ability to stabilize proteins (Dure, 1993; Honjoh et al., 2000), membranes (Ismail et al., 1999) and proteins that bring about ion sequestration (Zhang et al., 2000). However, the exact functional role of this group of proteins remains unknown. Based on conserved sequence motifs, LEAs have now been classified into 7 groups.

Group II LEA proteins (dehydrins) are highly hydrophilic with a variable structure. This structure includes one or more conserved 15-amino-acid lysine-rich sequences (the K-segment) with a consensus sequence, EKKGIMDKIKELP, that might form an amphipathic α -helix. There is immunological evidence for the existence of dehydrins in a wide range of photosynthetic organisms, including higher and lower plants and cyanobacteria (Close, 1997). Dehydrins have been found in the cytoplasm, nucleus and endomembrane system. Because of the sub-cellular localization of these proteins in the endomembrane system and the hydrophobicity of their K-element, Close (1997) proposed a membrane protection function. Studies of Dhn (Dehydrin) from cowpea show the formation of α -helical structures in the presence of SDS and a non-definite structure in the absence of SDS, suggesting that *in vivo* dehydrins may contain α -helical structure(s) in a lipid-bound state and have a membrane stabilization role (Ismail et al., 1999). Watkinson *et al* (2003) observed that the up-regulation of this group of LEAs was associated with photosynthetic acclimation.

Group III LEA proteins contain a repeat of an 11-mer amino acid motif with the consensus sequence TAQAAKEKAGE, possibly forming an amphiphilic α -helical structure. The arrangement of charged amino acids in the motif suggests a function in sequestering ions (Dure, 1993). Overexpression of HVA1 (a LEA III from barley) in rice results in improved tolerance to water and salt stress (Xu et al., 1996) and the expression of the same gene in *Saccharomyces cerevisiae* confers tolerance to high levels of KCl and NaCl (Zhang et al., 2000). Battista (2001) found that the deletion of a homologue of LEA 76 (which belong to LEA group 3), DR1172 in *Deinococcus radiodurans*, a non-spore forming bacterium, reduced tolerance to desiccation, highlighting the protective role of these proteins during water stress not only in plants but also in prokaryotes. The up-regulation of this class of LEAs was correlated with severe drought stress conditions in loblolly pine (Watkinson et al., 2003).

1.3.3. Heat shock proteins

HSPs, or molecular chaperones, are structurally diverse, but they all share the property of binding other proteins that are in non-native structural states, facilitating many structural processes such as folding, targeting and degradation. They are called heat shock proteins because the proteins were first discovered in abundance after heat stress. It is believed that during high temperature stress, they can prevent irreversible protein denaturation. HSPs are classified in 5 groups according to their approximate molecular masses.

The HSP 90 class

This class varies from 82 to 96kD. Proteins that belong to this class function as ATP-dependent chaperones that bind to highly structured folding intermediates, preventing aggregation. HSP90s can act alone or in concert with other proteins, forming for example the cytoplasmic chaperone heterocomplex (CCH). HSP70, HSP90 and an FK506 binding protein (FKBP, a peptidyl prolyl isomerase) have been identified as components of wheat CCH (Reddy et al., 1998). Another member of the heterocomplex is HOP (HSP70 and HSP90 organizing protein). There are three HOPs in Arabidopsis and studies in soybean have shown that HOP is part of the CHH in plants (Zhang et al., 2003).

Studies in *A. thaliana* have shown that HSP90 might have a “buffering” activity regulating the expression of genes, which generates different phenotypes. HSP90 activity was inhibited pharmacologically using geldanamycin (GDA), revealing altered phenotypes in treated plants compared to those grown without GDA in different *A. thaliana* ecotypes and recombinant inbred lines (RI, homozygous in almost all loci). The same altered phenotypes were observed when plants were grown at elevated temperatures. The fact of observing similar altered phenotypes in plants with reduced HSP90 activity and plants treated with a heat stress suggested that HSP90 can store and release genetic variation. Similar experiments reducing HSP90 activity in *Drosophila* showed phenotypes that might be described as “monstrous”, but in *A. thaliana* these

altered phenotypes show characteristics (i.e. purple pigment accumulation) that could be useful for the plant during stress conditions such as heat stress. (Queitsch et al., 2002). It has been suggested that the HSP90 complex performs this “buffering” activity by the activation/folding of signaling proteins that have variable domains such as R proteins (Sangster and Queitsch, 2005), which are part of the disease resistance response in plants.

The HSP90 family in Arabidopsis has 7 members: one in each organelle (chloroplast and mitochondria), one in the cytosol, three very similar in the endomembrane system¹ and one in the endoplasmic reticulum (ER) (Krishna and Gloor, 2001). Their protein structure includes an ATPase domain (N-terminal), a middle domain (probably for client protein binding) and a C-terminal domain for binding with co-chaperones containing the MEEVD motif, which is believed to interact with the TPR domain of FKBP peptidyl prolyl isomerases. The C-terminal domain is also a dimerization domain, since this protein forms an homodimer (Wegele et al., 2004) (Figure 1-1).

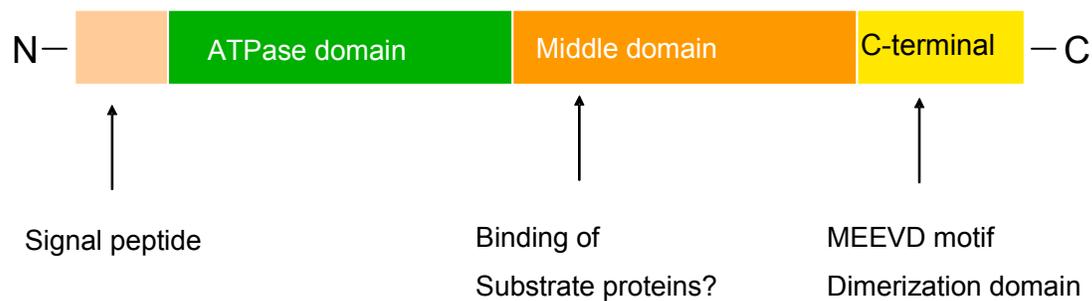


Figure 1-1 HSP90 protein structure

So far only two co-chaperones: SGT1 and RAR1 have been found for the cytosolic HSP90 (At5g56240) (Takahashi et al., 2003; Liu et al., 2004). Few target proteins have been identified for HSP90s in plants. RPM1 is a client protein of

¹ The 3 endomembrane system localized gene products were previously considered to be in the cytosol but after analysis with TargetP, they were considered to be in the endomembrane system, and that is the location for those genes in TAIR.

At5g56030, one of the endomembrane located HSP90s (Hubert et al., 2003)². The other client proteins are clavata (CLV) proteins, which regulate the shoot and floral meristem. CLV proteins are clients of the ER resident HSP90 (At4g24190) (Ishiguro et al., 2002). Recent studies in the *shd* (shepherd) mutant, which has a T-DNA insertion 16bps upstream of the first codon of the ER resident HSP90, have shown that it is not a real knockout, being expressed in roots under normal conditions, and induced in leaves after tunicamycin application (Klein et al., 2006). ER resident HSP90s, also known as GRP94 (glucose regulated protein 94) in mammals are induced by the Unfolded Protein Response (Kozutsumi et al., 1988). A GRP94 homolog in *X. viciosa*, a desiccation tolerant plant, is responsive to drought stress (from 74% to 7% RWC) and to rehydration (34% to 93% RWC) (Walford et al., 2004).

HSP90s have mostly not been found to be responsive after “shock” drought treatments. Among the only one responsive is At5g56030 (endomembrane located HSP90) which was called earlier ERD8 (early response to dehydration 8), being responsive after 1 hour of drought (Kiyosue et al., 1994).

The HSP70 class

This class varies from 68 to 110kD. Some members are constitutively expressed (HSC) whereas others are expressed only under environmental stresses. They cannot act by themselves and work with two co-chaperones namely DnaJ homologs and HSP40 in eukaryotes in an ATP dependent reaction. The major function of this protein seems to be protein folding. The binding of DnaJ homologs to HSP70 activates its ATPase domain. This binding is specific since the binding of a DnaJ that is not the co-chaperone of the HSP70 does not activate the ATPase function of the protein (Diefenbach and Kindl, 2000). Another function is folding and transport of proteins into the chloroplast and the mitochondrion (Zhang and Glaser, 2002).

The Arabidopsis HSP70 family has 18 members, 9 in the cytosol, 3 in the chloroplast, 2 in the mitochondrion, and 4 in the ER (Lin et al., 2001; Sung et al., 2001).

² At5g56030 is part of the 3 HSP90s that were considered to be in the cytosol at the time of the publication (2003).but then were relocated to the endomembrane system.

The HSP70 family is divided in two subfamilies. The DnaK subfamily, homologs to the bacterial HSP70 (DnaK), has 14 members in Arabidopsis. The HSP110/SSE subfamily, homologs to mammalian HSP110, which are larger and more divergent from HSP70s has 4 members in Arabidopsis (Lin et al., 2001).

The protein domain structure of HSP70s is very similar to the HSP90s (

Figure 1-2), but the site of binding with the co-chaperone seems to be under the ATPase domain in a cleft between its two subdomains and at or near the pocket of substrate binding (Suh et al., 1998).

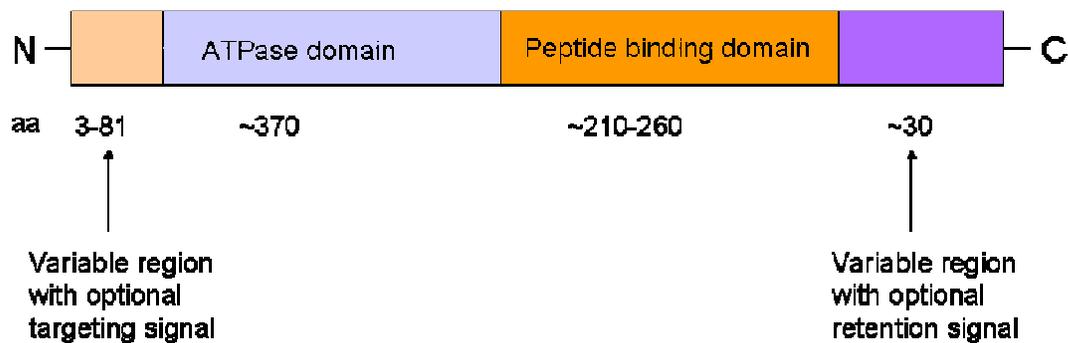


Figure 1-2 Domain structure of HSP70s

ER-resident HSP70s, are also called BiPs (luminal Binding Proteins). Overexpression of SoyBiPD (soybean BiP) in tobacco conferred tolerance to water stress after a reduction of its relative water content to 65% (Alvim et al., 2001). The plants overexpressing BiP also had a lower SOD activity compared with the control plants (empty plasmid) and antisense plants, which exhibited much more stress. This might indicate that BiP overexpressing plants were more tolerant to drought because, by an unknown mechanism, BiP reduced the ROS stress caused by the reduced water availability.

Overexpression of cytosolic NtHSP70-1 in tobacco conferred drought stress tolerance, but again the mechanism whereby this tolerance was achieved is not known. (Cho and Hong, 2006).

Small heat shock proteins

Small heat shock proteins (sHSPs) have a size between 15-30kD. They are the most diverse group of HSPs and can be found in the cytosol, mitochondria, chloroplast, and ER. *In vitro* studies suggest that they can act as molecular chaperones, preventing thermal aggregation of proteins by binding non-native intermediates. Lee and Vierling (2000), working with Luc protein (firefly luciferase), found that prevention of aggregation of heat denatured Luc was more effective with small HSPs than with other HSP70 or DnaK systems. The prevention of aggregation was more enhanced in the presence of HSP70 and homologs. On the basis of these results, Lee and Vierling (2000) proposed a model, where sHSPs bind substrate protein in a non-dependent ATP fashion, to then transfer them to the HSC70 complex. This energy-independent mode of action provides the cell with an efficient way to protect non-native proteins under stress conditions.

There are 19 sHSPs in Arabidopsis: 13 localized in the cytosol, 3 in plastids, 2 in the mitochondria, and 1 in the ER (Scharf et al., 2001).

The levels of sHSP17.4 in desiccation tolerance seeds were higher than in non-tolerant seed. This finding, plus the specific regulation during seed maturation (desiccation stage) in Arabidopsis, suggests that this sHSP has a protective role during desiccation (Wehmeyer and Vierling, 2000).

In vitro experiments with mitochondrial sHSP have shown that the protein protects the NADH:ubiquinone oxidoreductase complex during heat stress in plants (Downs and Heckathorn, 1998). Later experiments with maize mitochondrial sHSPs showed also that they can improve mitochondrial electron transport during salt stress, again protecting the NADH: ubiquinone oxidoreductase activity (Hamilton and Heckathorn, 2001).

1.3.4. Antioxidant proteins/molecules

Since water deficit induces stomatal closure, this reduces CO₂ assimilation, which results in NADPH accumulation in the chloroplast. This accumulation produces a leakage

of e^- towards O_2 , increasing the production of ROS (Asada, 1999). In plants, ROS are always produced in chloroplast, mitochondria and peroxisomes, whenever electron transport takes place. Therefore production and removal of ROS must be strictly controlled. But under abiotic stresses, such as drought the equilibrium between production and scavenging is altered, more ROS are produced which can result in damage to membranes (lipid peroxidation) and enzymes (protein oxidation).

Antioxidant molecules and scavenging enzymes

Molecules known to remove reactive oxygen species (ROS) are glutathione (γ -glutamyl-cysteinyl-glycine; GSH) and ascorbic acid (Asc) (Noctor et al., 2000). GSH reacts with $\cdot OH$, while Asc also reacts with the superoxide anion (O_2^-) and the singlet oxygen (1O_2). An abundance of the reduced species of these molecules was observed after exposure to a mild drought stress in wheat seedlings (Selote and Khanna-Chopra, 2006).

Among the best known ROS scavenging enzymes are: superoxide dismutases (SOD), ascorbate peroxidase (APX), monodehydroascorbate reductase (MDAR) dehydroascorbate reductase (DHAR), glutathione reductase (GR), glutathione peroxidase (GPX), and catalase (CAT). Through the water-water cycle (SOD and APX), the ascorbate-glutahtione cycle (APX, MDAR, DHAR, and GR), the glutathione peroxidase cycle (GPX and GR), and the action of catalase (CAT), ROS are removed from the plant (Mittler, 2002).

Other antioxidant molecules are thioredoxin and thioredoxin peroxidases (Trx) and glutathione S transferases (GST) that could also act as peroxidases. Overexpression of a GST peroxidase in tobacco resulted in osmotic and chilling tolerant plants compared to wild types (Roxas et al., 1997).

Trx catalyzes the reduction of S-S bridges to $-SH$ groups. One of the targets of this protein is the enzyme peroxiredoxin (Prx), which in its reduced form can then react with H_2O_2 and remove it (Desikan et al., 2005).

Flavonoids and anthocyanins

The definition of antioxidant in its broad sense means that these molecules when present at low concentration compared to an oxidizable substrate significantly delay or prevent the substrate's oxidation. Phenolic compounds are excellent antioxidants, due to the electron donating activity of the 'acidic' phenolic hydroxyl group. Flavonoids are phenolic compounds that are ubiquitous in the plant and are best known as the characteristic red, blue, and purple anthocyanin pigments of plant tissue (Winkel-Shirley, 2001). A survey of the antioxidant properties of 30 flavonoids, compared with a Vitamin E analog, resulted in higher scavenging activity from flavonoids (Rice-Evans et al., 1996). Among flavonoids the highest scavenging activities were found for flavonol quercetin, anthocyanidins cyaniding, and delphinidin (Rice-Evans et al., 1996).

Dihydroflavonol -4- reductase (DFR), which is the first enzyme for the production of anthocyanins and proanthocyanidins, has shown to be up-regulated during dehydration in a drought-resistant cowpea (Iuchi et al., 1996). Up-regulation of DFR and other genes for the synthesis of dihydroflavonols was observed in loblolly pine seedlings that showed photosynthetic acclimation after an exposure to mild stress (Watkinson et al., 2003).

1.4. Justification

Knowledge of mechanisms involved in tolerance and resistance to drought stress is still meager. Families of genes involved in "protection and repair" such as heat shock proteins are not fully characterized and their role remains unknown. Characterization of particular members of gene families that play a large role in drought tolerance will yield candidate genes for breeding or for reduction of the losses due to drought stress not only in pine trees and potatoes but also in other crops.

1.5. Objectives

In previous drought experiments in loblolly pine (Heath et al., 2002; Watkinson et al., 2003) differential expression of protection and repair proteins was observed. Under mild treatment where photosynthetic acclimation was detected up-expression of genes homologous to dehydrins (LEA group2), HSPs (90s, 70s and 40s) and peptidyl prolyl isomerases (PPiases) was observed; while under severe treatment up-expression of genes homologous to LEA76 (LEA group3), HSPs (70, 90) and PPiases was observed.

Based on these results my objectives will be:

1. Using microarrays and Expresso, an integrated solution to microarray experiment management and data analysis, further characterize responses to drought in loblolly pine needles and potato subjected to different levels of stress.
2. Use the results of analysis of gene expression patterns to propose roles for individual protection and repair genes in drought stress responses.
3. Search for homologs of pine drought responsive protection and repair genes in *A. thaliana* and look for upstream regulatory sequences.
4. Identify drought responsive genes in potato and the pathways in which they participate.

Chapter 2 Differential Expression of Heat Shock Protein Genes in Preconditioning for Photosynthetic Acclimation in Drought-Stressed Loblolly Pine

2.1. Introduction

Water stress affects a large range of species causing substantial losses in many crops (Valliyodan and Nguyen, 2006). It affects every aspect of plant growth, modifying anatomy, morphology, physiology, and biochemistry.

Growth is closely related to water availability and growth inhibition during drought is caused by different factors. Among these are reduced availability of CO₂ which affects photosynthesis, generation of reactive oxygen species (ROS) which may cause lipid membrane peroxidation, inactivation of enzymes and intracellular loss of water due to movement of solutes that can change pH, increasing protein instability

In early drought stress experiments, it was found that ABA accumulates under water deficits (Mizrahi et al., 1971; Boussiba et al., 1975). Many genes that are expressed after ABA application are also expressed under drought stress (Bray, 1997; Bray, 2002; Chaves et al., 2003; Yamaguchi-Shinozaki and Shinozaki, 2006) suggesting that ABA acts as a signaling molecule under drought stress conditions. Later microarray experiments, showed that there were genes that are up-regulated by drought stress but not after ABA application (Ishitani et al., 1997; Shinozaki and Yamaguchi-Shinozaki, 1997; Zhu et al., 2002) suggesting the existence of ABA alternative signaling pathways. ABA also signals stomatal closure in guard cells by ion efflux (Schroeder et al., 2001; Israelsson et al., 2006). Recent studies have shown that phospholipase D alpha-1 mediates this response (Mishra et al., 2006). ABA is also associated with decreased shoot growth during drought stress, although there is speculation that this is due to ABA interaction with ethylene, resulting in reduced shoot and root growth (Sharp and LeNoble, 2002; LeNoble et al., 2004).

The genes that are expressed under drought stress can be classified into two groups. The first group consists of genes that are related to signaling, such as transcription factors, kinases, enzymes related to phospholipid metabolism and calcium signaling molecules such as calmodulin binding proteins and 14-3-3 proteins. The over expression of some of these genes has generated drought stress tolerant plants, i.e. DREB1A:Arabidopsis (Kasuga et al., 1999), wheat (Pellegrineschi et al., 2004), and rice (Oh et al., 2005).

The second group are genes related to tolerance to drought stress, such as LEA proteins (Dure et al., 1989; Welin et al., 1994; Close, 1996; Nylander et al., 2001), enzymes for metabolism of osmolytes (Yoshida et al., 1997; Satoh et al., 2002), antioxidant proteins (Mittler and Zilinskas, 1994; Jiang and Zhang, 2002; Pastori and Foyer, 2002; Walz et al., 2002), and chaperones or HSPs (Vierling, 1991; Rizhsky et al., 2002; Rizhsky et al., 2004; Wang et al., 2004). Overexpression of some of these genes has also generated drought stress tolerant plants such as HVA1, barley LEA3 protein in rice (Xu et al., 1996) and BIP, soybean luminal binding protein in tobacco (Alvim et al., 2001).

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/or the recovery response, are not fully understood. Furthermore, the drought stress imposed has mostly been in the form of a “shock” treatment, while drought stress in the field takes place in days or weeks. Under prolonged drying conditions, plants have the opportunity to improve their water relations and photosynthesis by gene expression and modification of physiological function and morphology. There have been many physiology studies of prolonged drought stress. However, the effects of prolonged drought on gene expression has only been analyzed in a few cases: *A. thaliana* (Rizhsky et al., 2004), tobacco (Rizhsky et al., 2002), and barley (Talame et al., 2007). Moreover, these studies focus on the changes of gene expression under drought stress for one cycle of drought and did not investigate the consequences of consecutive cycles of drought stress, which is what occurs in nature.

Loblolly pine (*Pinus taeda*) is one of the most widely planted pine species in the world. Among plantations, cutover forest, and abandoned farmland, it covers

approximately 134,000 Km² (Shultz, 1999). Like other species, loblolly pine is affected by drought stress, being the most common cause of pine mortality in the USA (Southern Industrial Forestry Research Council Report, 1986). Research to understand drought tolerance and find genes related to it have been done in many species already, but only a few studies have been conducted in forest trees, and fewer yet in conifers (Chang et al., 1996; Dubos and Plomion, 2003). A recent study, have identified water stress responsive genes in loblolly pine roots using EST libraries using roots of plants that were exposed to a severe level of drought stress (-1.75Mpa) (Lorenz et al., 2006).

Consecutive cycles of drought stress can result in drought acclimation depending on the level of stress, as we observed in loblolly pine seedlings under mild stress conditions (Watkinson et al., 2003). We showed that specific expression patterns of genes encoding heat shock proteins (HSPs) are correlated with photosynthetic acclimation under mild drought stress.

The main known function of HSPs is protein folding and association with activation, processing or trafficking of signaling proteins (Vierling, 1991; Pratt et al., 2001; Wang et al., 2004). HSPs are ubiquitous in the cell, being found in all subcellular locations. HSP proteins are classified according to their molecular size and are expressed constitutively or induced by stresses. The HSP90 family includes proteins from 82 to 96 kD. While other HSPs have a more general folding function, these proteins “finish” the work by activating and/or transferring the protein to their place of function (Pratt et al., 2001). In plants a few target proteins have been identified such as: R proteins that initiate the hypersensitive response (Hubert et al., 2003; Takahashi et al., 2003) and clavata proteins, that regulate the shoot and floral meristem (Ishiguro et al., 2002).

The HSP70 family includes proteins from 68 to 110kD. HSP70s have been found in all subcellular locations in *Arabidopsis* (Lin et al., 2001). A HSP70 belonging to the HSP100 subfamily has been shown to be essential for thermotolerance (Hong and Vierling, 2000; Queitsch et al., 2000) but not for development in the absence of stress (Hong and Vierling, 2001). Overexpression of a cytosolic HSP70 in tobacco (Cho and Hong, 2006) and of a soybean ER resident HSP70 (BIP) in tobacco (Alvim et al., 2001) conferred drought tolerance. sHSPs, with a size between 15-30kD, do not have a known specific folding role but it has been shown that associated with HSP70, they can help in

the repair of a denaturated protein (Lee and Vierling, 2000). The regulated expression during seed development of *Arabidopsis* sHSP17.4 suggested a protective role in desiccation tolerance (Wehmeyer and Vierling, 2000) and overexpression of a tomato mitochondrial sHSP in tobacco increased thermotolerance (Sanmiya et al., 2004). Therefore, there is some knowledge of the possible role of these proteins during stress responses, but little is known about their role and mode of action during drought stress responses and in gymnosperms.

In this study, we investigated the role of heat shock proteins during photosynthetic acclimation and different levels of drought stress in loblolly pine seedlings. For this we used gene expression methods (microarrays and Real Time PCR) 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 to cytosolic HSP70 families are regulated depending on the level of drought stress.

2.2. Materials and methods

2.2.1. Plant material and drought stress application

Rooted cuttings of *Pinus taeda* 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 to 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 drought stress, or one cycle of mild stress, followed by two cycles of severe stress (progressive treatment). 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). Once stressed plants had reached the desired

water potential, net photosynthesis measurements were taken and the plants were re-watered. Photosynthesis was measured at light saturation on a Li-Cor 6400 (LICOR Biosciences, Lincoln, NE). Needles and roots 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 (-10 for mild or -17 Mpa for severe), and 24 h after re-watering from both treated and well-watered, control seedlings (Figure 1). At each sampling time, needles were taken from two different seedlings.

2.2.2. Microarrays

926 ESTs were selected from the NCSU NSF Pine Genome collection (<http://pinetree.ccg.umn.edu/>). The selection was based on association of the homologous genes with stress responses in *A. thaliana*. An algorithm implemented by Shukla (Shukla, 2004) was used to search for pine ESTs that had the best homology to these identified *A. thaliana* genes. Using pine contigs, in this instance, the algorithm chooses the closest homolog and then chooses the EST that is closer to the 3'UTR and has a low cross hybridization score. (<https://bioinformatics.cs.vt.edu/genesieve/>). The set of ESTs represents: core metabolism, stress responsive genes, including those from the phenylpropanoid and polyamine pathways and other secondary metabolites.

Clone amplification, cleaning, and spotting were performed according to Stasolla et al. (2003). Each clone was replicated 4 times on the array and printed with at least two pins. Spiking controls of non-plant cDNAs were also included to normalize laser focus and intensity between channels across hybridizations.

2.2.3. RNA extraction and hybridization

RNA was extracted from needles and roots according to Watkinson et al. (2003). Each pair of RNAs (treated versus control at each time point) were reverse transcribed and labeled with Cy3 and Cy5 dyes (Stasolla et al., 2003). Each comparison was reciprocally labeled to control any dye effect. The hybridizations were carried out according to Stasolla et al. (2003). A modified loop design (Kerr and Churchill, 2001)

was used to compare between treated and control samples, resulting in a total of 16 replicate spots per treatment

2.2.4. Real Time PCR

Two step real time PCR was performed. Total RNA was DNase treated with the DNasefree kit (Ambion, Austin, TX) after cleaning, 2µg of RNA were reverse transcribed using Superscript II (Invitrogen, Carlsbad, CA).

Real time PCR was performed with 5 µl of cDNA (from a 20ng/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 sec 94°C, 30 sec 56°C, and 30 sec at 72°C, and a 20 min gradient from 60 to 90°C to obtain a melting curve. The data was 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 to 0.1 fg.

The adenosine kinase gene (AK), the expression of which was shown previously to remain unchanged during drought stress in loblolly (Watkinson et al., 2003) was used for normalization (EST NXSI_116_A09, Forward Primer (FP) ‘5-GTGAGTTCCAGTTGCCTTTG-3’, Reverse Primer (RP) 5’-GGGTGGGAGACTGACAATG-3’). 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, BIP, PDI, HSC70-1, HSC 70-3, HSC70-5, DnaJ, sHSP, chlsHSP, mtsHSP, Lea2 and Lea3 (Table 2-1). All primers pairs were tested for dimer formation before using them with the actual samples.

Gene Name	Pine EST	Arabidopsis homolog	Subcellular location	Forward Primer	Reverse Primer
GRP94	NXNV_149_E10	At4g24190	ER	5'-TGGAGGGTTGTTGGTTAGAG-3'	5'-AGGGACATCTAGCACACACC-3'
BIP	NXNV_158_D06	At1g09080	ER	5'-TGGGTGTTTTCTAATTG-3'	5'TCATCCTACACTATTTC-3'
PDI	NXCI_042_G05	At1g21750	ER	5'-TTTACCTTCTTCAAGTGGC-3'	5'-CTAATGGCAAAAGCGTTG-3'
DnaJ	NXNV_132_E06	At3g44110	Cyt	5'-GGCACGAGAGACAACCTTG-3'	5'-TGTTGCTGCTTCCTCTTAG-3'
HSC70-3	NXSI_117_C08	At3g12580	Cyt	5'-GTCCAAGATCGAGGAAGTG-3'	5'-CACGCAGGAGGTGATGAAG-3'
HSC70-1	ST17D07	At5g02500	Cyt	5'-TATTCTGCGTTGGGGTGTGGC-3'	5'-CTCCAATGAGTCGCTCAGTATC-3'
HSC70-5	NXNV_123_C06	At5g09590	Mit	5'-GTTATGACTGTTGACTCTGG-3'	5'-TGGACCATTTGAATAAGG-3'
Lea2	ST14D07	no homolog	Cyt	5'-GTTTGCCCTGAGGAAGC-3'	5'-CTGTGCGTGCGGTGAAG-3'
Lea3	PC08E04	At1g52690	Cyt	5'-GCATTACCGTTGAAGCAGAG-3'	5'-CTTGATTGTTCCGCCATCG-3'
chlsHSP	NXLV_066_C03	At4g27670	Chl	5'-ATGTGAAGGTCTCTGTGGTGGAGG-3'	5'-GTATGGTATCTGATGTGCGAAGGC-3'
mtsHSP	NXSI_067_H12	At4g25200	Mit	5'-AACAGAAATACAGTAGCCGCATCG-3'	5'-AGCACGCCATTCTCATCTG-3'
sHSP	ST40F04	At1g53540	Cyt	5'-CGAAGCATTACACATCTGG-3'	5'-AGTATTGGCTACGGCAGAAG-3'

Table 2-1 List of genes and primers used for real time PCR amplification.

2.2.5. Cloning of pine GRP94 cDNA

Primers were designed using the loblolly contig for the GRP94 homolog in TIGR (TC73014, FP: 5'-GGACCGATACAATGTGCC-3', RP: 5'-AACCCCTCCAAAATTCCTG-3'). A PCR reaction using cDNA as template was performed using 5 µL of cDNA (from a 20ng/µL dilution) and GoTaq green master mix (Promega, Madison, WI) in a 20 µLs 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 sec at 94°C, 30 sec at 56°C and 1min 30 sec at 72°C. The 5'UTR region was obtained using the 5'-full RACE Core Set (Takara, Madison, WI).

2.3. Results

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

Figure 2-1 shows the effect of different levels of drought stress on photosynthesis. After one cycle of mild drought stress (-0.8 MPa approx.) the trees “acclimated” and photosynthesis in treated trees was similar to control trees, as we observed previously (Watkinson et al., 2003).

In the 2nd cycle of the progressive experiment, which experienced a level of stress similar to the 2nd cycle of the severe treatment, photosynthesis was reduced compared to the control treatment but it was not completely inhibited as we observed for plants subjected to severe stress with no prior mild (preconditioning) drought stress (-0.20±0.03 µmolCO₂/m²/s, -0.28±0.012 µmolCO₂/m²/s and -0.28±0.013 µmolCO₂/m²/s for cycles 1, 2, and 3 respectively). Therefore, even though photosynthesis did not recover completely,

exposure to a mild level of drought resulted in the maintenance of a minimum level of photosynthesis.

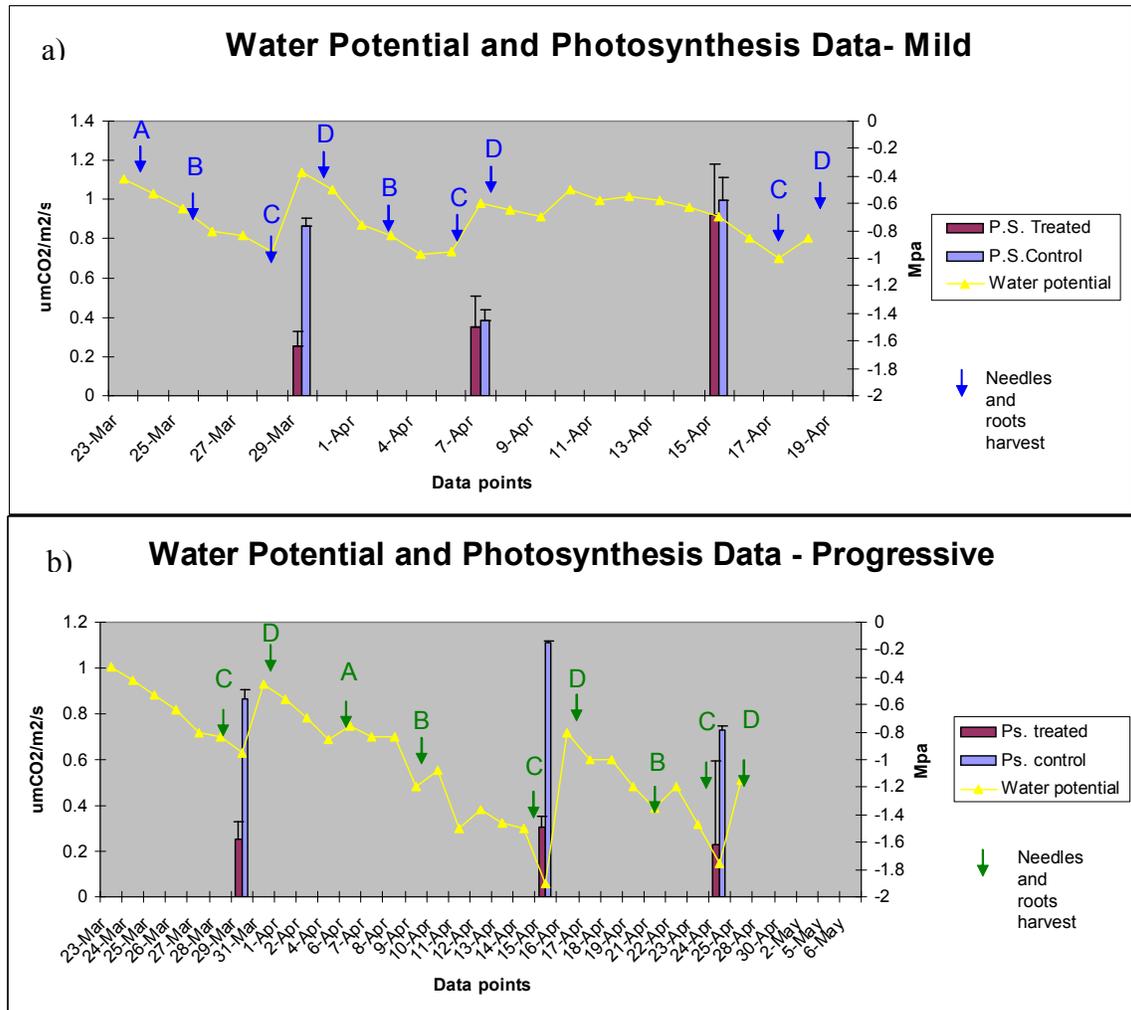


Figure 2-1 Effects of drought stress on water potential and photosynthesis.

Physiology results after application of drought stress in the mild treatment (a) and the progressive treatment (b). Net photosynthesis data (P.S) are represented as columns and water potential in 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 hours after rewatering (recovery). Arrows show the time of harvest of needles and roots.

2.3.2. Effects of preconditioning on gene expression

In order to see which genes are involved in preconditioning to drought stress a microarray experiment was carried out with needle samples from the second cycle of the progressive treatment and the severe treatment (Table 2-2).

We previously showed differential expression of specific members of individual HSP families in acclimation to drought stress at the end of the growing season (Watkinson et al., 2003). Our current data (Table 2-2) shows specific expression associated with the time of maximum stress (point c) and the time of recovery (point d). Two cytosolic members of the HSP70 family, HSC70-1, and HSC70-3, show up-regulation at the point of maximum stress, while an homolog to mitochondrial mtHSC70-2(HSC70-5) shows up-regulation at recovery (point D).

Members of the HSP90 gene family, the ER homolog (GRP94) and a cytosolic member (HSP90-1) showed up-regulation at recovery (point D) in the progressive treatment, while in the severe treatment a different HSP90 (HSP81-2) is up-regulated.

Arabidopsis GI	Pine ID	Annotation	Class	Progressive 2			Severe 2		
				PB	PC	PD	SB	SC	SD
At5g56030	NXNV_066_E07	HEAT SHOCK PROTEIN 81-2 (HSP81-2)	HSP90	0	0	0	0	0	+
At5g16650	NXPV_019_C12	DNAJ-like heatshock protein	HSP40	0	0	0	0	0	+
At4g25200	NXSI_067_H12	Arabidopsis mitochondrion-localized sHSP protein (AtHSP23.6-mito)	sHSP	0	0	0	0	0	+
At2g29960	ST38H03	peptidyl proline isomerase	Ppiase	0	0	0	0	0	+
At4g38740	ST37H09	cyclophilin (CYP2)	Ppiase	NA	0	0	NA	-	+
At1g22400	NXCI_054_F12	UDP-glucose glucosyltransferase	UDP-g	NA	0	0	NA	0	+
At5g59720	NXLV_049_G11	low-molecular-weight heat shock protein - like	sHSP	0	0	0	0	-	+
At2g20560	NXLV_073_C11	DnaJ homolog subfamily B member 5	HSP40	0	0	0	+	-	+
At5g64350	ST33E05	FKBP-type peptidyl-prolyl cis-trans isomerase, putative	Ppiase	0	-	0	+	0	0
At1g78270	NXCI_081_H03	UDP-glucose glucosyltransferase	UDP-g	NA	0	+	NA	0	0
At4g24190	NXNV_149_E10	clavata formation protein, putative nearly identical to SHEPHERD	HSP90	0	0	+	0	0	-
At5g09590	NXNV_123_C06	HEAT SHOCK 70 KDA PROTEIN, MITOCHONDRIAL	HSP70	0	0	+	0	0	0
At3g44110	NXNV_132_E06	heat shock protein-like protein dnaJ protein homolog Atj3	HSP40	0	0	+	0	0	0
At5g03160	NXRV_022_C02	DNAJ heat shock N-terminal domain-containing protein, similar to P58	HSP40	0	0	+	0	0	0
At1g53540	NXSI_041_C09	heat shock protein 17	sHSP	0	-	+	0	0	-
At5g52640	NXSI_011_G01	heat shock protein 90-1	HSP90	0	-	+	0	0	0
At5g49910	NXCI_075_D08	heat shock protein 70 / HSP70 (HSC70-7)	HSP70	0	-	+	-	+	-
At5g02500	ST17D07	heat shock cognate 70 kDa protein 1 (HSC70-1)	HSP70	0	+	+	-	+	0
At4g27670	NXLV_066_C03	mitochondrial heat shock 22 kd protein-like	sHSP	0	+	+	0	0	0
At3g12580	NXSI_117_C08	dnaK-type molecular chaperone hsc70.3	HSP70	0	+	0	0	0	-

Table 2-2 Effects of exposure to drought on gene responses in protection and repair categories in needles as reflected in microarray data

Gene expression data from microarray analysis in pine needles. PB or SB: point B (halfway through the stress), PC or SC: point C (Maximum stress), PD or SD: point D (24 hours after watering). Up-regulated genes are highlighted in green and down-regulated genes in red. Genes that are in bold were amplified with real time PCR to validate the microarray data.

2.3.3. Some HSPs are associated with photosynthesis preconditioning

Real time PCR was performed for some HSP genes (Table 2-1) that were up-regulated according to the microarray data and/or have been shown to be up-regulated during drought stresses in other plants.

From those results the expression of two genes were found to be associated with acclimation, ER HSP90 (GRP94) and ER PDI (Figure 2-2).

GRP94 and HSP70-1 were up-regulated only at the first cycle of severe stress, but after that their expression levels are lower (Figure 2-3). HSP70-1 is a homolog to an arabidopsis cytosolic member (At5g02500) in the HSP70 family.

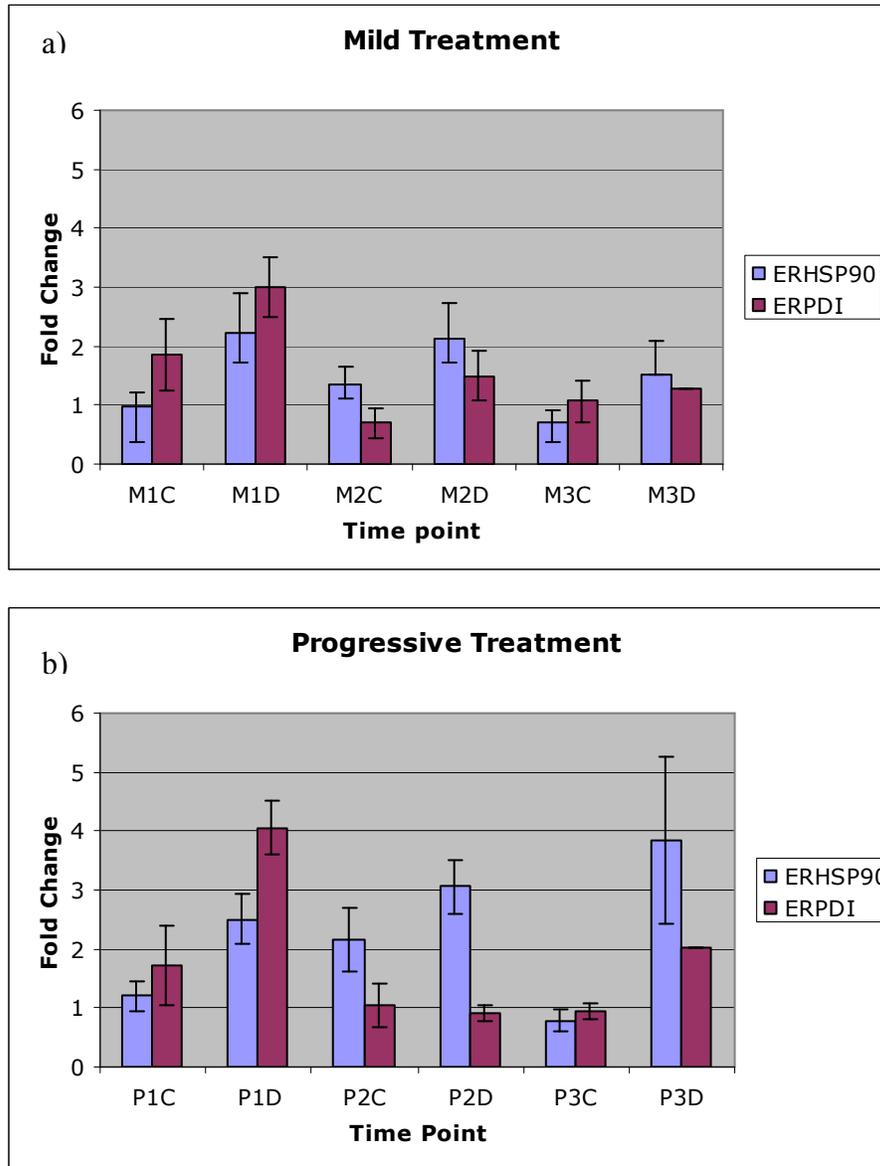


Figure 2-2 Relative expression of genes associated with photosynthetic acclimation

Real time PCR results from needles cDNA, in cases where acclimation occurred: 2nd and 3rd cycle of mild treatment (a) and 2nd and 3rd cycle of progressive treatment (b). a) M1C, M2C, and M3C are from samples taken at the maximum stress point of the 1st, 2nd, and 3rd cycle of respectively. M1D, M2D, and M3D are from samples taken at the “recovery” point (24 hours after watering) of the 1st, 2nd, and 3rd cycle respectively. b) P1C, P2C, and P3C are from samples taken at maximum stress point at the 1st, 2nd, and 3rd cycle respectively. P1D, P2D, and P3D are from samples taken 24 hours after watering at the 1st, 2nd, and 3rd cycle respectively.

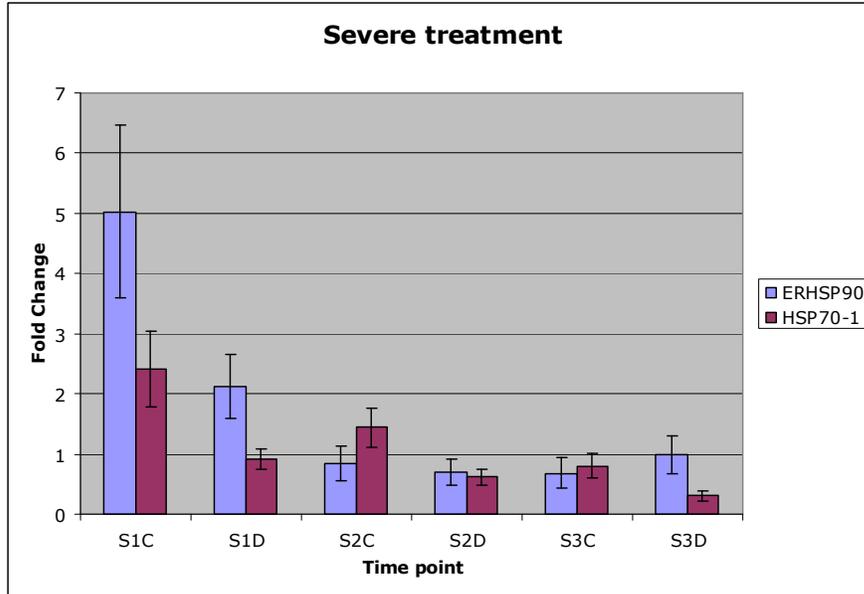


Figure 2-3 Relative expression of protection and repair genes that are highly induced at a severe drought stress but then are unchanged

Real time PCR results from needles cDNA during severe treatment. S1C, S2C, and S3C are from samples taken at maximum stress point of the 1st, 2nd, and 3rd cycle respectively. S1D, S2D, and S3D are from samples taken 24 hours after watering of the 1st, 2nd, and 3rd cycle respectively.

2.3.4. Other members of HSP families increase their expression after cycles of severe stress

Real time PCR results from severe treated samples showed an increased expression of mitochondrial HSP70 and sHSP, and BIP3 at each point of maximum stress (Figure 2-4).

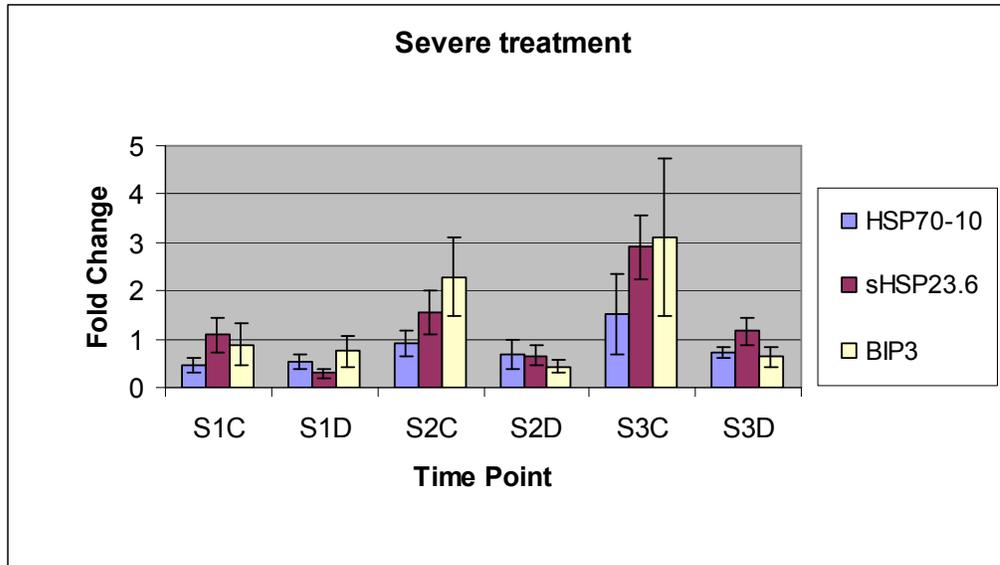


Figure 2-4 Relative expression of genes that increase their expression during each cycle of severe stress at the maximum stress point

Real time PCR results from needles cDNA samples obtained during severe treatment that increase their expression after each cycle of stress. S1C, S2C, and S3C are from samples taken at maximum stress point at the 1st, 2nd, and 3rd cycle respectively. S1D, S2D, and S3D are from samples taken 24 hours after watering at the 1st, 2nd, and 3rd cycle respectively. HSP70-10 and sHSP25.3 gene products localize to the mitochondria and BIP3 to the ER.

2.3.5. Pine GRP94 is closely related to the GRP94 from a resurrection plant

Since GRP94 showed up-regulation during the recovery of plants that showed photosynthetic acclimation, we obtained the full length cDNA sequence of this gene by doing PCR with primers based on the ESTs in the TIGR database. The translated sequence was compared with other plant ERHSP90 proteins using Clustal W (Higgins et al., 1996) (Figure 2-5). The pine protein is very similar to the other plant proteins: 76.3% similarity to the Arabidopsis ERHSP90, 77.7% to Barrel Clover, 78.9% to Cottowood, 79.2% to Maize, 79.2% to Rice and 80.6% to *X. viscosa*. The residues that are known to be involved in ATP binding and hydrolysis (Huai et al., 2005) are identical (Figure 2-5) among the 8 plant proteins and human GRP94 (data not shown), which suggest that pine ERHSP90 is an ATPase. Pine ERHSP90 is more closely related to the ERHSP90 *X. viscosa* (Figure 2-6) which has been shown to be upregulated during dehydration

(increasing its expression from 50% to 7% RWC) and during rehydration (34%, 79% and 93% RWC) in *X. viscosa* (Walford et al 2004).

The region that is most dissimilar in pine with respect to the other proteins is the C-terminal which is important for protein dimerization and contains the MEEVD domain for interaction with co-chaperones that contain the tetratricopeptide repeat domain (TPR) like cyclophilins (Young et al., 1998). However, the MEEVD domain has not been found in any ER resident HSP90s (Caplan, 2003) and Meunier et al. (2002) has found that GRP94 forms a large multichaperone complex that includes a luminal cyclophilin B that does not have a TPR domain. The co-chaperone interacting domains for GRP94 have not been identified yet.

Arabidopsis	MRNRRLTSLVSLVFLFSLFLF--LPDGGKRLHANAEEES--SDDVTPDPKVEEKI--GG--HGLSTDSQVHRESESMKKTLLRSNAEKFEFQAEVSRLLMDIIINSLSYNNKIDILFLNEIISNA	111
Barrel_Clover	MRKWTIIPSTLLLSLLSLLLE--LADGGKFGANAEGN--SDEIVDPKVEEKI--GG--VPHGLSTDSQVVKRESESIKRSLRSNAEKFEFQAEVSRLLMDIIINSLSYNNKIDILFLNEIISNA	112
Cottonwood	MRKWTIIPSTLLLSLLSLLSLLS--LADGGKRLHANAEEES--SDDVTPDPKVEEKI--GG--HGLSTDSQVHRESESMKKTLLRSNAEKFEFQAEVSRLLMDIIINSLSYNNKIDILFLNEIISNA	112
Maize	MRKWTIIPSTLLLSLLSLLSLLS--LADGGKRLHANAEEES--SDDVTPDPKVEEKI--GG--HGLSTDSQVHRESESMKKTLLRSNAEKFEFQAEVSRLLMDIIINSLSYNNKIDILFLNEIISNA	112
Rice	MRKWTIIPSTLLLSLLSLLSLLS--LADGGKRLHANAEEES--SDDVTPDPKVEEKI--GG--HGLSTDSQVHRESESMKKTLLRSNAEKFEFQAEVSRLLMDIIINSLSYNNKIDILFLNEIISNA	113
Tomato	MRKWTIIPSTLLLSLLSLLSLLS--LADGGKRLHANAEEES--SDDVTPDPKVEEKI--GG--HGLSTDSQVHRESESMKKTLLRSNAEKFEFQAEVSRLLMDIIINSLSYNNKIDILFLNEIISNA	112
X_viscosa	MRKWTIIPSTLLLSLLSLLSLLS--LADGGKRLHANAEEES--SDDVTPDPKVEEKI--GG--HGLSTDSQVHRESESMKKTLLRSNAEKFEFQAEVSRLLMDIIINSLSYNNKIDILFLNEIISNA	116
Pine	MRKWTIIPSTLLLSLLSLLSLLS--LADGGKRLHANAEEES--SDDVTPDPKVEEKI--GG--HGLSTDSQVHRESESMKKTLLRSNAEKFEFQAEVSRLLMDIIINSLSYNNKIDILFLNEIISNA	114
Arabidopsis	SDALDKI RFLALTDKQVLEGGDTAKLEI QI KLQKAKKI LSI RDGRI GMPKEDLI NKLGTI AKSGTSAPFVEKMQTSGDNLII GQFQVGFYSYVLYVADYVEVI SKHNDDQYVWESKADGAF	231
Barrel_Clover	SDALDKI RFLALTDKQVLEGGDTAKLEI QI KLQKAKKI LSI RDGRI GMPKEDLI NKLGTI AKSGTSAPFVEKMQTSGDNLII GQFQVGFYSYVLYVADYVEVI SKHNDDQYVWESKADGAF	232
Cottonwood	SDALDKI RFLALTDKQVLEGGDTAKLEI QI KLQKAKKI LSI RDGRI GMPKEDLI NKLGTI AKSGTSAPFVEKMQTSGDNLII GQFQVGFYSYVLYVADYVEVI SKHNDDQYVWESKADGAF	232
Maize	SDALDKI RFLALTDKQVLEGGDTAKLEI QI KLQKAKKI LSI RDGRI GMPKEDLI NKLGTI AKSGTSAPFVEKMQTSGDNLII GQFQVGFYSYVLYVADYVEVI SKHNDDQYVWESKADGAF	232
Rice	SDALDKI RFLALTDKQVLEGGDTAKLEI QI KLQKAKKI LSI RDGRI GMPKEDLI NKLGTI AKSGTSAPFVEKMQTSGDNLII GQFQVGFYSYVLYVADYVEVI SKHNDDQYVWESKADGAF	233
Tomato	SDALDKI RFLALTDKQVLEGGDTAKLEI QI KLQKAKKI LSI RDGRI GMPKEDLI NKLGTI AKSGTSAPFVEKMQTSGDNLII GQFQVGFYSYVLYVADYVEVI SKHNDDQYVWESKADGAF	232
X_viscosa	SDALDKI RFLALTDKQVLEGGDTAKLEI QI KLQKAKKI LSI RDGRI GMPKEDLI NKLGTI AKSGTSAPFVEKMQTSGDNLII GQFQVGFYSYVLYVADYVEVI SKHNDDQYVWESKADGAF	236
Pine	SDALDKI RFLALTDKQVLEGGDTAKLEI QI KLQKAKKI LSI RDGRI GMPKEDLI NKLGTI AKSGTSAPFVEKMQTSGDNLII GQFQVGFYSYVLYVADYVEVI SKHNDDQYVWESKADGAF	234
Arabidopsis	AVSEDTWNEPLGRGTETI RLHLRDEAGEYLEESKLELVKRYSEFI NFPPI SLWASKEVEVEVPDEE--ESAD--EE--T--ETTSTEE--KEEAAE--EDGEEKKQTKKVKETVYEWEL	340
Barrel_Clover	AVSEDTWNEPLGRGTETI RLHLRDEAGEYLEESKLELVKRYSEFI NFPPI SLWASKEVEVEVPDEE--ESAD--EE--T--ETTSTEE--KEEAAE--EDGEEKKQTKKVKETVYEWEL	344
Cottonwood	AVSEDTWNEPLGRGTETI RLHLRDEAGEYLEESKLELVKRYSEFI NFPPI SLWASKEVEVEVPDEE--ESAD--EE--T--ETTSTEE--KEEAAE--EDGEEKKQTKKVKETVYEWEL	344
Maize	AVSEDTWNEPLGRGTETI RLHLRDEAGEYLEESKLELVKRYSEFI NFPPI SLWASKEVEVEVPDEE--ESAD--EE--T--ETTSTEE--KEEAAE--EDGEEKKQTKKVKETVYEWEL	344
Rice	AVSEDTWNEPLGRGTETI RLHLRDEAGEYLEESKLELVKRYSEFI NFPPI SLWASKEVEVEVPDEE--ESAD--EE--T--ETTSTEE--KEEAAE--EDGEEKKQTKKVKETVYEWEL	339
Tomato	AVSEDTWNEPLGRGTETI RLHLRDEAGEYLEESKLELVKRYSEFI NFPPI SLWASKEVEVEVPDEE--ESAD--EE--T--ETTSTEE--KEEAAE--EDGEEKKQTKKVKETVYEWEL	343
X_viscosa	AVSEDTWNEPLGRGTETI RLHLRDEAGEYLEESKLELVKRYSEFI NFPPI SLWASKEVEVEVPDEE--ESAD--EE--T--ETTSTEE--KEEAAE--EDGEEKKQTKKVKETVYEWEL	344
Pine	AVSEDTWNEPLGRGTETI RLHLRDEAGEYLEESKLELVKRYSEFI NFPPI SLWASKEVEVEVPDEE--ESAD--EE--T--ETTSTEE--KEEAAE--EDGEEKKQTKKVKETVYEWEL	342
Arabidopsis	LNDVKAII WLRSPKEVTEDEEYTKFYHSLAKDFDEKPLAWSHFNAEGDVEFKAVLIVPPKAPHDLYESYNSKSNLKLTVRRVFI SDFDELLPKYLFKGLVDSQDPLPLNVSREMLQQ	460
Barrel_Clover	LNDVKAII WLRSPKEVTEDEEYTKFYHSLAKDFDEKPLAWSHFNAEGDVEFKAVLIVPPKAPHDLYESYNSKSNLKLTVRRVFI SDFDELLPKYLFKGLVDSQDPLPLNVSREMLQQ	464
Cottonwood	LNDVKAII WLRSPKEVTEDEEYTKFYHSLAKDFDEKPLAWSHFNAEGDVEFKAVLIVPPKAPHDLYESYNSKSNLKLTVRRVFI SDFDELLPKYLFKGLVDSQDPLPLNVSREMLQQ	464
Maize	LNDVKAII WLRSPKEVTEDEEYTKFYHSLAKDFDEKPLAWSHFNAEGDVEFKAVLIVPPKAPHDLYESYNSKSNLKLTVRRVFI SDFDELLPKYLFKGLVDSQDPLPLNVSREMLQQ	460
Rice	LNDVKAII WLRSPKEVTEDEEYTKFYHSLAKDFDEKPLAWSHFNAEGDVEFKAVLIVPPKAPHDLYESYNSKSNLKLTVRRVFI SDFDELLPKYLFKGLVDSQDPLPLNVSREMLQQ	462
Tomato	LNDVKAII WLRSPKEVTEDEEYTKFYHSLAKDFDEKPLAWSHFNAEGDVEFKAVLIVPPKAPHDLYESYNSKSNLKLTVRRVFI SDFDELLPKYLFKGLVDSQDPLPLNVSREMLQQ	463
X_viscosa	LNDVKAII WLRSPKEVTEDEEYTKFYHSLAKDFDEKPLAWSHFNAEGDVEFKAVLIVPPKAPHDLYESYNSKSNLKLTVRRVFI SDFDELLPKYLFKGLVDSQDPLPLNVSREMLQQ	464
Pine	LNDVKAII WLRSPKEVTEDEEYTKFYHSLAKDFDEKPLAWSHFNAEGDVEFKAVLIVPPKAPHDLYESYNSKSNLKLTVRRVFI SDFDELLPKYLFKGLVDSQDPLPLNVSREMLQQ	461
Arabidopsis	HSSLTKI KKKLI RKALDM RRLAEEPPDETD--REKKE--ETSS--D--D--EKKGGYAKFVWEEFGKSI KLGI I EDATNRNRLAKLRFETTKSDG--KLTSLDQYI SRMKSGQKDI FYI TQSSK	576
Barrel_Clover	HSSLTKI KKKLI RKALDM RRLAEEPPDETD--REKKE--ETSS--D--D--EKKGGYAKFVWEEFGKSI KLGI I EDATNRNRLAKLRFETTKSDG--KLTSLDQYI SRMKSGQKDI FYI TQSSK	579
Cottonwood	HSSLTKI KKKLI RKALDM RRLAEEPPDETD--REKKE--ETSS--D--D--EKKGGYAKFVWEEFGKSI KLGI I EDATNRNRLAKLRFETTKSDG--KLTSLDQYI SRMKSGQKDI FYI TQSSK	579
Maize	HSSLTKI KKKLI RKALDM RRLAEEPPDETD--REKKE--ETSS--D--D--EKKGGYAKFVWEEFGKSI KLGI I EDATNRNRLAKLRFETTKSDG--KLTSLDQYI SRMKSGQKDI FYI TQSSK	577
Rice	HSSLTKI KKKLI RKALDM RRLAEEPPDETD--REKKE--ETSS--D--D--EKKGGYAKFVWEEFGKSI KLGI I EDATNRNRLAKLRFETTKSDG--KLTSLDQYI SRMKSGQKDI FYI TQSSK	574
Tomato	HSSLTKI KKKLI RKALDM RRLAEEPPDETD--REKKE--ETSS--D--D--EKKGGYAKFVWEEFGKSI KLGI I EDATNRNRLAKLRFETTKSDG--KLTSLDQYI SRMKSGQKDI FYI TQSSK	580
X_viscosa	HSSLTKI KKKLI RKALDM RRLAEEPPDETD--REKKE--ETSS--D--D--EKKGGYAKFVWEEFGKSI KLGI I EDATNRNRLAKLRFETTKSDG--KLTSLDQYI SRMKSGQKDI FYI TQSSK	578
Pine	HSSLTKI KKKLI RKALDM RRLAEEPPDETD--REKKE--ETSS--D--D--EKKGGYAKFVWEEFGKSI KLGI I EDATNRNRLAKLRFETTKSDG--KLTSLDQYI SRMKSGQKDI FYI TQSSK	578
Arabidopsis	EQLKSPFLERLI KKGVEYI FFTDPVDEYLMQYLMQYMDYEDKFKQVSKGKGLKGDSDKDEKLFESFELTDPKPFASRI YDSVKTSLDI SPDATV--EEE--DETE--VEV--ES--ETK--EE--AP--A--SN	696
Barrel_Clover	EQLKSPFLERLI KKGVEYI FFTDPVDEYLMQYLMQYMDYEDKFKQVSKGKGLKGDSDKDEKLFESFELTDPKPFASRI YDSVKTSLDI SPDATV--EEE--DETE--VEV--ES--ETK--EE--AP--A--SN	699
Cottonwood	EQLKSPFLERLI KKGVEYI FFTDPVDEYLMQYLMQYMDYEDKFKQVSKGKGLKGDSDKDEKLFESFELTDPKPFASRI YDSVKTSLDI SPDATV--EEE--DETE--VEV--ES--ETK--EE--AP--A--SN	699
Maize	EQLKSPFLERLI KKGVEYI FFTDPVDEYLMQYLMQYMDYEDKFKQVSKGKGLKGDSDKDEKLFESFELTDPKPFASRI YDSVKTSLDI SPDATV--EEE--DETE--VEV--ES--ETK--EE--AP--A--SN	699
Rice	EQLKSPFLERLI KKGVEYI FFTDPVDEYLMQYLMQYMDYEDKFKQVSKGKGLKGDSDKDEKLFESFELTDPKPFASRI YDSVKTSLDI SPDATV--EEE--DETE--VEV--ES--ETK--EE--AP--A--SN	697
Tomato	EQLKSPFLERLI KKGVEYI FFTDPVDEYLMQYLMQYMDYEDKFKQVSKGKGLKGDSDKDEKLFESFELTDPKPFASRI YDSVKTSLDI SPDATV--EEE--DETE--VEV--ES--ETK--EE--AP--A--SN	696
X_viscosa	EQLKSPFLERLI KKGVEYI FFTDPVDEYLMQYLMQYMDYEDKFKQVSKGKGLKGDSDKDEKLFESFELTDPKPFASRI YDSVKTSLDI SPDATV--EEE--DETE--VEV--ES--ETK--EE--AP--A--SN	696
Pine	EQLKSPFLERLI KKGVEYI FFTDPVDEYLMQYLMQYMDYEDKFKQVSKGKGLKGDSDKDEKLFESFELTDPKPFASRI YDSVKTSLDI SPDATV--EEE--DETE--VEV--ES--ETK--EE--AP--A--SN	696
Arabidopsis	NKGAYMRGKRVLEI NRPNPI I KELKDR I ASQPEDESVKETAQLMYQTAL I ESGFELTDPKPFASRI YDSVKTSLDI SPDATV--EEE--DETE--VEV--ES--ETK--EE--AP--A--SN	806
Barrel_Clover	NKGAYMRGKRVLEI NRPNPI I KELKDR I ASQPEDESVKETAQLMYQTAL I ESGFELTDPKPFASRI YDSVKTSLDI SPDATV--EEE--DETE--VEV--ES--ETK--EE--AP--A--SN	803
Cottonwood	NKGAYMRGKRVLEI NRPNPI I KELKDR I ASQPEDESVKETAQLMYQTAL I ESGFELTDPKPFASRI YDSVKTSLDI SPDATV--EEE--DETE--VEV--ES--ETK--EE--AP--A--SN	803
Maize	NKGAYMRGKRVLEI NRPNPI I KELKDR I ASQPEDESVKETAQLMYQTAL I ESGFELTDPKPFASRI YDSVKTSLDI SPDATV--EEE--DETE--VEV--ES--ETK--EE--AP--A--SN	796
Rice	NKGAYMRGKRVLEI NRPNPI I KELKDR I ASQPEDESVKETAQLMYQTAL I ESGFELTDPKPFASRI YDSVKTSLDI SPDATV--EEE--DETE--VEV--ES--ETK--EE--AP--A--SN	799
Tomato	NKGAYMRGKRVLEI NRPNPI I KELKDR I ASQPEDESVKETAQLMYQTAL I ESGFELTDPKPFASRI YDSVKTSLDI SPDATV--EEE--DETE--VEV--ES--ETK--EE--AP--A--SN	798
X_viscosa	NKGAYMRGKRVLEI NRPNPI I KELKDR I ASQPEDESVKETAQLMYQTAL I ESGFELTDPKPFASRI YDSVKTSLDI SPDATV--EEE--DETE--VEV--ES--ETK--EE--AP--A--SN	798
Pine	NKGAYMRGKRVLEI NRPNPI I KELKDR I ASQPEDESVKETAQLMYQTAL I ESGFELTDPKPFASRI YDSVKTSLDI SPDATV--EEE--DETE--VEV--ES--ETK--EE--AP--A--SN	814
Arabidopsis	I E A E PVEEQ E E N KDEL	823
Barrel_Clover	I E A E PVEEQ E E N KDEL	818
Cottonwood	I E A E PVEEQ E E N KDEL	823
Maize	I E A E PVEEQ E E N KDEL	808
Rice	I E A E PVEEQ E E N KDEL	812
Tomato	I E A E PVEEQ E E N KDEL	812
X_viscosa	I E A E PVEEQ E E N KDEL	812
Pine	I E A E PVEEQ E E N KDEL	834

Figure 2-5 Protein alignment of ER HSP90 from pine with related plant ERHSP90s.

The conserved residues are shaded in yellow. The black boxes indicate the HSP90 family signature (NKDIL) and the ER retention signal (KDEL). The red boxes indicate the residues that are required for ATP binding and hydrolysis: E109, N113, D116, D157, M162, N170, V200, G201, G202, T249, K371 in the pine sequence. The GenBank accession numbers for the sequences are: Q8SB39 for rice (*O. sativa*) and AAN34791 for *X. viscosa*. For maize (*Z. mays*) the translation of the GenBank sequence AY103537 was used. The cottonwood (*P. trichocarpa*) sequence was obtained from the Poplar genome web page (http://genome.jgi-psf.org/Poptr1/Poptr1_home.html) and the ID is ESTEXT_FGENESH1_PM_V1.C_LG_V0560. The Arabidopsis sequence was obtained from the Arabidopsis Genome web page (www.arabidopsis.org) and the gene ID is At4g24190. 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.

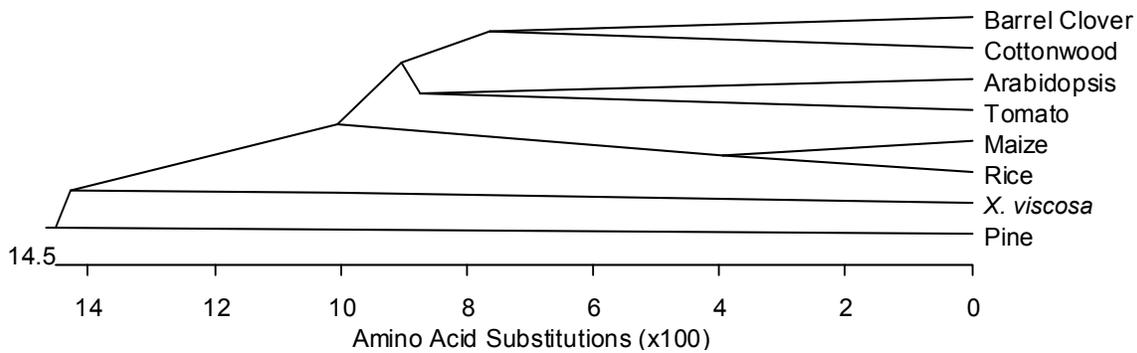


Figure 2-6 Phenogram of plant ER resident HSP90

The alignment used to generate the phenogram is the same as is shown in Figure 2.5.

2.4. Discussion

2.4.1. Possible role of heat shock proteins in preconditioning to drought

Exposure to a mild drought stress enabled the plant to “condition”, or perform better when exposed to a second cycle of more severe stress. We have observed that certain HSPs are up-regulated during recovery when photosynthetic acclimation occurs e.g. HSC70-1 and HSC70-3. These genes also showed up-regulation at maximum stress during the first cycle under mild stress conditions (data not shown). Overexpression of HSC70-1 in Arabidopsis plants resulted in increased thermotolerance (Sung and Guy, 2003). Silencing of the gene did not produce viable plants illustrating the importance of HSC70-1 under stress and normal conditions (Sun and Guy, 2003). 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 show a general response to the accumulation of unfolded proteins in the cytosol, an event similar to the unfolded protein response in the ER (Aparicio et al., 2005). Drought stress could also cause protein accumulation and these genes might be up-regulated in pine for this reason.

A recent study in *N. tabacum* showed that over-expression of an homolog to HSC70-3 (NtHSP70-1) confers drought-stress tolerance in 3 week old tobacco seedlings (Cho and Hong, 2006). Moreover, plants overexpressing the gene were able to maintain a lower water potential compared to the empty vector and antisense plants. The over-expressing 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 drought tolerance. HSP70-1 also showed up-regulation at maximum stress in the first cycle of the severe treatment (Figure 2-3.) and then it did not show more expression. This differential response of the same gene might imply different roles at different levels of stress. Since severe levels of drought stress causes higher oxidation and therefore more protein damage, cytosolic pine HSC70-1 might have a protein folding/repair function, while during recovery after a mild stress it might have a water flux regulator role.

The other genes that were up-regulated 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 up-regulated after tunicamycin treatment (Martinez and Chrispeels, 2003; Kamauchi et al., 2005). It has been shown that homologs to these genes in mammalian systems are part of a protein complex (Meunier et al., 2002) that binds to nascent proteins. 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.

2.4.2. Possible role of heat shock proteins under severe drought stress conditions

Pine mitochondrial HSPs such as mitochondrial HSP70 and mitochondrial sHSP (Figure 2-4) seem to have a role during high levels of stress, since their expression increased at each cycle of severe stress. During severe stress, when the levels of ROS are higher (Selote et al., 2004), the increased expression of mitochondrial HSP70 might be due to an increase in refolding/transport of antioxidant proteins to the mitochondria. The mitochondrial sHSP is homolog to the mitochondrial tomato sHSP23.6 that upon overexpression confers thermotolerance to tobacco plants (Sanmiya et al., 2004). Moreover, it has been shown that mitochondrial sHSP protects the NADH:ubiquinone oxidoreductase complex during heat stress in plants (Downs and Heckathorn, 1998). Since heat stress also causes ROS production in the mitochondrion (Polla et al., 1996) 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.

The pine BIP3 that also showed gradual up-regulation after each cycle of severe stress is closely related to a soybean BIP3 that, when overexpressed in tobacco, conferred tolerance against severe levels of drought (Alvim et al., 2001). Since BIP3 is responsive to tunicamycin which induces accumulation of proteins and activates the unfolded protein response (Koizumi, 1996), the increasing expression of this gene might be related to an increased accumulation of unfolded protein due to the severe drought stress applied.

2.4.3. Importance of pine GRP94 during drought stress responses

Pine GRP94 was one of the genes that showed up-regulation 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; some integrins (Randow and Seed, 2001); or proteins that are secreted such as IgGs (immunoglobulin chains) (Melnick et al., 1994). In Arabidopsis one of the targets that has been identified is a clavata protein, which regulates meristem development (Ishiguro et al., 2002). Pine GRP94 is more similar to the *X. viscosa* GRP94 protein than to the Arabidopsis protein. *X. viscosa* GRP94 is responsive to water stress (from 74% to 7% RWC) and to rehydration (34% to 93% RWC). It is also responsive to heat and to salt stress, stresses that share protein denaturation effects. It did not show response to cold stress and high light (Walford et al., 2004). I propose that pine GRP94 has a similar role under drought stress, protection/repair of denaturated proteins. But since HSP90s target specific proteins, GRP94 may repair specific proteins that help to protect the photosynthetic machinery. Protein interaction experiments could help find the target proteins of this gene.

Chapter 3 Combinatorial Analysis of Cis-elements and Co-regulated Genes of *A. thaliana* Heat Shock Protein 90 Genes

3.1. Introduction

The binding of transcription factors to promoter regions controls gene expression. The binding sequence of these cis-elements usually is found by mutational experiments of the candidate target region and *in vitro* or *in vivo* studies of the interaction of the protein with the DNA region. Many cis-elements in plants have been discovered using these approaches and they are available in databases such as PLACE (Higo et al., 1999) which had 469 plant elements annotated as of 01/08/07. Among the other techniques that can be used, genome wide chromatin immunoprecipitation arrays (ChIP) allowed the analysis of the interactions of 106 transcription factors in yeast (Lee et al., 2002). In *A. thaliana*, ChIP experiments have helped in the identification of genes regulated by 15 AGAMOUS-like MADS domain proteins (Wang et al., 2002), but genome wide data is not available yet.

There are more than 1500 putative genes annotated as transcription factors in Arabidopsis (Riechmann et al., 2000). Chen et al (Chen et al., 2002) analyzed expression matrices of 402 of these transcription factors under different developmental stages and environmental stresses. Cluster analysis showed that the induction or repression of some of these transcription factors was not specific to any one stress. Before Chen et al., work (Chen et al., 2002), *AtERF1* was reported only to be responsive to ethylene and wounding (Fujimoto et al., 2000). Chen et al., (2002) showed that *AtERF1* is induced by pathogens that include bacteria, fungi, oomycetes and viruses. Further analysis showed that in fact *AtERF1* integrates the ethylene and jasmonate pathways in plant defense (Lorenzo et al., 2003). Therefore gene expression under different stresses is regulated by the combinatorial arrangement of different stress responsive transcription factors.

Different techniques have been used for the analysis of cis-elements in the promoter region. The most widely used method is the counting of a motif (enumerative method) in order to determine the overrepresentation of that motif on a group of genes.

This has been applied to the promoter regions of yeast (van Helden et al., 1998; Jensen and Knudsen, 2000); and to the 5'UTR (Hulzink et al., 2002) and promoter regions of plants (Hudson and Quail, 2003). Hudson and Quail (2003) used microarray expression data to find motifs regulated by phytochrome A from *phyA* mutant/wild type comparisons. They searched for one to 10 mer motifs that were shared among the genes that were not affected in the mutant genotype. The presence of the motifs in these genes was then compared with the rest of the genes included in the microarray, in order to find the ones that were overrepresented. Enumerative methods have the disadvantage of generating many false positives, due to the presence of repetitive sequences in the promoters. To overcome this problem, they considered only the motifs that were statistically overrepresented by raw counting and per promoter basis. This kind of analysis addresses the need of find binding sites for transcription factors, but does not take into account that the expression of a set of co-regulated genes depends not only on one transcription factor but the combination of many of them. The use of microarray data from cell cycle, sporulation and a variety of stresses allowed the identification new regulatory networks in yeast (Pilpel et al., 2001). First they identified all genes that contain motif pairs from a dataset of 356 motifs and then a measure of the co-expression of the group of genes (expression coherence score) was obtained using the microarray data. The pairs that gave statistically significant scores were considered synergistic combinations. Their results were correlated with previous findings but also new combination pairs, that suggested cross-talk among processes was discovered. This approach was improved later by including CHiP data to the microarray data to find specific motif pairs associated with the stages of cell cycle in yeast (Kato et al., 2004). This kind of analysis has not been done for Arabidopsis yet.

Gene expression data from microarrays for Arabidopsis is available at several database sites (NASC, TAIR). These data may be used to find co-regulated genes with more precision than using only one set of experiments. These co-regulated genes will probably share similar cis-elements in their promoter regions. However the data need to be classified by treatment or organ in order to give precise association among genes. A pair of genes might be strongly associated only in a subset of experiments but not at all under different conditions. Lee et al., (2000) used microarray data among other data

sources to build a network of yeast genes. When the microarray data were subdivided according to the kind of experiment the accuracy of its results improved significantly.

Microarray data analysis combined with promoter analysis can also be used to predict candidate interacting proteins under different stresses for large gene families. The finding of specific motifs in the upstream region of a gene could help to identify experimental conditions that could be used to associate a phenotype to a gene, on a plant that have a gene mutated with no obvious phenotype. Heat shock proteins (HSPs) in plants are an example of an unexpectedly large families compared with other organisms. These proteins are known to form complexes in order to achieve their chaperone function (Krishna and Gloor, 2001). In mammals and other eukaryotic organisms HSP90 forms heterocomplexes with other proteins, called co-chaperones, for the targeting and/or activation of target proteins such as the glucocorticoid receptor (Pratt et al., 2001). These co-chaperones are HSP70 and HSP90 organizing protein (Hop), HSP70, HSP40 (J-domain protein), HSP70 interacting protein (HIP), immunophilin and p23 (Krishna and Gloor, 2001). Homologs of these co-chaperone genes are present in *A. thaliana*, and they are present in multigene families too. The HSP70 family has 18 members (Lin et al., 2001), the J-domain proteins has 89 members (Miernyk, 2001), the Hop family has 3 members (Krishna and Gloor, 2001; Zhang et al., 2003), and the immunophilin family has 52 members (He et al., 2004). These large families may have arisen as a result of adaptation of plant cells to a range of stresses since HSPs are induced not only under heat stress but also under different environmental stresses, such as drought stress. This adaptation may involve formation of specific complexes for different stresses. Therefore we will expect similar cis-element arrangements among HSP90s and their co-chaperones.

Exposure to mild drought stress can “pre-condition” the plant photosynthetic machinery, “acclimating” it to more successfully defend itself against subsequent drought stresses. We have shown that specific expression patterns of genes encoding several heat shock proteins (HSPs) are correlated with photosynthetic acclimation under mild drought stress in loblolly pine (Watkinson et al., 2003; Vasquez-Robinet, In preparation). The experiments showed that a homolog of Arabidopsis HSP90-7 was up-regulated after rehydration coincident with acclimation (Vasquez-Robinet, In preparation). HSP90-7 is a member of the HSP90 gene family that in mammals forms a complex with co-chaperones

involved in activation, processing or trafficking of signaling proteins. We found that the HSP90-7 homolog was co-expressed with candidate co-chaperones such as HSP70-3, and a HSP40 homolog.

Studies in *A. thaliana* have shown that HSP90 might have a “buffering” activity regulating the expression of genes, since HSP90 inhibition with geldanamycin generated different phenotypes, and they were ecotype specific (Queitsch et al., 2002). The HSP90 family in Arabidopsis has 7 members: one in each organelle (chloroplast and mitochondria), one in the ER, one in the cytosol, and three very similar members in endomembrane system. Studies in the hypersensitive response in plants have shown two co-chaperones for the cytosolic HSP90 (At5g56240): SGT1 and RAR1 (Takahashi et al., 2003; Liu et al., 2004)

XCisClique (Pati et al., 2006) is a new tool that integrates expression data by correlation analysis and combinatorial arrangement of groups of known cis-elements in the promoter region of co-regulated genes. The possibility of finding candidate co-chaperones for the HSP90 genes and its function under different abiotic stress situations was analyzed using *XCisClique*.

3.2. Materials and methods

3.2.1. Microarray expression data

We used microarray expression data from abiotic stress experiments posted by the AtGenExpress project in the Nottingham database (<http://www.arabidopsis.org.uk/>). Using the Affymetrix Microarray Analysis Suite 5.0 (Affymetrix Inc.) with the Affymetrix MAS 5.0 Scaling Protocol the data available have been analyzed and normalized so the mean is equal to 100. The hybridizations were carried out on the Affymetrix ATH1 Arabidopsis Genome Array. 272 slides were included, comprising two biological replicates and two tissues (shoots and roots) in a time course for control and treated plants subjected to following stresses: salt, drought, genotoxic conditions, oxidative stress, UV-B, wounding, heat, cold and osmotic stress. We have selected 5 time points that were included in all the experiments: 0:5h, 1h, 3h, 6h and 12h. Details of the experiments are available at the AtGenExpress consortium webpage <http://web.uni-frankfurt.de/fb15/botanik/mcb/AFGN/atgenex.htm>.

The data was processed in a tissue-wise manner since the response in roots will probably have a different regulation compared to the shoots. In order to evaluate the quality of the biological replicates the Spearman Rank Coefficient was calculated between replicates. The result of this test showed a correlation greater than 0.9 between all replicates, so the simple average was used to build the vectors for the correlation analysis matrices among genes.

3.2.2. Promoter and cis-element data

Promoter sequences for the *A. thaliana* genome were downloaded using Perl scripts that interact with the *e-utilities* interface at NCBI. This data was then stored in a local pSQL database, which includes gene, protein, annotation and references among other information.

Cis-element sequences and annotations were obtained from the PLACE website (Higo et al., 1999). The consensus sequence for the heat shock element (HSE) is NGAAN (where N is any nucleotide) arranged in continuous inverted repeats such as -NGAANNTTCNNGAAN-. Mutational analysis has indicated that the G/C bp (G and complementary C) at position one of the unit is more important than the base in the third position (Barros et al., 1992), therefore a set of 38 regular expressions that represent the different possible mutations and negative or positive strands was generated. A new HSE recently found in yeast (Yamamoto et al., 2005) was included in the cis-elements database.

3.2.3. Analysis with *XCisClique*

XCisClique (Pati et al., 2006) has been implemented in Perl. The inputs for the package are: a set of gene identifiers corresponding to a co-regulated gene set, the set of all promoters of the entire genome and a list of regulatory motifs. *XCisClique* does not use the translation start site as the starting point of the gene promoter, instead a library of existing and putative TATA-box patterns in plants have been included and the cis-elements are searched upstream from the TATA-box. The regulatory motifs can be fed as

regular expressions, simple strings, or consensus sequences. The script extracts a user specified length sequence from the *promoters* database to be analyzed in both foreground (input gene set) and background (whole genome). The cis-elements are then searched in each set. In the case of palindromes that result in two occurrences for the same motif, the script recognizes this duplication and considers only one occurrence. The significance of the elements found it is assessed by two tests. A chi-square independence test is calculated per motif:

$$X^2 = \sum_{i,j} \frac{(E_{i,j} - O_{i,j})^2}{E_{ij}} \quad (1)$$

where O and E are the observed values and expected values of a cis-element in the promoters. E and O are two contingency tables, where the columns indicate presence or absence and the rows the foreground and background data sets. Therefore E is calculated by:

$$E_{i,j} = \frac{T_i \times T_j}{N} \quad (2)$$

where T_i is the total across row i in the contingency table for observed values, T_j is the total across column j , and N is the total number of observed values .

The second statistical test is made for combinations of cis-elements that are significantly overrepresented in the foreground gene set. The presence or absence of a cis-element on a gene is represented in a binary matrix, where the rows represent the genes and columns the cis-elements. Then the *Apriori* algorithm (implemented in C++) finds all maximal groups of presences in this binary matrix (Agrawal, 1994). A set is called maximal when no more rows can be added without removing columns or viceversa. Each motif combination obtained with *Apriori* is called a biclique. The presence of a motif combination in a group of genes is treated as a hypergeometric distribution and a p-value is generated per biclique:

$$H_{tail}(N, C_M, n, c) = 1 - \sum_{i=1}^c \left(\frac{C(n, c) \cdot C(N-n, C_M-c)}{C(N, C_M)} \right) \quad (3)$$

Where: N is the number of genes in *A. thaliana* genome, n is the set of genes in the biclique (G), M represents the motifs of a biclique, C_M is the number of genes in *A. thaliana* genome containing all the motifs in the set of motifs of biclique, and c is the number of genes in G containing all elements of M. The p-values obtained from the hypergeometric distribution that are less than 0.05 are considered significant and then FDR (false discovery rate) correction of 10% it is applied to the significant bicliques.

The gene expression data has been modeled as a gene expression vector (V_g) with 45 components (9 treatments and 5 points per treatment).

$$V_g = (e_{g,1,1}, e_{g,1,2}, e_{g,1,3}, e_{g,1,4}, e_{g,1,5}, e_{g,2,1}, \dots, e_{g,9,4}, e_{g,9,5}) \quad (4)$$

Where, $e_{g,k,t}$ is the ratio of treated and control expressions for gene g, a particular treatment k, at time point t. Then the correlation between two gene expression vectors (V_{g1} and V_{g2}) with ranks (rg1, rg2) is calculated using the Spearman Rank Correlation Coefficient, which is then used to calculate the correlation among the genes in a biclique. The biclique is evaluated for tightness of correlation of gene expression using the p value of the Sum of Absolutes Values (SAV) Statistics. Since a negative correlation may be as biologically significant as a positive correlation, we use the absolute value of ρ (vg1,vg2) to avoid unwanted cancellation of negative and positive correlations. Therefore SAV is defined as:

$$S(G) = \sum_{g_1, g_2 \in G} |\rho(g_1, g_2)| \quad (5)$$

then the p-value of an observed SAV value P_{(S(G))} is calculated from a precomputed distribution of SAV for a geneset of size |G|.

The final p-value of a biclique is obtained using the p-value from the hypergeometric distribution and the SAV p-value from the expression analysis.

$$H_{tail}(N, C_M, n, c).p_r(S(G)) \quad (6)$$

The statistically significant resulting bicliques (combined p-value less than 0.0025) are post processed to generate a motif match file.

The output from *XCisClique* feeds directly into the visualization tool ‘MotifSee’, a PHP driven web-based interface that accepts txt files with the following fields format: GeneID, Motif, Sequence, Start Position, End Position, TATA-start, TATA-end.

3.3. Results

3.3.1. Significant motif combinations for the different HSP90 members

To test our hypothesis, analysis of correlation using the Spearman Coefficient was performed. After the analysis between 50 and 130 top-ranked genes with the highest correlation to each HSP90 were chosen for each HSP90. Then each set of genes was analyzed by *Xcis-clique* using the hypergeometric evaluation in order to find significant motif combinations and the number of significant combinations found per gene set and the motifs present in those combinations.

Gene Name*	At Number	Sub-celular location	Number of genes analyzed**	Significant Bicliques	Significant Bicliques including HSP90
HSP90-1	At5g52640	Cytoplasm	124 (0.60)	77	3
HSP90-4	At5g56000	Endomembrane System	48 (0.55)	104	7
HSP90-5	At2q04030	Chloroplast	63 (0.69)	78	1
HSP90-6	At3q07770	Mitochondrion	79 (0.63)	47	6
HSP90-7	At4g24190	Endoplasmic Reticulum	106 (0.50)	165	97

Table 3-1. Number of genes co-regulated with HSP90 family members under nine abiotic stresses

* HSP90-2 and 3 are 98% similar at the nucleotide level, i.e., are represented by the same probe in ATH1. Thus, no specific expression data were available to determine co-regulated genes [gene nomenclature: Krishna and Gloor (2001).

**Numbers in parentheses show the Spearman correlation cutoff in a set of genes.

Xcis-clique creates bicliques (sets of genes (G) that shares the same motifs (M)). Statistically significant bicliques that included an HSP90 were analyzed (Table 3-1). HSP90-2, HSP90-3 and HSP90-4 share at least 96% similarity at the protein level (Krishna and Gloor, 2001). At the nucleotide level (coding region) they also show more than 95% similarity. Therefore only the gene, HSP90-4, that has a specific probe in the Affychip was used in the analysis (At5g56000). Table 3-2 shows the significant motifs found for each group of genes coregulated with the HSP90s. Previous studies, using heat shock (HS), determined HSP90-1 as the most highly induced member of the HSP90 family (Yabe et al., 1994; Haralampidis et al., 2001). Other cytosolic family members are HS-induced to a lower degree (Miloni and Hatzopoulos, 1997). The finding of an HSE without mutations in HSP90-1 and an HSE with mutations in HSP90-4 (where the HSF might not bind as efficiently (Barros et al., 1992) may explain this response (Table 3-2).

This analysis also suggests putative motifs that might explain the response to other stresses already reported for these HSPs besides heat shock, such as water stresses (Kiyosue et al., 1994; Krishna et al., 1995), biotic stresses (Hubert et al., 2003; Takahashi et al., 2003), or embryogenesis (Prasinos et al., 2005). Analysis with XCisClique also identifies motifs for responses that have not been reported yet for HSP90 genes, such as the circadian cycle related motifs CCA1ATLHCB1 (Wang et al., 1997) and EVENINGAT (Harmer et al., 2000). These motifs together with the light responsive elements TBOXATGAPB (Chan et al., 2001), HDZIP2ATATHB2 (Ohgishi et al., 2001) are present in the organellar HSP90s (HSP90-6 and 7), which suggests that these genes might have a role in the folding/activation of proteins that regulate these responses.

Biclique ID	Gene name	Heat	Light	Circadian Cycle	ABA, Drought, Cold	GA	UPR	Biotic stress	Organ Development
Biclique11040_3_7	HSP90-1	PerfectHSE1-end(1)			MYB1AT,				
Biclique4137_3_6	HSP90-4	2-nuIPerHSE1-5end(1)			MYB1AT, MYB2CONSENSUSAT, MYGCONSENSUSAT, DPBFCOREDDCC3, DRECRICOREAT, LTREATLIT78	GAREAT		WRKY-like(2)	
Biclique1139_11_3	HSP90-5		CCA1ATLHCB1, TBOXATGAPB						L1BOXATPDF1
Biclique9_12_2	HSP90-6		CCATATLHCB1, TBOXATGAPB, HDZIF2ATATHB2	EVENINGAT	MYB1AT, AMyb4 (2),	GADOWNAT		WRKY-like(2), TGA1(2)	CARGCW8GAT
Biclique12054_7_8	HSP90-7				MYB1AT, ABRE-like(2), DPBFCOREDDCC3		UPRMOT IFIAT		

Table 3-2 Significant motif combinations of HSP90 isoforms and their co-regulated genes

(1) The consensus sequence for the heat shock element (HSE) is nGAAn arranged in continuous inverted repeats such as -nGAAnnTTcnnGAAn-. Mutational analysis has indicated that the G/C at position one of the unit is essential for heat response (Barros et al. 1992). A set of 38 regular expressions that represent the different possible mutations on the 2nd and 3rd nucleotide was used for the analysis.

(2) Motifs curated from Mahalingam et al. 2003

Biclique ID	Arabidopsis GI	Gene Name	HSP90-location	HSP70	Ppiase	DnaJ	sHSP	Other Stress related proteins	Non Stress related proteins
Biclique11040_3_7	A15g52640	HSP90-1	Cytoplasm	A13g12580 (Cyt), A15g02490(Cyt)			A13g46230(ND), A11g53540(ND)	A12g26150 (ATHstA2, Nu), A13g24170 (Glutathione reductase. cvt)	
Biclique4137_3_6	A15g56000	HSP90-4	Endomembrane System	A13g12580(ES)	A13g25230 (ND)			A13g12050 (Activator of HSP90, ND), A14g30490 (AFG1- Like ATP Ase family, Mt)	A13g53550 (Hypothetical protein, ND)
Biclique1139_11_3	A12g04030	HSP90-5	Chloroplast						A15g61170 (40s Ribosomal protein, ND), A14g18730 (60s Ribosomal protein, Cyt)
Biclique9_12_2	A13g07770	HSP90-6	Mitochondrion	A15g49910 (Chl)					
Biclique12054_7_8	A14g24190	HSP90-7	Endoplasmic Reticulum	A14g16660(ER)		A13g62600(ES)		A11g56340 (calreticulin, ER), A12g02810(PDI, ER), A12g02810(UDP- galactose/UDP- glucose transporter, ER), A15g61790 (calnexin, ES)	A11g56330 (GTP-binding protein, ER)

Table 3-3 HSP90 genes and their putative co-chaperones

Abbreviations in parenthesis indicate the subcellular localization predicted found in TAIR (ER: Endoplasmic reticulum, ES: Endomembrane system, Mit: Mitochondrion, Chl: Chloroplast, Cyt: Cytoplasm, ND: Not determined)

3.3.2. Putative co-chaperones

Xcis-clique discovered at least one putative co-chaperone for each of 4 HSP90 genes (Table 3-3). The putative co-chaperones had similar co-expression and shared similar motif combinations. At least two co-chaperones were found for each of HSP90-4 and HSP90-7. Co-chaperones associated with HSP90-1, HSP90-4 and HSP90-7 localize to the same cellular compartment as predicted by TargetP (Emanuelsson, 2000) for each of them.

The same agreement is not found in the case of HSP90-6 which is predicted to localize to the mitochondrion (Krishna and Gloor, 2001). Proteomic analysis of mitochondria has localized HSP90-6 in the mitochondria (Heazlewood et al., 2004), while its putative co-chaperone is predicted to localize to the chloroplast. However, there are, as yet, no experimental data that addresses the localization of this putative co-chaperone.

We were not able to find a co-chaperone for HSP90-5. This might be because the motifs that co-regulate HSP90-5 and its co-chaperone have not yet been discovered, or that this HSP90 does not form a complex with a co-chaperone.

3.3.3. Other stress genes present in the bicliques

Other candidate genes were present in the significant bicliques, such as sHSPs for HSP90-1. Lee and Vierling (2000) have shown cooperation between sHSPs and HSP70s in the folding of heat denatured proteins *in vitro*. These genes appear together with HSP90-1 in a biclique that contains a perfect HSE. HSP90-1 is the most heat inducible HSP90 (Haralampidis et al., 2001). Therefore, it is possible that the primary role of this protein is exerted under heat stress together with HSP70s and sHSPs.

Genes that are related to the unfolded protein response, such as protein disulfide isomerase, calreticulin and calnexin (Table 3-3) appear to be co-regulated with HSP90-7. All these genes share the unfolded protein response motif UPRMOTIFIAT found in genes up-regulated after ER stress induction by tunicamycin (Martinez and Chrispeels, 2003). The mammalian ER HSP90 complex was isolated using protein cross-linking (Meunier et al., 2002). Homologs to most genes found in that study are included in the biclique of HSP90-7 validating the results obtained with *Xcis-clique* (Table 3-4). Another

gene co-regulated with HSP90-7 (Table 3-3) is UDP-glucose/UDP-galactose transporter. This transporter together with calreticulin and calnexin are known parts of a second ER folding system, the calnexin/calreticulin system. All genes have the unfolded protein response motif (UPRMOTIF IIA) (Table 3-2) in common.

3.4. Discussion

3.4.1. Functional redundancy of endoplasmic HSP90s?

The protein and nucleotide coding regions of HSP90-2, HSP90-3, and HSP90-4 show more than 90% similarity (Krishna and Gloor, 2001). However the alignment of the 5'UTR and 500bp promoter region of these genes (Figure 3-1) do not show that much similarity (less than 50% in both cases). Therefore these genes are probably a consequence of gene duplication, however the promoter region has not been conserved, suggesting that each of them is expressed under different situations, space or time. A point mutation in HSP90-2 that can impair the transduction of the hypersensitive response pathway in Arabidopsis (Hubert et al., 2003) can be explained if HSP90-3 or HSP90-4 (that are very similar) are not being expressed at that point. Therefore experimental analysis of the expression of these genes should be done to get more clues about the functional roles of these proteins. This analysis can be done by real time PCR by designing primers in the regions that are not homologous such as the 5' UTR, and quantifying the expression of these genes under abiotic and biotic stresses.

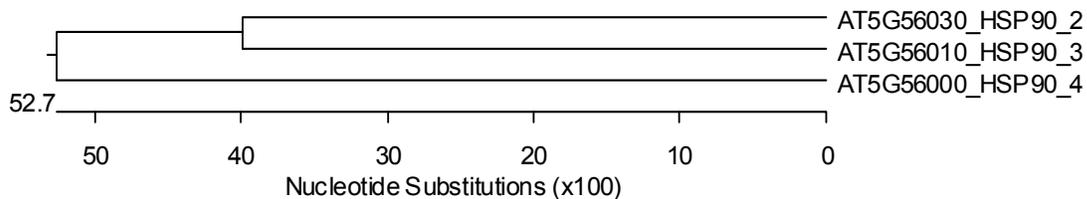


Figure 3-1 Phylogenetic tree promoter region of HSP90-2, HSP90-3 and HSP90-4

The alignment was done using the default parameters in Clustal W

Species	HSP90	HSP70 (HSP110 subfamily)	HSP70 (DnaK subfamily)	Protein disulfide isomerase	DnaJ	Ppiase	UDP-glucosyltransferase	O-mannosyltransferase
Mammals	GRP94	GRP170	BiP	Erp72, CaBP1, PDI	ERdj3	Cyclophilin B	glucosyltransferase	SDF2-L1
Arabidopsis	HSP90-7 (At4g24190)	GR170 homolog (At4g16660)	-	Erp72 homolog (At1g21750)	ERdj3 homolog (At3g62600)	-	-	-

Table 3-4 Comparison between the known members of the mammalian ER HSP90 complex and the members found for Arabidopsis by Xcis-clique

The members of the mammalian ER HSP90 complex described in Meunier et al., 2002., were compared to the whole set of genes available in the Arabidopsis database with blast and the first hits obtained are the same genes that were obtained with Xcis-clique.

3.4.2. Organellar HSP90s old and new roles

It has been shown that HSP90-5 is induced by light and that a mutation in this gene (*cr88*) affects the chloroplast development (Cao et al., 2003). This mutant is defective in other aspects of photomorphogenesis. The significant motifs found for this gene include the light responsive element (TBOXGAPB) and the L1BOXATPDF1 motif, which is found specifically in the L1 layer expressed PROTODERMAL FACTOR1 (PDF1) gene. These reported motifs can explain induction by light and the defects in photomorphogenesis found by Cao et al., (2003).

Cao et al., (2003) also has shown that this gene is induced by heat stress, but it does not confer higher thermo tolerance to the wild type if compared to the mutant genotype (*cr88*). Notably *Xcis-clique* did not find an HSE, among the significant motifs for this gene. Therefore a role in light responses for this gene is possible. No functional genomics work is yet available for HSP90-6. Besides the light and circadian cycle motifs, CARGCW8GAT, binding site of AGAMOUS LIKE 15 protein (Tang and Perry, 2003), appears as part of a significant motif combination. Therefore from the significant motifs found for this gene, a prediction could be made that a mutated or overexpressing HSP90-6 genotype could have altered light, circadian cycle responses and flower development. A mutation in HSP90-7 produced an altered flower development phenotype (Ishiguro et al., 2002). Ishiguro et al., (2002), by functional complementation demonstrated that the mutant phenotype arose because the *CLAVATA3* gene, that regulates flower development, was a target of this HSP90. Therefore a role in flower development for HSP90-6 cannot be discarded.

Chapter 4 Can Arabidopsis Acclimate to Drought Stress? Photosynthetic Responses in Wild Type Columbia and two At4g24190 (HSP90-7) Promoter Insertional Mutants During two Cycles of Drought Stress

4.1. Introduction

Most drought stress experiments in Arabidopsis have been done using the quick drying method (Urao et al., 1993; Kiyosue et al., 1994; Seki et al., 2001), but drought stress in the environment occurs in a gradual way. There have been drought stress experiments in Arabidopsis that use slow drying down to 70% of RWC (Rizhsky et al., 2004) and others that have gone down to 30% (Gigon et al., 2004). Recently Talame et al., (2007) have compared gene expression responses between low and fast drying in barley. Those authors concluded that only ~10% of the genes that were induced under the fast drying were induced in the slow drying plants. Moreover the magnitude of expression change in slow drying plants was low, compared to the fast drying plants. Therefore they suggest that fast drying experiments could be helpful to find genes related to signaling and recognition of the stress, but not to find genes related to acclimation that might provide long term protection.

Drought acclimation has been studied in other organisms such as wheat (Selote et al., 2004; Selote et al., 2006), Japanese mountain birch (Kitao et al., 2003), and in some cases gene expression analyses have been done, i.e.: pine (Watkinson et al., 2003) and potato (Watkinson et al., 2006).

We have shown previously a specific correlation of the responses of heat shock protein gene expression with photosynthetic acclimation in loblolly pine (Watkinson et al., 2003). Moreover, a homolog of HSP90-7 (At4g24190) in Arabidopsis was up-regulated when photosynthetic acclimation occurred (Vasquez-Robinet et al., unpublished). This gene is the homolog of GRP94 in mammals, which have been shown to be responsive during the unfolded protein response (Kozutsumi et al., 1988). Moreover

experiments with tunicamycin in *Arabidopsis* (which can induce the unfolded protein response) have shown the up-regulation of this gene (Martinez and Chrispeels, 2003; Kamauchi et al., 2005). The GRP94 homolog gene has been shown to be induced during systemic acquired resistance (Wang et al., 2005). It has also been shown to be responsive under cold stress (Bae et al., 2003), but there is no report of its expression during drought stress responses in *Arabidopsis*. This gene has been shown to be responsive to drought stress in the desiccation tolerant plant *X. viscosa* (Walford et al., 2004).

In order to see if this gene has a similar function in *Arabidopsis*, the drought responses of putative loss of function T-DNA mutants were evaluated, together with wild type plants (Columbia ecotype), to determine (1) if *Arabidopsis* can acclimate to drought stress and (2) HSP90-7 has a role in photosynthetic acclimation during drought stress.

4.2. Materials and methods

4.2.1. Plant material and mutant screening

The drought stress experiments were performed in Col-0 *A. thaliana* ecotypes and in Col- T-DNA insertion lines from the SALK collection.

Six T-DNA insertion lines on At4g24190 gene were screened SALK016310, SALK048558, SALK076127, SALK145452, SALK088837 and SAIL227_C07. The primers for detection of homozygous lines were designed using the SALK primer tool on <http://signal.salk.edu/tdnaprimers.2.html> and are listed in Table 4-1. The T-DNA left border primer used for the SALK lines was Lba1: 5'- TGGTTCACGTAGTGGGCCATCG-3', and for the SAIL line was Lb1: 5'-GAAATGGATAAATAGCCTTGCTTCC-3'. The localization of the insertion was confirmed by sequencing of the PCR bands obtained in homozygous lines. DNA was isolated using the DNeasy Plant Mini Kit (Qiagen, Valencia, CA).

T-DNA Line	Left Primer (LP)	Right Primer (RP)
SALK048558	5'-TCCTTCACACTCTCATCCTGAG-3'	5'-GGTATCAATCCACTTTGACCG-3'
SALK076217	5'-TCCATGTATCCTCGGAAACAG-3'	5'-TATCGACGGATTCCGATGTAG-3'
SALK016310	5'-TTTGGTGGAGAAATTAAGTTTGG-3'	5'-GCAAGTGACCCAGAGGTAATA-3'
SALK145452	5'-CTGTTTCATGGAAGAAAGCAGC-3'	5'-CAAGATTCAAGCAATCCAAGC-3'
SAIL227C07	5'-AGGAGGTCTCTGGTGAGGAAG-3'	5'-CAAGATTCAAGCAATCCAAGC-3'
SALK088837	5'-GCCTGAAAATCACCAACACAG-3'	5'-AAAATATAGCCGTCGATCGTC-3'

Table 4-1 Primers used for genotyping of T-DNA lines

4.2.2. Evaluation of embryo lethality

To evaluate whether At4g24190 was important for embryo formation, since homozygous lines with insertions in an exon were not found, seeds of heterozygous plants were counted following the guidelines in Seed Genes (www.seedgenes.org).

4.2.3. Drought stress application

The method of Mane et al. (2007) was followed with some modifications. *A. thaliana* seeds were imbibed for 3 days in the dark at 4°C. They were then sown on moist soil (Sunshine Mix 1), covered with plastic wrap and then transferred to a growth chamber set to 8 hours light, 100% fluorescent light at 22°C, 12 hours dark, at 18°C, and a constant relative humidity of 75% (Convion 4030, Winnipeg, MB. Canada). The flats were watered every two days. After three weeks, the plants were transplanted to trays with 6 plants per 6” x 4” pot. Mutant plants and wild type plants were planted together to ensure both were experiencing the same level of stress (Figure 4-1)



Figure 4-1 Drought stress experiment setup in growth chamber

Wild type plants have a green tag, while mutant plants have a purple tag. Well watered plants (controls) have an additional yellow tag. The plants were 5 weeks old.

After that, plants were fertilized every week with a half strength solution of Peter’s professional soluble plant food (Scotts-Sierra Horticultural Products, Marysville, OH). They were allowed to grow for 3 more weeks. To one group of plants drought stress was imposed by withholding water for approximately 2 weeks (1st cycle), re-watered, and then 1 more week (2nd Cycle) of no water. Another group of plants was stressed only during the 2nd cycle (Non acclimated: NAC). Figure 4-2 shows a diagram of the drought stress experiment. Photosynthesis was measured at noon (3 hours after the lights came on). This time point was defined as “maximum stress”. Photosynthesis measurements were taken using a Li-6400 portable Photosynthesis System (Li-Cor Inc. Lincoln, NE). Immediately after photosynthesis was measured plants were watered to full capacity. Four hours later photosynthesis was measured again, “recovery”. Sampling and photosynthesis measurements were performed at the end of each cycle (Figure 4-2). Three replicates were sampled for each time point and each replicate was made up of two plants that came from different pots. Only the aerial part (rosette) was harvested. Half the sample was used for relative water content (RWC) measurements and the other half was flash frozen in liquid nitrogen and stored at -80°C for RNA isolation.

Relative water content was measured as follow: the plants were weighed (fresh weight, FW), then put in a Petri dish containing distilled water and stored at 4°C overnight. The next day they were weighed again (turgid weight, TW) and then dried in a oven overnight at 90°C. After drying they were weighed again (dry weight, DW). RWC was calculated with the following formula: $RWC\% = (FW - DW) / (TW - DW)$.

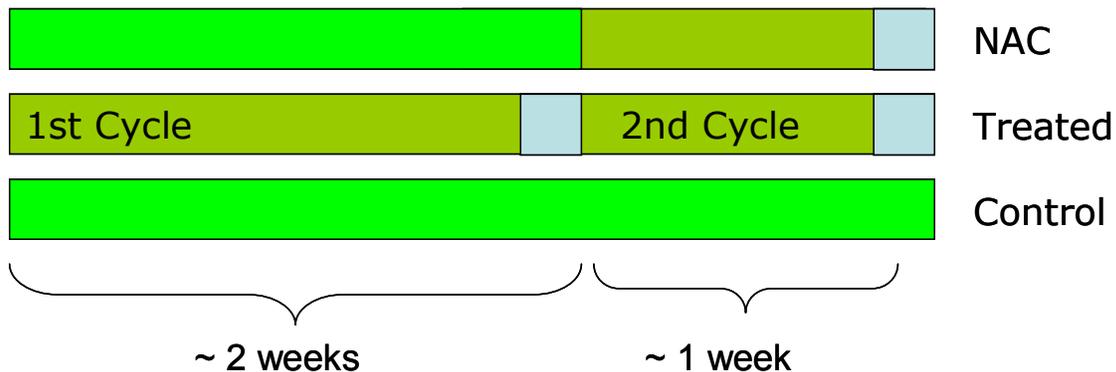


Figure 4-2 Diagram of drought stress experiments.

Turquoise blocks indicate the day of sampling which was at noon (maximum stress) and 4 hours after watering (recovery).

4.2.4. RNA isolation

Leaf tissue was ground to a fine powder under liquid nitrogen using a previously cooled mortar and pestle. The fine ground powder was then transferred to two 1.5 ml Eppendorf tubes containing 1 volume of grinding buffer (0.18M TRIS, 0.09M LiCl, 4.5mM EDTA and 1% SDS; pH 8.2), 1 volume of phenol pH 4.7 (Sigma, St Louis, MO), 1/10th volume of 2M NaOAc pH 4.0 and 10 μ l of β -mercaptoethanol/ml extraction buffer. The sample was thoroughly mixed on an orbital shaker (300 rpm) for 10 min. The sample was then centrifuged for 10 min at (9000 x G) at 4°C. The supernatant was transferred to a new Eppendorf tube and 1 volume of phenol:chloroform:isoamyl alcohol (25:24:1) pH 6.6 (Sigma, St Louis, MO) was added. The sample was then centrifuged for 10 min at (9000 x G) at 4°C. The supernatant was transferred to a new Eppendorf tube and an equal volume of chloroform was added. The sample was then centrifuged for 10 min (9000 x G) at 4°C. The supernatant was transferred to a new Eppendorf tube and 1/2 volume 10 M LiCl was added. The sample was incubated at -20°C overnight for RNA precipitation. The RNA was then pelleted for 30 min (10 000 x G) at 4°C. The pellet was washed with 80% ethanol and then resuspended in RNA Storage Solution (Ambion, Austin, TX)

4.2.5. Real time PCR

Two step real time PCR was performed. Total RNA was DNase treated with the DNasefree kit (Ambion, Austin, TX). After cleaning, 2 μ gs of RNA were reverse transcribed using the cDNA archive kit (Applied Biosystems, Foster City, CA) and oligo dT₁₈V primers. Real time PCR was performed with 5 μ l of cDNA (from a 20 ng/ μ l dilution) using SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA) in a 25 μ l reaction volume on an ABI Prism 7700 Sequence Detection System (Applied Biosystems), with 0.5 μ M primer final concentration and the following cycling steps: initial denaturation for 10 min. at 94°C, followed by 34 cycles with 15 sec 94°C, 30 sec 56°C and 30 sec 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). Primers to obtain larger amplicons (700-900bp) were designed from *A. thaliana* sequences from the TAIR

database. The products of the reaction were run through electrophoresis and isolated from agarose. Ten fold dilutions of each product between 10 pg and 0.1 fg were used to generate a standard curve for each gene. Chloroplastic GAPDH (At3g26650, Forward: 5'-CGTGATCTAAGGAGAGCAAGA-3', Reverse: 5'-TTCCCTTGAGGTTAGGGAGC-3') was used as a control gene. Two sets of primers were designed for the At4g24190 gene one in the 3'UTR (F1: 5'-ACCCGTTGAGCAACAAGAAGAG-3' and R1: 5'-CGAGTAAAACGATGTTCTGCTT-3') and the 2nd in the C-terminal (F1:5'-TGGATGGAGTGCTAATATGGAG-3'and R2:5'-GCAAGTAGTGCGTTTACATAATCC-3'). Both primer pairs gave similar results. The results showed here are from the 2nd primer pair.

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.

4.3. Results

4.3.1. At4g24190 mutants genotyping results

At the time of the experiment there were 3 lines available containing insertions in exons of the gene: SALK016310, with an insertion in the last exon, SALK048558 with an insertion on exon 13 and SALK076127 with an insertion in exon 4 according to TAIR. Genotyping of the seedlings resulted in three homozygous mutants for SALK016310 and no homozygous mutants for the two other lines. Reverse transcription was done from RNA from leaves from the 3 homozygous SALK016310 lines, and semi-quantitative PCR was done to check the level of expression of the gene and to verify that they were not expressing the gene (Figure 4-3), further analysis of the insertion place in the SALK website (<http://signal.salk.edu/cgi-bin/tdnaexpress>) showed that the insertion was in the 3'UTR.

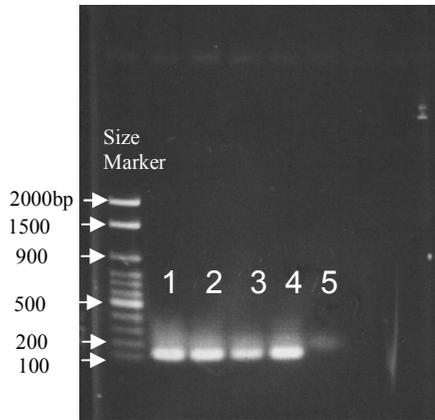


Figure 4-3 Gene expression in T-DNA insertion lines in At4g24190 as revealed by semiquantitative RT-PCR

Lanes 1, 2 and 3 are the 3 SALK016310 homozygous lines. Lane 4 has Col-O and lane 5 is the negative control. PCR was performed using cDNA from each genotype and using primers that amplify the 3' UTR (F1 and R1).

Since it was not possible to find homozygous mutants in the other lines, it was suggested that a complete knockdown of the gene may be embryo lethal. In mammalian cells it has been observed that a basal amount of ER resident HSP90 is needed for survival (Gorza and Vitadello, 2000). To check if the gene was important for embryo/seed development, seeds from an heterozygous plant (SALK048558). A mutant phenotype was observed in only 3.4% of total seeds counted (29 of 845 seeds) in the mutant line. No comparable aberrant phenotype was observed in siliques from wild type plants. Mutant seeds also appeared smaller and darker than the wild type seeds. Most mutant seeds were localized close to the tip of the silique, in the region closest to the stigma surface. The number of observed mutant seeds was much smaller than the 25% expected Mendelian (3:1 ratio) if a homozygous mutation of At4g24190 had resulted in embryo lethality.

The only mutant report of the ER resident HSP90 in Arabidopsis is a mutant in the Wassilewskija ecotype (*shd:shepherd*), where the insertion is 16bp upstream from the coding sequence (Ishiguro et al., 2002). Therefore, a mutant that has the insertion in the promoter may be viable, where expression was reduced, but not suppressed completely. Three insertion lines were screened: one with the insertion just before the first exon (SALK145452), two with insertions approximately 300bp upstream of the 5'UTR (SALK088837 and SAIL227_C07). Homozygous lines were obtained with SALK145452 and SAIL227_C07. The SALK 145452 mutants produced two PCR products after PCR with LbA and the left and right primers (LP and RP) for that region. The bands had a

difference in size of 20bps approximately (Figure 4-4), so a PCR was performed with LbA and LP and another one with LbA and RP, suggesting a tandem tail to tail insertion. This was confirmed by sequencing both products (Figure 4-5) and it was not in the 5'UTR. Instead, the insertion was upstream 254 bp from the first exon (Figure 4-5, Figure 4-6). The SAIL227_C07 mutant gave only one band after PCR with the LB1 primer and its respective LP and RP primers. After sequencing of the product, the insertion proved to be ~70bps closer than the location given by SALK, 240bp upstream from the first exon (Figure 4-5, Figure 4-6).

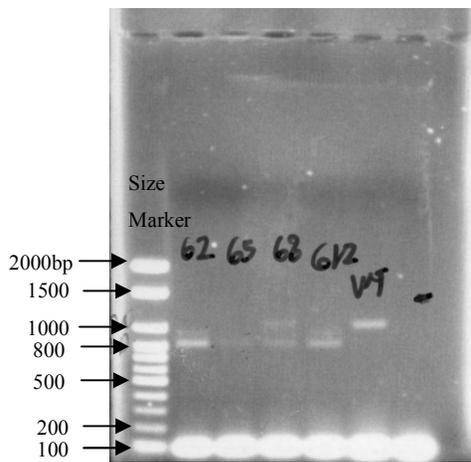


Figure 4-4 PCR results of screening for homozygous SALK145452 mutants

G2, G5, G8 and G12 are SALK145452 lines (lanes 1,2,3,4). Line 5 has the Col-O control and the last line is the negative control.

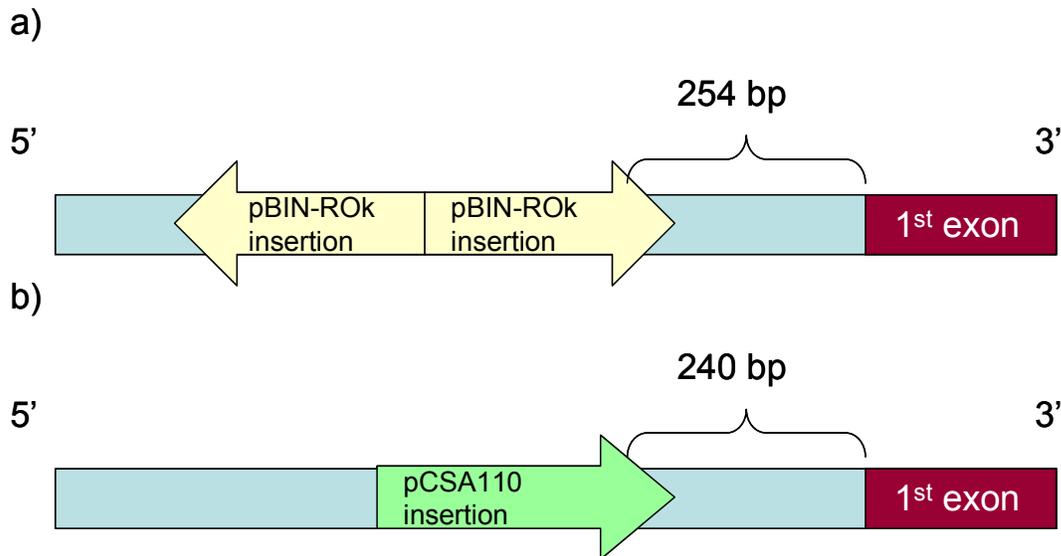


Figure 4-5 Location of the T-DNA insertions in the SALK145452 (a) and SAIL227_C07 (b) homozygous lines.

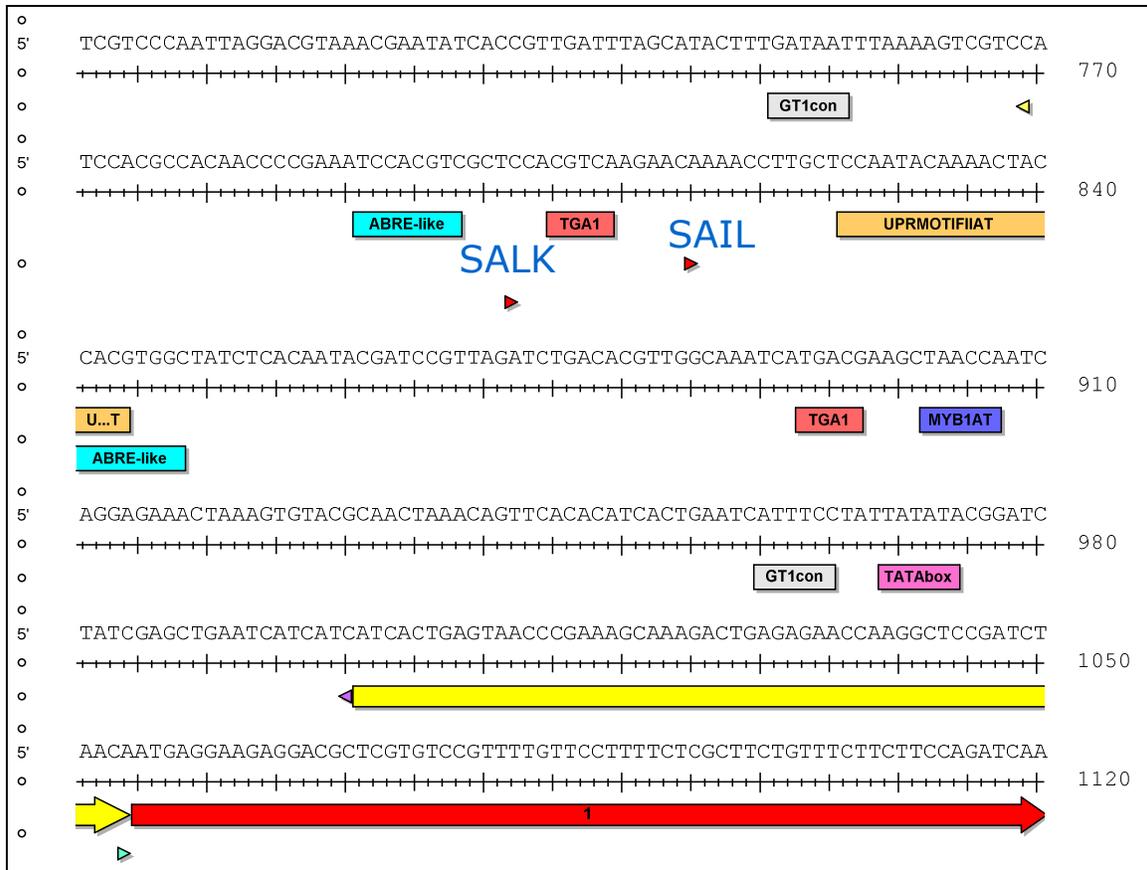


Figure 4-6 Location of T-DNA insertions in the upstream sequence of At4g24190 (AtHSP90-7) for SALK145452 and SAIL227_C07

The localization of the insertions of the SAIL: mutant (SAIL227_C07) and SALK mutant (SALK145452) were confirmed by sequencing the bands obtained using a left border T-DNA primer and an At4g24190 primer, designed by the SALK primer tool (<http://signal.salk.edu/tdnaprimers.2.html>). The small colored boxes highlight the location of cis-elements and the insertions are just before the unfolded protein response element (UPRMOTIFIAT). The purple and green triangle mark the location of the insertions from SALK database (purple: SAIL227_C07 and light green SALK145452) and the red triangle marks the actual place of the insertion found by sequencing. The light yellow box represents the 5'UTR of the gene and the red arrow part of the first exon.

Real time PCR was carried out with cDNA from the SALK145452 and SAIL227_C07 homozygous lines. The primers used (primer pair 2) localized to the C-terminal part of the gene (exon 12). Gene expression in the mutants was compared with levels of gene expression in Col-0 plants growing in the same pot at the same conditions (well watered, 8 hours of light, 21C light, 18C dark) showing that the gene expression was reduced on the SALK145452 line and reduced for the SAIL227_C07 line (Figure 4-7).

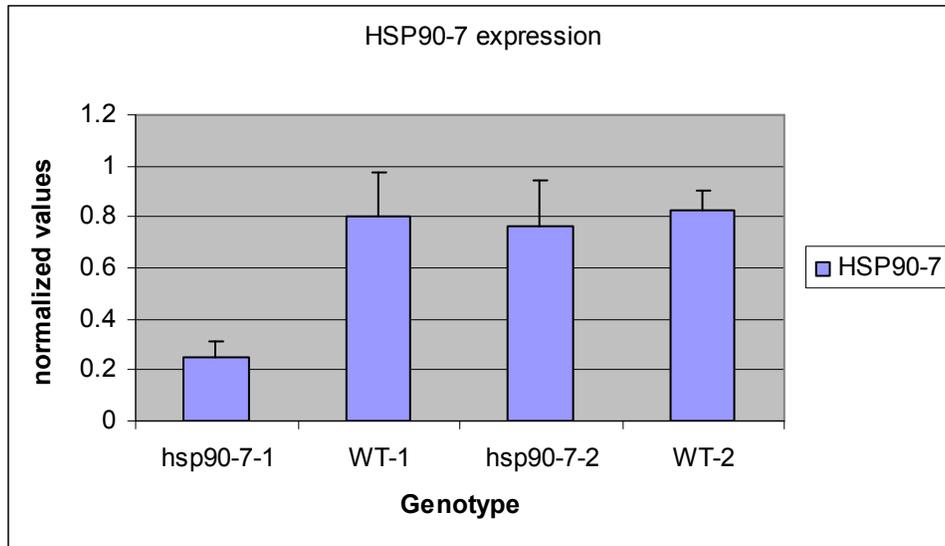


Figure 4-7 Expression of HSP90-7 gene in mutant homozygous lines and wild type (Col-O) plants

Real time PCR was done in cDNA from 3 replicates (each replicate made of two plants), with 3 technical replicates. WT-1 indicate the results from wild type plants that were growing in the same pot that *hsp90-7-1* and WT-2 indicate the results from wild plants that were growing in the same pot that *hsp90-7-2*. The expression data was normalized with chloroplastic GAPDH.

4.3.2. Mutant plants acclimated after one cycle of mild stress while wild type plants did not

There were no morphological differences between the wild type and mutant lines before and after the exposure to drought stress (Figure 4-8). After one cycle of mild stress, both mutants SALK14542 (hereafter referred to as *hsp90-7-1*) and SAIL227_C07 (hereafter referred to as *hsp90-7-2*) have a RWC similar to the wild type (Figure 4-9 A and C, Table 4-2) and they recovered after 4 hours in a similar way (Figure 4-9 B and D, Table 4-2). Their photosynthetic behavior was similar, although it seemed that the *hsp90-7-1* mutant has a higher photosynthesis rate (WT= 3.3 $\mu\text{molCO}_2/\text{cm}^2/\text{s}$, *hsp90-7-1*=4.0 $\mu\text{molCO}_2/\text{cm}^2/\text{s}$) than the wild type (Figure 4-9 C).

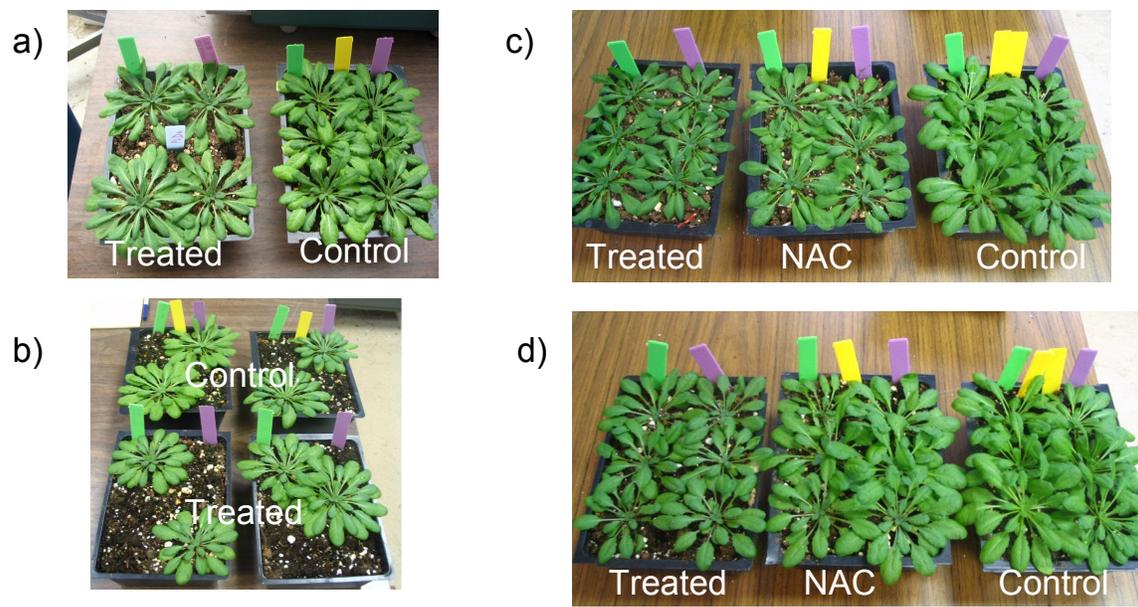


Figure 4-8 Effect of drought stress on WT and mutants after two cycles of stress and recovery

4-8 a and 4-8 b illustrate the appearance of the plants from the 1st experiment (*hsp90-7-1*) and 4-8 c and 4-8 d from the 2nd experiment (*hsp90-7-2*). There are no obvious phenotypic differences between the wild type and mutant plants after the drought stress application (a and c) or after the recovery (b and d) both recovered on a similar way.

Treatment	Maximum stress				Recovery			
	1st Experiment		2nd Experiment		1st Experiment		2nd Experiment	
	WT	<i>hsp90-7-1</i>	WT	<i>hsp90-7-2</i>	WT	<i>hsp90-7-1</i>	WT	<i>hsp90-7-2</i>
1st cycle (Control)	94.5±0.5	96.0±1.6	96.5±2.8	97.7±1.7	91.3±0.7	94.2±1.2	90.6±2.0	90.1±0.4
1st cycle (Mild)	90.5±1.2	91.3±0.4	86.8±0.7	85.2±1.4	94.1±0.7	92.0±1.8	88.0±2.9	90.4±1.0
2nd cycle (Control)	93.5±2.3	95.7±0.9	97.5±0.5	92.7±1.9	94.6±1.0	94.9±1.2	96.3±1.5	94.9±1.0
2nd cycle (Severe)	72.3±0.3	69.3±0.3	60.5±0.1	61.3±2.2	96.6±0.1	94.8±2.7	88.3±1.5	84.6±1.9
NAC(mild)	96.6±1.7	94.5±0.8	88.3±0.9	84.9±0.6	96.5±1.9	95.7±3.3	89.3±1.8	91.7±1.1
NAC(severe)	-	-	74.6±2.0	71.9±3.3	-	-	87.7±1.3	86.2±2.0

Table 4-2 Relative water content (RWC%) in mutant and wild type plants.

The RWC at maximum stress after two cycles of drought stress are in bold, and the RWC of the NAC that experienced a mild stress are in italics.

After the 2nd cycle of drought stress the mutant plants had a higher photosynthetic rate compared to wild types (Figure 4-9 A and C), moreover both wild type and mutant genotypes were experiencing the same low levels of water stress at that time (similar RWC Table 4-2). In the first experiment (WT vs *hsp90-7-1* mutant) the RWC was around 70% and in the 2nd experiment (WT vs *hsp90-7-2* mutant) it was around 60%. In the 2nd experiment there were mutant NAC plants that had a RWC around 74% (Figure 4-9 C, yellow arrow) and had a lower PS rate compared to the ones that were previously exposed to a mild stress and had an even lower RWC of 60% (Figure 4-9 C, yellow bubble). The NAC WT plants had a RWC around 72%, and their photosynthetic rate was

slightly higher than the WT plants that had a previous exposure to a mild stress and a RWC of 61% (Figure 4-9 C).

The recovery rate after the 2nd cycle was similar among the mutants and wild type, although since WT and *hsp90-7-2* were at a lower RWC in the 2nd experiment (Table 4-2) compared to the 1st experiment (~70%), they (WT and *hsp90-7-2*) did not recover to a RWC close to normal values (~95%) moreover, the *hsp90-7-2* mutant recovered to a slightly higher RWC compared to the wild type (*hsp90-7-2* RWC= 88 +/- 1.5%, WT RWC =84+-1.8%).

The NAC mild plant in the 1st experiment were not as stressed (RWC ~94%, Table 4-2) as the NAC mild plants in the 2nd experiment (RWC 85%) which explains the differences in photosynthetic rates observed (Figure 4-9 A and C) where NAC mild plants from the 1st experiment have higher photosynthetic rates compared to NAC mild plants from the 2nd experiment.

In the 1st experiment it seemed that the mutant had a higher photosynthetic rate compared with the wild type, so I thought that maybe they were at a different developmental stage, because this mutant also took one to two weeks more to bolt compared to the wild types (Figure 4-10). From a total of 8 plants of each type, 7 WT flowered while only 1 mutant plant flowered. The WT and mutant plants were growing in the same pots and therefore were exposed to same conditions.

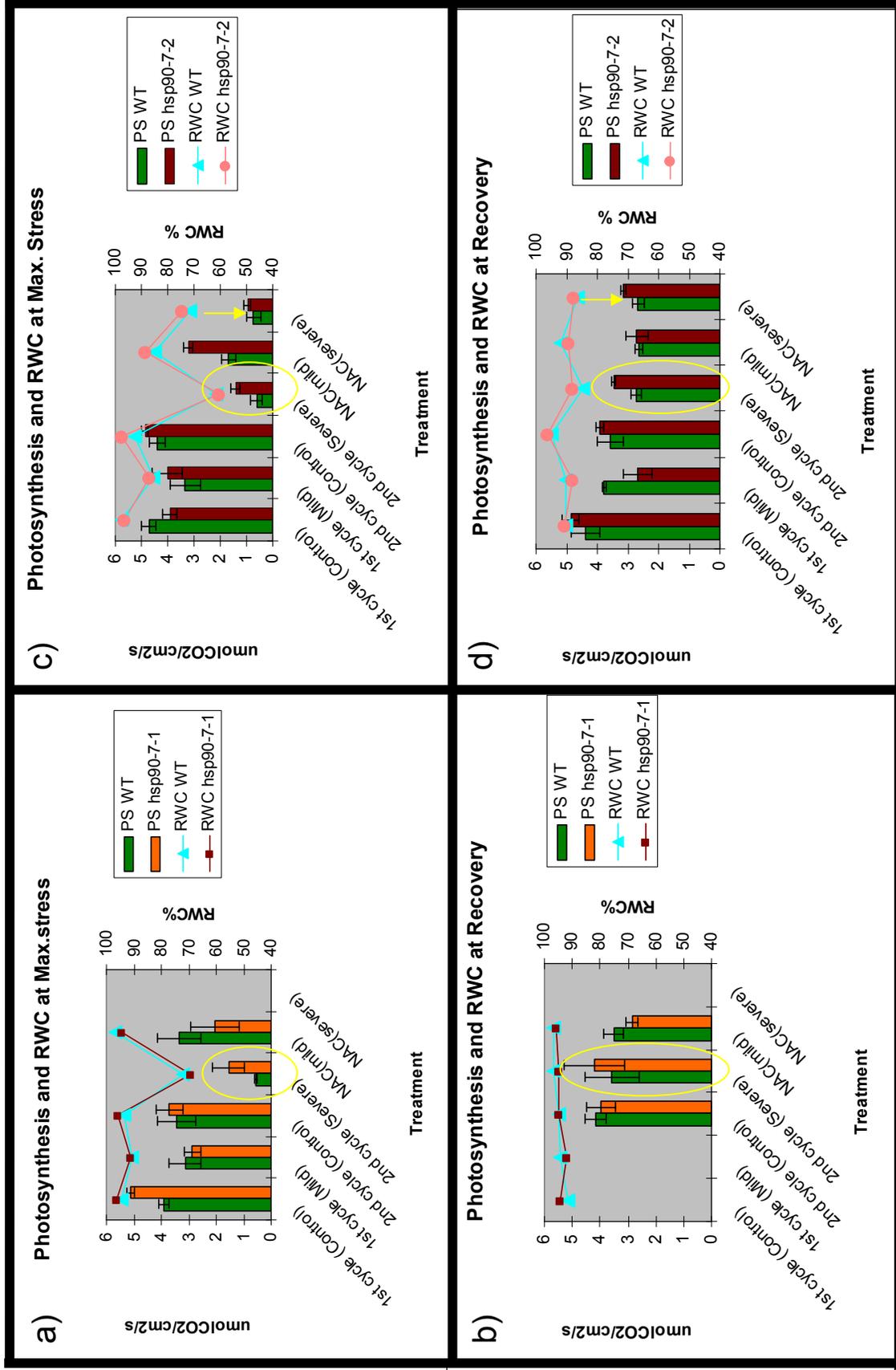


Figure 4-9 Effects of Two Successive Cycles of Drought Stress on Photosynthesis and Relative Water Content in wild type Col and *hsp90-7-1* and *2*

A and B are the results of the 1st experiment (Fall 2006) and C and D are the results of the 2nd experiment (Spring 2007). Photosynthesis data is represented in columns and RWC in lines. In both experiments mutant genotypes acclimate to the drought stress while the wild types did not (A and C, yellow bubbles and yellow arrow) and they were experiencing the same level of water stress. This effect is also seen after 4 hours of rehydration (recovery B and D).

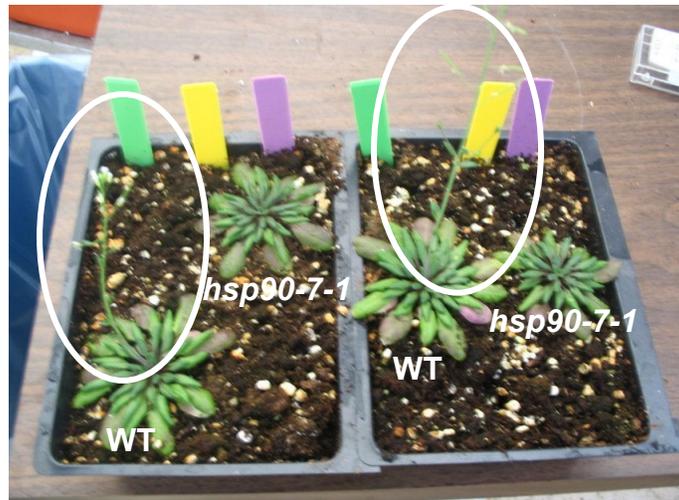


Figure 4-10. Time of flowering in unstressed WT and *hsp90-7-1* plants

Wild type plants (WT) that were grown together with mutant plants (*hsp90-7-1*) for eight weeks under the same conditions flowered earlier (white oval).

4.3.3. Effect of T-DNA insertion in the expression of HSP90-7 in mutant lines

Since both mutant lines had acclimated while the wild type did not, expression of At4g24190 may have been altered. However, since the insertion was close to the ER stress responsive element (UPRMOTIFIIAT, Figure 4-6) and HSP90-7 may be important for the event similar to the Unfolded Protein Response that may occur during severe drought stress, it was possible that the gene expression level would be high, and that could explain why the mutants acclimate. We tested this hypothesis by measuring the expression level of the At4g24190 in the *hsp90-7-2* mutant and wild type plants. Figure 4-11 shows the results after real time PCR (data not available for *hsp90-7-1* mutant). As can be seen in Figure 4-11, the *hsp90-7-2* mutant had a lower level of expression of HSP90-7 compared to the WT. Moreover the WT NAC severe plants (Figure 4-11, light turquoise bar) have their expression a little higher than the plants previously exposed to a mild stress (Figure 4-11, maroon bar).

In both cases (*hsp90-7-2* and WT) the levels of expression after rehydration were not as high compared to the maximum stress point (Figure 4-11).

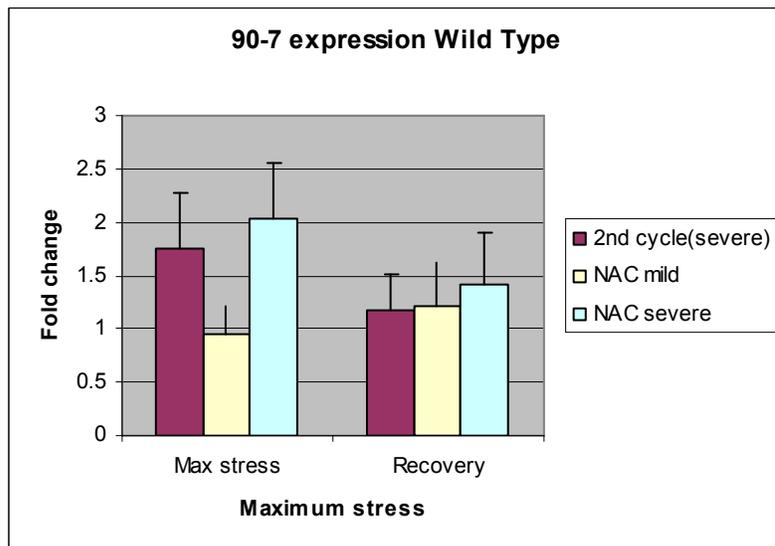
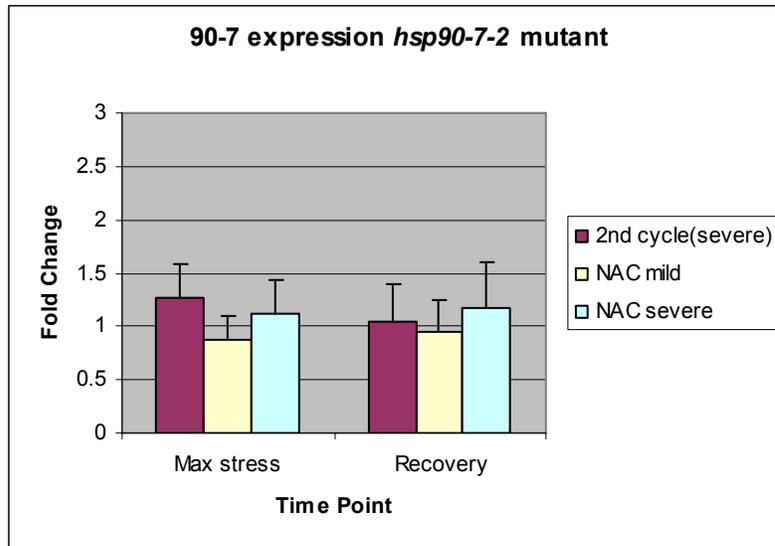


Figure 4-11 Effects of Drought Stress on the Relative expression of AtHSP90-7 in the *hsp90-7-2* Mutant and Wild type

Real time PCR was carried out on cDNA obtained from mRNA from drought stressed leaves from the *hsp90-7-2* mutant and wild type plants during the 2nd cycle, and from corresponding well watered plants. The relative expression of HSP90-7 in samples from plants that experienced 2 cycles of stress (1st cycle: mild stress, 2nd cycle: severe stress) are in maroon, from plants that were non acclimated (NAC) and were experiencing a mild stress are in yellow, and from non acclimated plants that were experiencing a severe stress are in pale turquoise.

4.4. Discussion

4.4.1. AtHSP90-7 may be an essential gene for pollen germination or pollen tube formation

Segregation ratios obtained from mutant seeds in a heterozygous line SALK048558, 20:2 deviated from the expected Mendelian value (3:1). This result suggested that AtHSP90-7 action is not related directly to embryo development. Moreover, the fact that the mutant seeds are located close to the tip of the silique, suggests that the mutant allele might have an effect on pollen germination. Segregation studies on the *shd* a knockout of the At4g24190 gene in the Wassilewskija ecotype, resulted in a reduced inheritance of the *shd* mutation when the homozygous male plants were crossed with heterozygous plant, while that did not happen when the homozygous plant was the female donor, thus male gametophytes were affected by the mutation (Ishiguro et al., 2002). Further studies were done where attaching pollen grains from *shd* flowers on to wild type stigmas and *dad1* mutants stigmas (where self pollination is inhibited since their anthers cannot dehisce at flower opening), showed that the pollen grains germinated but the pollen tube was rarely formed (Ishiguro et al., 2002). Thus, one documented action of the AtHSP90-7 gene is to enable pollen tube germination.

Recent work on the *shd* mutant have shown that it is, in fact, not a complete knockout, since the gene can be induced after tunicamycin treatment (also used for unfolded protein response induction) and is expressed in the roots (Klein et al., 2006).

4.4.2. Lower expression of AtHSP90-7 is needed for photosynthetic acclimation in Arabidopsis

It appears that the difference in net photosynthetic rates between the two mutants in the unstressed conditions might be due to the different placements of the mutants. Different cis-elements may be active in this mutant that activate the gene differently, producing a different photosynthetic phenotype than in the controls (*hsp90-7-1* controls have higher photosynthesis rates, while *hsp90-7-2* controls are similar to wild type

controls). However, from the real time data in the *hsp90-7-2* mutant it seems that a reduction in the level of HSP90-7 expression might be necessary for photosynthetic acclimation. Klein et al., (2006) observed that the reduction of expression of this gene in the *shd* mutant in proliferating tissue increased the protein steady state levels of BIP, therefore BIP compensates HSP90-7. They did not observe the same compensation in mature leaves. However, when the mRNA levels were measured they could not find a difference concluding that this higher accumulation in *shd* is due to increased protein stability. The steady state levels of BIP protein might also be higher in the *HSP90-7-2* mutant and might have contributed to the photosynthetic acclimation observed.

It seems that At4g24190 is induced under low RWC (Figure 4-7) as can be seen in WT plants with a RWC between 60% and 70%. This was seen previously in real time expression analysis from Anti-PLD alpha plants that experience a severe stress compared to wild type plants (Mane et al., 2007). Under low RWC drought stress, protein unfolding can occur, due to the high level of ROS species. The activation of antioxidant genes and the occurrence of high levels of H₂O₂ under severe water condition has been shown in wheat seedlings (Selote et al., 2004; Selote and Khanna-Chopra, 2006). Selote et al., (2006) observed that an exposure to a mild stress induced a coordinated antioxidant response that was correlated with drought acclimation in wheat seedlings.

Therefore it seems that HSP90-7 has a different role in Arabidopsis than in pine, since it is induced at low water levels (60-70% RWC) but it does not appear to be related to photosynthetic acclimation.

Chapter 5 Physiological and Molecular Adaptations to Drought in Andean Potato Genotypes

5.1. Introduction

Plants have different ways to respond to drought stress, they can avoid the stress or they can develop mechanisms of tolerance. These mechanisms are not exclusive and plants might use a range of response types. A “tolerance“ mechanism implies ways to avoid tissue dehydration, while maintaining the water potential, or tolerating a low tissue water potential. This can be accomplished by minimizing water loss, which is mostly achieved by the closing of stomata, but the plant can also adopt morphological changes to avoid water loss: such as decreasing canopy leaf area through reduced growth and shedding of older leaves (Chaves et al., 2003). The closing of stomata reduces CO₂ availability, therefore affecting photosynthesis and yield.

During drought stress ABA biosynthesis is stimulated. The accumulation of ABA (Mizhari et al., 1971, Boussiba et al., 1975, Radin 1981, Robertson et al., 1985), initiates a series of signaling events that help the plant tolerate the stress. The advances of molecular biology have allowed the identification of many of the genes that are involved in the transduction of this response. At least two pathways have been identified, one ABA dependent and, the other, ABA independent (Ishitani et al., 1997, Shinozaki and Yamaguchi-Shinozaki 1997; Zhu, 2002). ABA is responsible for stomatal closure in guard cells (Schroeder 2001, Israelson et al., 2006) and phospholipase D alpha-1 and ROS mediate this response (Mishra et al. 2006). ABA also increases/activates transcription factors such as MYBs, DREBs, bZIPs that activate the expression of other genes (reviewed by Yamaguchi-Shinozaki and Shinozaki, 2006). However ABA levels are high only a few hours after the perception of the stress, after that it is metabolized. The *abi 1-1* and *abi2-1* mutants are not responsive to ABA (ABA insensitive mutants). The mutation in these mutants are in type 2 C protein phosphatases (PP2C), that are known as negative regulators of ABA signaling (Leung et al., 1997). Recent studies with *Arabidopsis* plants at low water potential has found that these mutants accumulate ABA,

but to a higher levels compared to wild type plants, therefore Verslues and Bray (2006) proposed that PP2C is involved in ABA catabolism/degradation signaling.

After the first ABA-responsive transcription factors are induced, the expression of other genes follows, that help to protect the cell such as heat shock proteins (Vierling 1991, Rizhsky et al., 2002, Rizhsky et al., 2004, Watkinson et al., 2003, Wang et al., 2004), antioxidant proteins (Mittler and Zilinskas, 1994; Jiang and Zhang, 2002; Pastori and Foyer, 2002; Walz et al., 2002), and osmolyte metabolic enzymes (Yoshiba et al., 1997, Satoh et al., 2002).

Potato is the fourth most important food crop in the world, with an annual production approaching 300 million tons (CIP, 1998) and like other crops it is also affected by drought stress. Moreover, the specie of potato that it is mostly consumed, *S. tuberosum* is highly susceptible to drought stress (Weisz et al., 1994). The domesticated tetraploid *S.tuberosum* subsp *tuberosum* comes from the Chiloé Island in Chile, and originally from Andean stock (Hawkes, 1990). *S. tuberosum* subsp. *andigena* is a tetraploid Andean potato cultivated from the Andes from Venezuela to Northern Argentina (Huaman and Ross, 1985), being cultivated at an altitude as high as to 3500 m.o.s.l (Terrazas et al., 1996) and therefore adapted to harsh climate situations. Also, since it is also a tetraploid, it is possible to create hybrids with *S. tuberosum* subps *tuberosum* (Tai and Tarn, 1980; Kumar and Kang, 2006). Therefore *S. tuberosum* subps *andigena* (hereafter referred to as Andigena) is an ideal candidate to study genes related to drought tolerance, that in the future could be used for breeding or biotechnology improvement of *S. tuberosum* sbps *tuberosum*.

Previous experiments with Andigena accessions showed that the accession that adapted the best, had a higher, constitutive level of expression of genes encoding enzymes of the flavonoid pathway suggesting that the flavonoids are important to adaptation (Watkinson et al., 2006).

In the present study we evaluated the physiological and phenotypic response to drought stress of 3 Andigena genotypes, one Andigena x Tuberosum hybrid and Atlantic (*S. tuberosum* subps *tuberosum* cultivar). We were able to identify resistant and tolerant Andigena genotypes, based on photosynthetic performance after 25 days of stress. We further evaluated gene expression of two of the Andean genotypes, using a 44k feature

oligo microarray from Agilent Gene expression analysis suggested that the better performance in the resistant genotype is correlated with up-regulation of heat shock protein genes and antioxidant genes located in the chloroplasts, suggesting a better protection of the chloroplast against the ROS species that might have arisen due to drought stress.

5.2. Materials and methods

5.2.1. Plant material and drought stress application

Three *S.tuberosum* spp *andigena* genotypes: Leona, Negra Ojosa and Sullu, one breeding variety (adg x tbr) Costanera and *S. tuberosum* (Atlantic) (Figure 5-1,**Error! Reference source not found.**) were clonally propagated *in vitro* and rooted in rooting media. They were transferred to 9 cm square pots containing Promix BX (Premier Horticulture, Quakertwon, PA) in the greenhouse and grown under natural light (July, Blacksburg, VA, latitude 37.229N. longitude -80.414W) at (20-25C) for two weeks. Tip cuttings were then taken, dipped in Rootone® F brand (Bayer Cropscience, Research Triangle Park, NC), transferred to 9 cm square pots with a mix of 2 parts of Pro Mix BX and one part of sand, and kept at high humidity for two days in the dark and then grown under the same conditions. After 4 weeks, plants were transferred to 28cm x 19 cm containers containing the same potting mix (2 Pro Mix BX: 1 sand) and grown under the same conditions for 6 more weeks. After that time water was withheld (September, Blacksburg, VA). Every week the plants were fertilized with a half strength solution of Peters professional soluble plant food (Scotts-Sierra Horticultural Products, Marysville, OH).The plants were monitored by photosynthetic measurements (LiCor 6400, Lincoln, NE). Control plants were watered every 10 days. Photosynthesis averaged between 8 and 12 $\mu\text{mol CO}_2/\text{m}^2/\text{s}$ throughout the experiment. Samples were harvested when photosynthesis was reduced to 50 to 60% in the most susceptible genotype (18 days after water stress imposition) and then when it was reduced to 70% in the other genotypes (25 days after water stress imposition). After that point, the plants were rewatered and photosynthesis measurements were taken 24h later. Photosynthesis was monitored every 3 -4 days on the 4th fully expanded leaf and when one genotype reach 50% to 60% photosynthesis, photosynthesis was monitored every other day. The 4th fully expanded

leaf was harvested at the three time points (18 and 25 days after withholding water and at the 24 hour recovery point), they were then flash frozen in liquid nitrogen and stored at -80C. Half of each sample was used for RNA isolation and the other half was used for metabolite analysis. Tubers were also harvested at each point, counted, weighed and flash frozen in liquid nitrogen. At each point 3 treated and 3 control plants were harvested.



Figure 5-1 Negra Ojosa, Leona, Sullu y Costanera plants after 18 days of stress

It can be observed that at this point Costanera is already wilting compared to the other genotypes, and its photosynthesis was 50% reduced compared to control plants.

5.2.2. RNA isolation

RNA was isolated from approximately 2 g of leaf tissue. RNA was isolated using a phenol-based method as instructed by TIGR

http://www.tigr.org/tdb/potato/microarray_SOPs.shtml.

5.2.3. Microarrays

RNA extracted from leaves at the point of maximum stress (25 days after water withhold) were hybridized on custom made Agilent OligoMicroarrays, with 44 K features. All the reagents were bought from Agilent and the hybridization was done following the Agilent protocol:

<http://www.chem.agilent.com/scripts/LiteraturePDF.asp?iWHID=46782>.

USDA num	Cip num	Cultivar name	Spp	Biological Status	Country of Origin	Predominant skin color	Predominant skin flesh color	Altitude	Longitude	Latitude
									Coord.	Coord.
Q44027	379706.27	Costanera	Adg x Tbr	Bred variety	PER	White-cream	White			
Q44030	701997	Sullu	Adg	landrace	PER	Yellow	Light yellow	3700	-75.38	-11.82
Q44031	704058	Leona	Adg	landrace	ECU	Dark purple-black	Purple			
Q44032	704143	Negra Ojosa	Adg	landrace	ARG	Purplish red	Light yellow	3440	-65.47	-22.12
NA	NA	Atlantic	Tbr	landrace	CHILE	White-cream	White			

Table 5-1 Information about *S. andigena* landraces used in the drought experiment.

5.2.4. Statistical analysis and data mining

The microarray analysis platform TM4 has been used (Saeed et al., 2003). The following normalization steps were carried out on MIV (median intensity values) using the MIDAS pipeline: total intensity normalization, lowess normalization, standard deviation regularization and low intensity filtering. The output of the MIDAS pipeline was used to identify differentially expressed genes using one class *t*-test in Multi Experiment Viewer (MEV) module of TM4. The details of the microarray analyses are described in (Sioson et al., 2006). The physiology data was analyzed in R (<http://www.R-project.org>) using an ANOVA model where the main and interaction effects of genotype and treatment on tuber number, average tuber weight and root weight was analyzed.

5.2.5. Real Time PCR

Two step real time PCR was performed. Total RNA was DNase treated with the DNasefree kit (Ambion, Austin, TX). After cleaning, 4µgs of RNA were reverse transcribed using the cDNA archive kit (Applied Biosystems, Foster City, CA) and oligo dT₁₈V primers. Real time PCR was performed with 5 µL of cDNA (from a 20ng/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 sec 94°C, 30 sec 56°C and 30 sec 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). Primers to obtain larger amplicons (700-900bp) were designed from *S. tuberosum* sequences from the TIGR potato ESTs database. The products of the reaction were run through electrophoresis and isolated from agarose. Ten fold dilutions of this products in concentration between 10 pg and 0.1 fg were used to generate a standard curve.

An 183 bp amplicon of adenosine kinase served as an internal control since its expression level does not change in potato during drought stress (Watkinson et al., 2006). Real time primers were designed from cloned sequences from candidate genes (Table 5-2). All primers pairs were tested for dimer formation before using them with the actual samples.

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.

Gene Name	Subcellular location	Forward Primer	Reverse Primer
HSP40	Chl	5'-GCACACAGCATAGCATGTTTCATC-3'	5'-TCATCGCCCTTCTCGTTACC-3'
Ppiase	Chl	5'-ATCCAATTCATCTTCCCGCC-3'	5'-TACCGCCACCAATCGAGCTG-3'
HSF	Nu	5'-AACCAAGTCCAAGAAAGTATGTCGG-3'	5'-ACAGCAGGTGACAATCAAGCC-3'
HSC70-3	Cyt	5'-TGTGCAAGCAGCCATTCTTAGTG-3'	5'-TCCAGCAGTTTCGAGTCCTAGC-3'
BIP	ER	5'-CAAGGTCTATTCTCATCTCCG-3'	5'-GGAGAAATGTCGTAATCAGG-3'
HSP90(GRP94)	ER	5'-ATCAAGGTCGAAAGATACAGG-3'	5'-TAAACCATTAGGAACAGCACC-3'
Glutathione Synthetase	Chl	5'-AGCTCCATATCTGAAGGAAGTGACC-3'	5'-GGGGTAACAAAGAGTCCAAACGC-3'
Glutathione S transferase	Cyt	5'-GGGAAAGAACTAACAGGAACC-3'	5'-GGAGTTGGAGTACCATGTTG-3'
Thioredoxin H	Cyt	5'-TCCAATTATCAATGACTTTGCTTCC-3'	5'-GCATAGCTTGAACCTCGTATTCCA-3'

Table 5-2 Primers used for Real Time PCR

Cyt: Cytosol, Chl: Chloroplast, Nu: Nucleus and ER: Endoplasmic Reticulum

5.3. Results

5.3.1. Effect of drought stress on root weight, tuber number and average tuber weight

Figure 5-2 shows the differences in root weight under stress and non-stress conditions (control). The Atlantic (*S. tuberosum*) and Costanera (adg x tbr) appear to have responded to drought by increasing their root mass, while Sullu, Leona and Negra Ojosa (*S. andigena* landraces) showed little change in root mass when exposed to drought. Sullu and Negra Ojosa also have similar sizes of root mass.

There were also differences in the number of tubers that were produced under stress and under control conditions (Figure 5-3). Sullu and Negra ojosa drastically reduced their number of tubers under stress, while the other genotypes (Atlantic, Costanera and Leona) had a slight increase in the tuber number. The average tuber weight (Figure 5-4) was reduced for most of the genotypes, but not for Sullu, which is probably a consequence of the mild effect that this drought stress caused in this genotype as we will see in the next section.

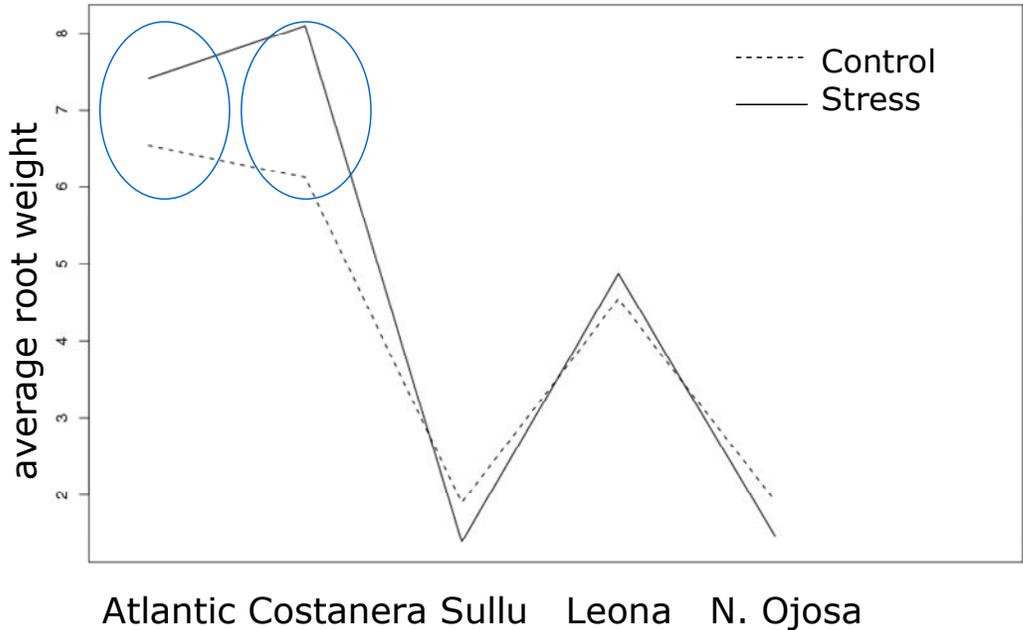


Figure 5-2 Effect of drought stress on root weight.

Mean root weight of control and stressed genotypes averaged across all times was compared. Atlantic (non Andigena genotype and Costanera (adg x tbr) showed a different behavior under stress conditions: more root growth during stress (circled in blue) compared to the Andigena genotypes

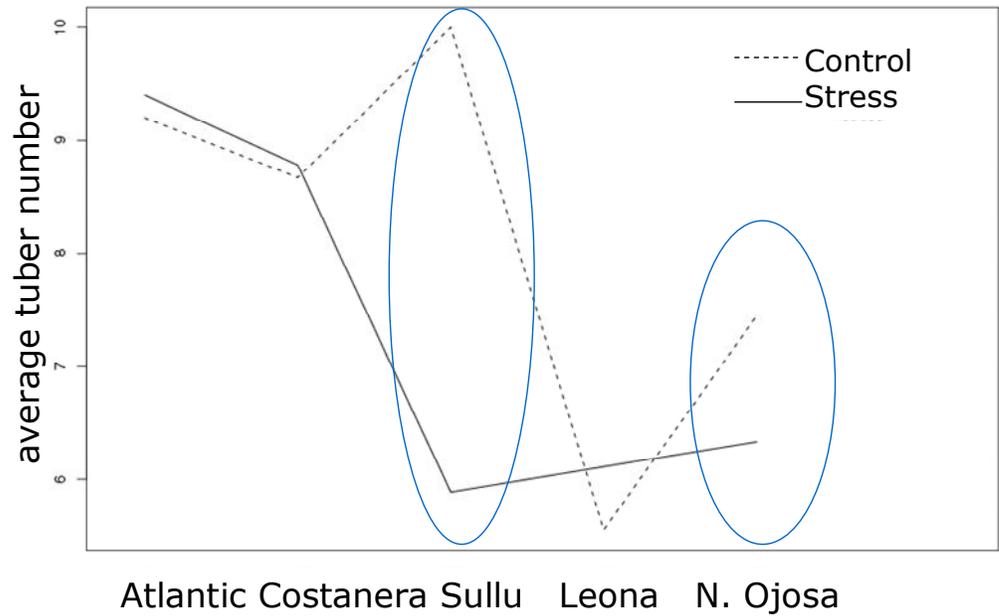


Figure 5-3 Effect of drought stress on average tuber number

Average tuber number of control and stressed genotypes averaged across all times was compared. Atlantic (non Andigena genotype), Costanera (adg x tbr), and Leona showed little change in tuber number while tuber number was reduced in Sullu and Negra Ojosa under drought stress (blue ovals).

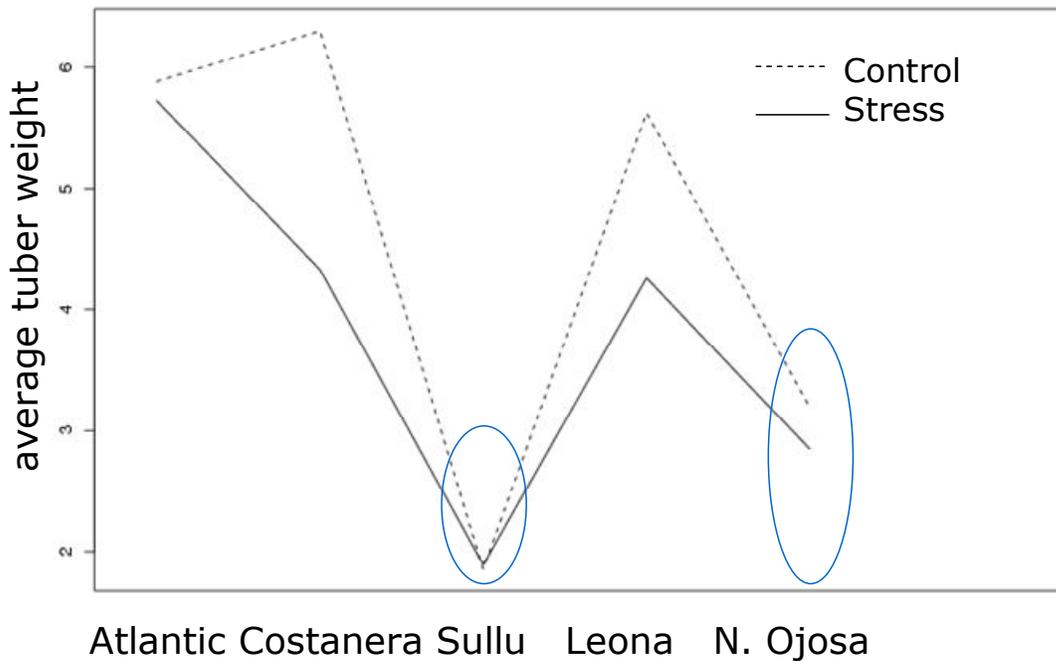


Figure 5-4 Effect of drought stress on average tuber weight

Average tuber weight of control and stressed genotypes averaged across all times was compared. Sullu and Negra Ojosa had smaller tubers (blue ovals) than the other genotypes. The magnitude of the differences in tuber weight between control and treated plants in Sullu and Negra Ojosa was less than the drought-mediated differences observed in the other genotypes.

5.3.2. *S. andigena* landraces are more tolerant to drought stress than is compared to *S. tuberosum*

S. tuberosum (Atlantic) was employed as a control to measure the level of drought stress in the plants analyzed, but after 17 days of drought stress Atlantic treated plants had their photosynthesis reduced to almost 80% and Costanera (which comes from a *S. tuberosum* and *S. andigena* cross) showed photosynthesis reduced to 80% after 22 days. Atlantic leaves were bigger than Sullu and Negra Ojosa leaves, which have a similar leaf size (Figure 5-1), while Costanera leaves were not as big as those of Atlantic, but still two times bigger than Sullu and Negra Ojosa. However, Negra Ojosa was more affected than Sullu, showing a 40% reduction while, in Sullu, drought stressed plants exhibited photosynthetic rates close to those of the control plants (Figure 5-5). By day 25 photosynthesis in Negra Ojosa had been reduced 80% while, in Sullu plants only a 56% reduction was observed.

24 hours after rewatering, Atlantic and Sullu recovered 80% of photosynthesis, while Negra Ojosa only recovered to 23% of the control rate. Therefore, even though it could stand water deficits longer than Atlantic it could not recover from that level of stress. Leona, the other *S. andigena* genotype showed photosynthetic behavior similar to that of Negra Ojosa (photosynthetic reduction to 80%), but it recovered to 70% after rewatering (data not shown).

The relative water content of the andigena landraces at maximum drought stress was between 70-80%.

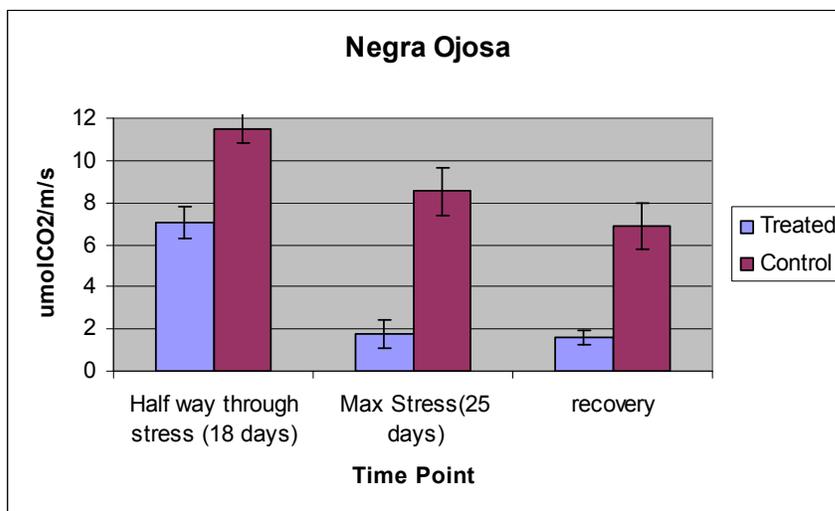
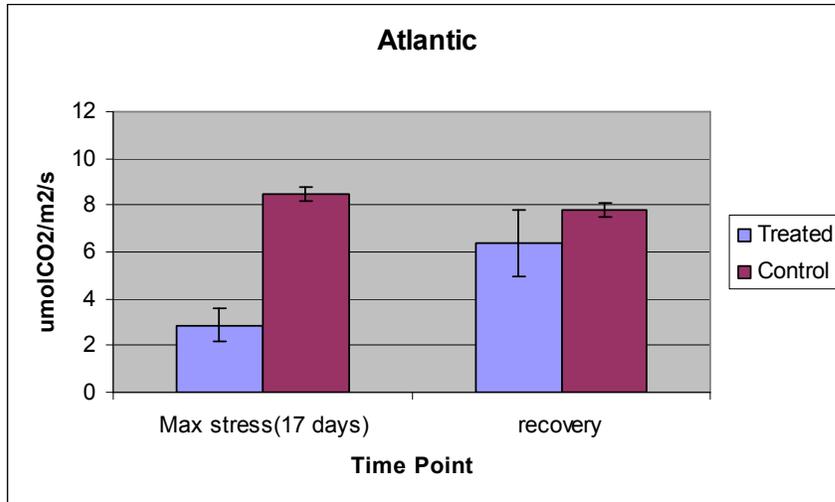


Figure 5-5 Effects of drought stress on photosynthesis in Atlantic, Negra Ojosa and Sullu

Net photosynthesis during drought stress is showed, halfway trough stress, at the point of maximum stress and 24 hours after watering (recovery). All the measurements were done at noon.

5.3.3. Differential expression of chaperone genes at maximum drought stress in Sullu and Negra ojosa

Since Negra Ojosa and Sullu have similar root mass and tuber mass and Sullu and Negra Ojosa exhibited two extremes of drought stress tolerance among the 3 *S. andigena* landraces studied (Sullu being the most tolerant and Negra Ojosa the most susceptible), gene expression analysis (microarrays) was performed on RNA samples from both genotypes. Table 5-3 shows the genes that were expressed differently in Sullu and Negra Ojosa. Many of the chaperone genes that are up-regulated in Sullu, are located in the chloroplast, moreover, 3 of them (Cpn 60 or chaperonin 60) are involved on the folding of Rubisco (Barraclough and Ellis, 1980; Viitanen et al., 1995).

Real time PCR confirmed the results found with microarrays (Figure 5-6). Other chaperone genes not included/not detected in the microarray were also analyzed: an homolog of BIP3 (luminal binding protein 3) and an homolog of GRP94 (ER resident HSP90). The general response of non-chloroplast HSPs during maximum stress of Negra Ojosa is higher than that of Sullu, most of the chaperones have a higher fold change compared with Sullu, probably because Negra Ojosa was experiencing more stress. During recovery most of the genes analyzed did not show any change with the exception of HSP70, and a homolog of the cytosolic HSC70-3 of tobacco, which is highly up-expressed in Sullu and therefore might have helped in the recovery of this plant after watering.

Location	At homolog	Description	Type	Sullu_Exp	Negra_Exp
	AT3G4411	DnaJ-like protein [Lycopersicon esculentum]	HSP40 (N, central and C terminal)	0	-
	AT1G7670	DnaJ Heat Shock N-terminal domain-containing protein	HSP40(N_terminal)	0	-
chloroplast	AT5G0613	Chaperone protein DnaJ related, DnaJ central domain (4 repeats)	HSP40(central)	0	-
	AT5G1545	HSP100/CipB, putative [Arabidopsis thaliana] pir T51523 cipB heat shock protein	HSP100	0	+
chloroplast	AT4G2428	pir T08899 dnaK-type molecular chaperone HSC70-9, chloroplast - spinach	DnaK Hsp70	0	+
nucleus	AT4G1398	pir G71400 probable heat shock transcription factor - Arabidopsis thaliana	HSF(A5)	0	+
chloroplast	AT5G1937	peptidyl-prolyl cis-trans isomerase-like protein [Oryza sativa (japonica cultivar-gr)]	Ppiase	0	+
	AT1G2412	DnaJ heat shock protein putative, similar to altered response to gravity (A. thalian)	HSP40(N_terminal)	0	+
	AT5G2359	DnaJ Heat Shock N-terminal domain-containing protein, low similarity to CA11 from	HSP40(N_terminal)	0	+
	AT3G5734	Similar to SP:Q9QYI4 DnaJ homolog subfamily B member 12 Mus musculus	HSP40(N_terminal)	-	+
chloroplast	AT5G5522	AT5g55220/ ref NP_200333.2 trigger factor type chaperone family protein [Arabidopsis thaliana]	Ppiase (ATTIG) **	+	+
chloroplast	AT4G0799	DNAJ heat shock N-terminal domain-containing protein [Arabidopsis thaliana]	HSP40 (N-terminal)	-	0
cytosol	AT3G1258	heat shock protein 70-3 [Nicotiana tabacum]	DnaK HSP70	-	0
nucleus	AT4G3699	heat stress transcription factor [Lycopersicon peruvianum] pir S12361 heat shock transcription factor HSF24 - Peruvian tomato	HSF(B1)	-	0
cytosol	AT5G5264	Heat Shock Protein 81-1 (HSP81-1)	HSP90	-	0
chloroplast	AT1G5549	probable chaperonin 60 beta chain precursor, chloroplast (clone potbchap1) - potato gb AAB39827.1 chaperonin-60 beta subunit	HSP60	+	0
chloroplast	AT2G2800	Identical to SwissProt:P21238- Rubisco subunit binding-protein alpha subunit, chloroplast precursor (60 KDA Chaperonin alpha subunit, CPN-60 alpha) (A.	HSP60	+	0
chloroplast	AT3G6021	chloroplast chaperonin 10	Chaperonin10	+	0
	AT2G3800	thaliana	HSP40(central)	+	0
mitochondrion	AT1G7994	DnaJ Heat Shock N-terminal domain containing protein , similar to SP:Q9UGP8 translocation protein SEC63 homolog	HSP40 (N-terminal)	+	0
cytosol	AT3G4623	17.4 kDa Class I heat shock protein (HSP17.4-Cl), (Arabidopsis thaliana)	SHSP	+	0

Table 5-3 Differential expression of chaperone genes in Sullu and Negra Ojosa (maximum stress).

Genes highlighted in yellow were used for real time validation of the data. ** The fold change response of the Ppiase homolog (ATTIG) had a positive fold change in Negra Ojosa , but it did not pass the statistical test.

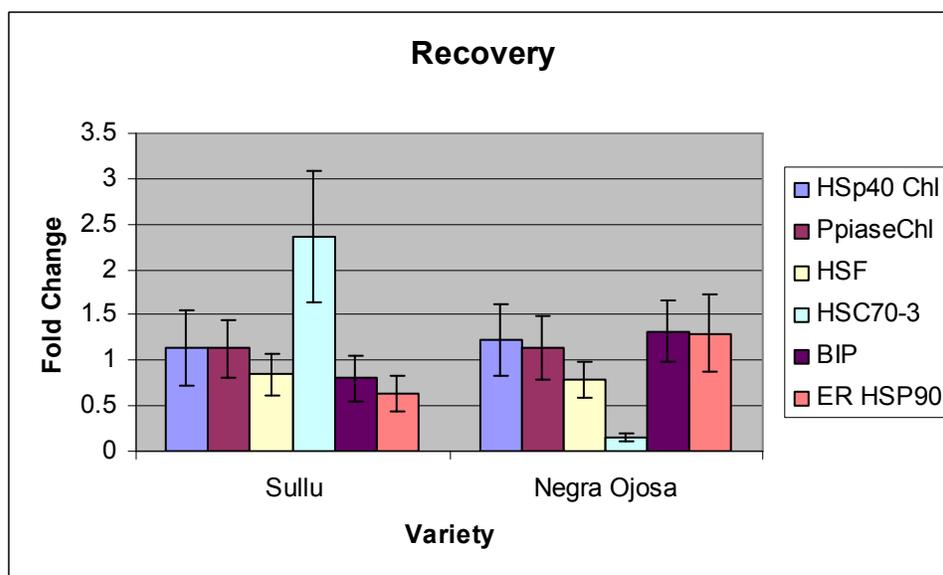
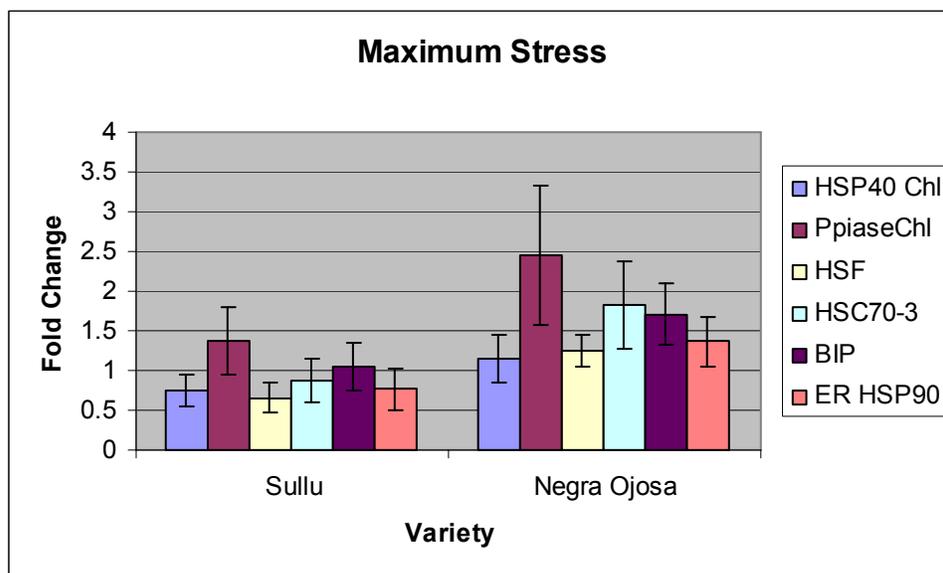


Figure 5-6 Real time PCR results of selected chaperone genes at maximum stress and recovery

Real time PCR was carried out on cDNA from leaf samples from Sullu and Negra Ojosa at maximum stress and 24 hours after watering (recovery). HSP40 Chl. and Ppiase Chl products are located in the chloroplast.

5.3.4. Differential expression of ABA responsive genes in Sullu and Negra Ojosa

Some genes that are known to be responsive to ABA (TAIR database) were responsive in Negra Ojosa and Sullu (Table 4). Many genes involved in ABA signaling

were responsive in Negra Ojosa including 3 MYB transcription factors, 1 HD-zip transcription factor, 2 phosphatases, 2 kinases (1 MAPKK and 1 CDPK) and 1 lipoxigenase. From these set, 2 of the MYB genes and the kinases were downregulated and the other one was up-regulated. In Sullu only 3 genes that were ABA responsive, responded: one COR gene and a Ring Zinc Finger transcription factor were downregulated and a double stranded RNA binding protein showed up-regulation (Table 5-4).

AGI homolog	Description	Type	Sullu_Exp	Negra_Exp
AT3G45640	mitogen-activated protein kinase 3 [Lycopersicon esculentum]	MAPK	0	-
AT4G23650	Encodes calcium dependent protein kinase 3 (CPK3), a member of the Arabidopsis CDPK gene family.	CDPK	0	-
AT4G34990	transcription factor [Lycopersicon esculentum] pir S69189 myb-related protein TMH27 - tomato	MYB32	0	-
AT3G47600	transcription factor [Lycopersicon esculentum] pir T07398 myb-related transcription factor THM6 - tomato	MYB94	0	-
AT5G16600	myb-related transcription factor [Lycopersicon esculentum] pir T07393 myb-related transcription factor - tomato	MYB43	0	+
AT3G61890	homeobox-leucine zipper protein ATHB-12 [Arabidopsis thaliana] emb CAB71896.1	ATHB12	0	+
AT3G11410	protein phosphatase 2C [Nicotiana tabacum] emb CAC84141.2 protein phosphatase 2C [Nicotiana tabacum]	Phosphatase	0	+
AT2G04550	dual specificity protein phosphatase family protein [Arabidopsis thaliana]	Phosphatase	0	+
AT1G55020	lipoxigenase [Solanum tuberosum]	LOX	0	+
AT1G75750	cold-regulated LTCOR12 [Lavatera thuringiaca]	COR	-	0
AT1G63840	At1g63840/T12P18_14 [Arabidopsis thaliana] pir E96663 probable RING zinc finger protein	RING zinc finger	-	0
AT1G09700	Encodes a nuclear dsRNA binding protein. Involved in mRNA cleavage. The mutant is characterized by shorter stature, delayed flowering, leaf hyponasty, reduced fertility, decreased rate of root growth, and an altered root gravitropic response. It also exhibits less sensitivity to auxin and cytokinin.	dsRNA binding	+	0

Table 5-4 Differential expression of ABA responsive genes in Sullu and Negra Ojosa

5.3.5. Differential expression of antioxidant genes in Sullu and Negra Ojosa

Negra Ojosa seem to be experiencing a more severe stress compared with Sullu, since they exhibited lower photosynthesis (Figure 5-5) which was also reflected in the differential expression of heat shock proteins and ABA responsive genes. So since there was not a differential expression on proline biosynthesis or other osmotic related genes (data not shown) the expression of genes related to oxidative stress was investigated, since high levels of drought stress also causes oxidative stress.

Table 5-5. shows the differential expression of genes directly related to antioxidant responses (Mittler 2004). Most of them are up-regulated in Sullu, while they

are downregulated or unchanged in Negra Ojosa. Moreover, most of the genes up-regulated in Sullu encode proteins that are located in the chloroplast (1 glutathione synthetase, 1 dehydroascorbate reductase, 3 thioredoxins and 3 glutathione S transferases), which is probably related to the better photosynthetic performance under drought stress in Sullu.

Real time PCR was performed on 3 antioxidant genes: Thioredoxin H, glutathione S transferase and glutathione synthetase, for point of maximum stress and recovery. In both cases these genes are up-regulated in Sullu, compared to Negra Ojosa, where they are downregulated or non changed (Figure 5-7), confirming the microarray results, and also adding the information that antioxidant genes are up-regulated during recovery in Sullu, especially Glutathione S transferase. The homolog of this gene in Arabidopsis (AT3G03190), has been shown to be responsive to oxidative stress (Richards et al., 1998).

Subcellular location	At homolog	Description	Gene Name*	Sullu_Exp	Negra_Exp
cytosol	AT1G17180	2,4-D inducible glutathione S-transferase [Glycine max] pir T06239 probable glutathione transferase (EC 2.5.1.18), 2,4-D inducible - soybean	GSTU25	-	0
cytosol	AT3G09270	Q03664 GTX3_TOBAC probable glutathione S transferase (Auxin induced protein PCNT103)	GSTU8	0	-
cytosol	AT2G02380	glutathione S-transferase [Capsicum annuum]	GSTZ2	0	-
cytosol	AT5G39950	thioredoxin [Glycine max]	TrxH2 **	+	-
chloroplast	AT5G27380	glutathione synthetase [Lycopersicon esculentum] (GSH synthetase) (GSH-S)	GSH2	+	-
chloroplast	AT5G06430	thioredoxin-related, contains weak similarity to Swiss-Prot:Q9SEU7 thioredoxin M-type 3, chloroplast precursor (TRX-M3)	Trx	+	0
chloroplast	AT2G41680	putative thioredoxin reductase [Arabidopsis thaliana] gb AAL32557.1 putative thioredoxin reductase [Arabidopsis thaliana]	Trx red	+	0
chloroplast	AT5G06290	thioredoxin peroxidase [Nicotiana tabacum]	PrxR B	+	0
cyt/chl	AT1G75270	dehydroascorbate reductase [Nicotiana tabacum]	DHAR	+	0
cytosol	AT1G78380	putative glutathione S-transferase T5 [Lycopersicon esculentum]	GSTU19	+	0
cytosol	AT3G03190	glutathione S-transferase [Petunia x hybrida]	GSTF11	+	0
-	-	phospholipid hydroperoxide glutathione peroxidase [Lycopersicon esculentum]	GPX	+	0
ER	AT3G63080	probable glutathione peroxidase [Arabidopsis thaliana] gb AAO50670.1	GPX5	+	0

Table 5-5 Differential expression of ROS scavenging/antioxidant genes

Genes highlighted in yellow were used for real time validation of the data.

* Gene names for Glutathione transferases are according to Wagner (Wagner et al., 2002) and gene names for antioxidant genes are according to Mittler (Mittler et al., 2002).

** The fold change response of the thioredoxin homolog (TrxH2) had a positive fold change in sullu, but it did not pass the statistical test.

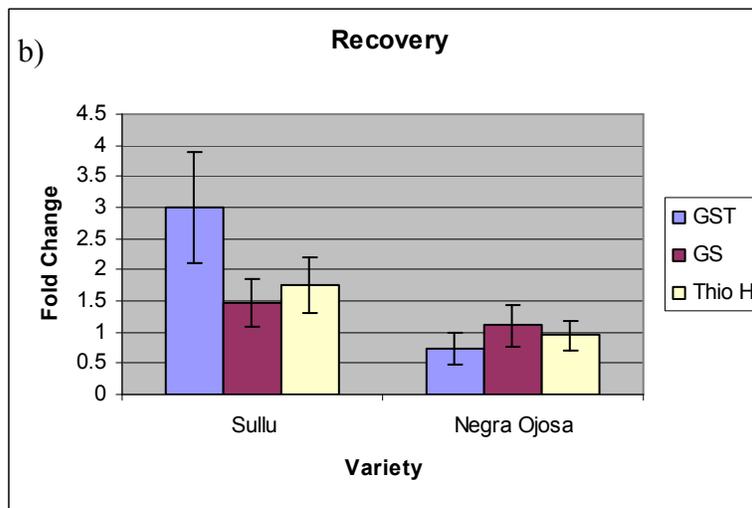
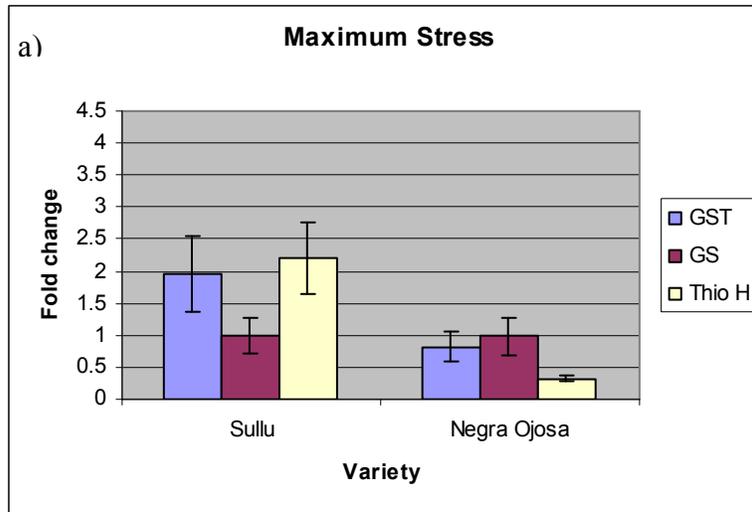


Figure 5-7 Real time PCR results of selected antioxidant genes at maximum stress and recovery.

Real time PCR was carried out on cDNA from leaf samples from Sullu and Negra Ojosa at maximum stress (a) and 24 hours after watering (recovery, b). GST: Glutathione-S-transferase , GS: glutathione synthetase and Thio H: thioredoxin H

5.4. Discussion

5.4.1. *S. tuberosum* spp *andigena* genotypes are adapted to low water conditions compared to *S. tuberosum*

The *S. tuberosum* spp *andigena* genotypes performed better under drought stress conditions compared to *S. tuberosum*. However as it was pointed out earlier, the andigena genotypes showed a different phenotype (i.e, smaller leaves, Figure 5-1), and therefore a direct comparison with *S. tuberosum* was not possible. Nevertheless there was a different

response among the andigena genotypes (who have the same, small leaf, phenotype, Fig1), since not all of them have the same degree of drought stress resistance and we were able to identify two extreme genotypes, a resistant one: Sullu and a susceptible one: Negra Ojosa.

Another phenotypic difference observed between *S. tuberosum* and andigena genotypes, is that *S. tuberosum* (and the hybrid tbr x adg Costanera) increased their root mass greatly during drought stress, while the andigena genotypes had a root mass similar to the control plants (Figure 5-2), which probably is also related with the adaptation of the andigena genotypes to low water conditions.

5.4.2. Heat shock genes that may contribute tolerance to drought stress in Sullu

As shown in Table 5-3, many of the genes that are up-regulated during maximum stress in Sullu encode heat shock proteins that are localized in the chloroplast (7 genes up-regulated, 4 in the chloroplast, 1 in the mitochondria and 2 with unknown location, probably the cytosol). The up-regulation of these genes might explain why Sullu can cope with the stress better than Negra Ojosa. More over, the chaperonins up-regulated are involved in the folding of Rubisco (Viitanen et al., 1995). Many more HSP40-DnaJ genes are up-regulated in Negra Ojosa than in Sullu. The subcellular location of this proteins is unknown, therefore it is assumed that to be cytosolic. The family of HSP40 genes in *A. thaliana* is large with close to 90 members (Miernyk, 2001). They play a role in mammalian and plant systems in the activation of the ATPase activity of HSP70/DnaK. There are only 17 HSP70s in *A. thaliana* (Lin et al 2001), and to date it is not known why there are so many HSP40/DnaJ genes in plants. Recent studies suggest that the DnaJ domain it is very important for the recognition of HSP70, and swapping of domains only sometimes affects this interaction (Hennessy et al., 2005). One way to classify HSP40 genes is by the position of the DnaJ domain in the protein. Most of the N-terminal HSP40 proteins are up-regulated in Negra Ojosa (Table 5-3), while in Sullu only one gene with a N-terminal domain is up-regulated, and it is one localized in the mitochondrion, while the other ones are in the cytosol. The HSP40s with N-terminal domain may be helping Negra

Ojosa to cope with the higher level of drought stress, while, in Sullu, which is not as stressed, the activation of these HSP40s does not occur.

It is interesting that the differential expression of the heat shock factors, HSFA5, which is up-regulated in Negra Ojosa, belongs to the A class of HSFs (Nover *et al* 2001) and HSFB1, which is down-regulated in Sullu belongs to the B class of HSFs. The B class of HSFs can activate or suppress expression (Czarnecka-Verner *et al.*, 2000), and recent studies with soybean HSFB1 has shown that this suppression might occur through interaction with TFIIB (transcription factor B) (Czarnecka-Verner *et al.*, 2004). Therefore probably Negra Ojosa is increasing the expression of certain HSP genes (i.e HSP40s), while Sullu is avoiding the suppression of gene expression of another class of genes, which might be the chaperonin genes.

5.4.3. Negra Ojosa experiences a severe drought stress, compared to Sullu.

ABA signalling genes are up-regulated primarily in Negra Ojosa

ABA accumulates during drought stress and since photosynthesis was severely affected in Negra Ojosa (Figure 5-5) after 25 days (maximum stress), ABA accumulation may already have been at its peak, and therefore many ABA signaling genes were up-regulated in this genotype (Table 5-4), while in Sullu the few that responded were down-regulated. In Negra Ojosa 2 MYBs that were downregulated are responsive to salt stress, jasmonic acid (JA), salicylic acid (SA) and CdCl₂ stress as well as to ABA (Yanhui *et al.*, 2006). The other MYB, MYB43, that shows up-regulation in Negra Ojosa is responsive only to ABA (Yanhui *et al.*, 2006). The other transcription factor up-regulated in Negra Ojosa (ATHb12) has been shown to be induced also by water loss to 50% in *A. thaliana* (Olsson *et al.*, 2004). Therefore the TFs up-regulated in Negra Ojosa, are probably specifically regulated by ABA under water loss, while the other TFs have a function under other ABA mediated stress responses.

The other genes that are up-regulated in Negra Ojosa are phosphatases. Moreover, one of them is an homolog of 2 C protein phosphatase (At3g11410), a known negative regulator of ABA signaling (Leung *et al* 1997). Recent studies in Bray's laboratory

(Verslues and Bray, 2006), have proposed that this gene regulates ABA responses, by regulating genes that catabolize/degrade ABA, since ABA accumulation under low water potential in the PP2C mutants (*abi1* and *abi2*) are higher than wild type plants, probably the ABA peak occurred earlier in Negra Ojosa, and PP2C was up-regulated to continue with the downstream events, such as the expression of MYB43 and AThB12, which are not considered as part of the early transcription factors activated by ABA.

5.4.4. Antioxidant genes help Sullu tolerate drought stress

As shown in Table 5-5 and Figure 5-7, most antioxidant genes that show a response in this experiment, are up-regulated in Sullu, while they are not responsive or down-regulated in Negra Ojosa. Moreover, most of these gene products are localized in the chloroplast, which probably helps to reduce ROS that might have arisen due to drought stress, while Negra Ojosa, did not have this extra protection.

There are two GST genes that are up-regulated in Sullu, homologs to GSTU19 (former GST8) and GSTF11 in *A. thaliana*. Drought stress (slow and fast) experiments in *A. thaliana* have shown up-regulation of this gene (Bianchi et al., 2002), moreover the expression pattern was similar to the peroxiredoxin ATPRX33 (At3g49110) which was found to be induced by ozone previously (Sharma and Davis, 1994). Therefore GSTU19, might have helped the plant to scavenge ROS generated by drought stress. The other GST homolog to GSTF11, is also an homolog of the petunia GST *An9* (79% similarity). In maize and petunia, this protein seems to be necessary for conjugation of anthocyanins before they can be transferred to the vacuole (Alfenito et al., 1998). Several genes related to anthocyanins production were up-regulated in Sullu (Data not shown) and GSTF11, might have been involved in the transport of anthocyanins in addition to its antioxidant function was finished.

In conclusion, Andigena genotypes are more resistant to drought stress than *S. tuberosum* subsp *tuberosum* mostly due to morphologic differences. However there is a diverse response to drought among Andigena genotypes, which are independent of morphology/habit. We propose that the higher drought stress resistance found in Sullu, is correlated with concerted up-regulation of heat shock proteins and antioxidant genes encoding proteins located in the chloroplast.

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