FINAL TECHNICAL REPORT

DOE/ER/20208--T1

The following Final Report is submitted in reference to the grant "Role of HSP100 Proteins in Plant Stress Tolerance" # DE-FG-95ER20208. All work cited was performed in my laboratory, unless indicated otherwise. Work accomplished is presented first as a brief summary with figures, and then further details of the experiments are provided in a subsequent section.

PUBLICATIONS - * = Appended

*Vierling, E. Plant HSP100/ClpB. In: Handbook of Molecular Chaperones and Protein Folding Catalysts. M.J. Gething, ed. Sambrook and Tooze Publications at Oxford University Press. pp.253-255. 1997.

*Vierling, E. Chloroplast-localized Clp proteins. In: Handbook of Molecular Chaperones and Protein Folding Catalysts. M.J. Gething, ed. Sambrook and Tooze Publications at Oxford University Press. pp.255- 258. 1997.

S-W. Hong and E. Vierling. Identification of T-DNA insertions in Arabidopsis homologs of the yeast thermotolerance gene, HSP104. 9th International Conference on Arabidopsis Research, Madison, WI. pg. 128. (abstract). 1998.

S-W. Hong and E. Vierling. Expression of Arabidopsis homologs of the yeast thermotolerance gene Hsp104. In preparation.

SUMMARY

• We isolated the genomic copy of the gene encoding Arabidopsis HSP101, a protein that can confer thermotolerance in yeast. Approximately 1000 bp of the promoter was sequenced (Fig. 1). This gene has not yet been sequenced by the genome project.

Conclusions: *The promoter contains canonical heat shock elements. The gene contains multiple introns, which is surprising as mRNA splicing has been reported to be impaired during heat stress; we have no evidence that this is the case AtHSP101.*

• We have found that *AtHSP92.7*, a gene encoding a homolog of *AtHSP101* (71% identity at the amino acid level - Fig. 2) that was recently sequenced by the Arabidopsis genome project, is not expressed in vegetative tissues in the absence of heat stress. However, like *AtHSP101*, it is expressed in response to high temperature stress, although at a lower level (Fig. 3).

Conclusions: *AtHSP92.7 is member of the ClpB subfamily of HSP100 proteins. The AtHSP92.7 gene also contains multiple introns. Like AtHSP101 it may also be critical to thermotolerance; its function may be complementary or redundant with AtHSP101.*

• We have isolated T-DNA insertion alleles of *AtHSP101* and *AtHSP92.7* (Figs. 1, 4& 5). The *AtHSP101* insertion is 143 bp 5' to the putative transcription start site; it causes reduced expression of *AtHSP101* (Fig. 6). The *AtHSP92.7* insertion is within the second exon and represents a null allele.

Conclusions: *Homozygous plants of either mutant are viable and fertile, and analysis of other phenotypes is discussed below.*

• A quantitative assay for thermotolerance of hypocotyl growth has been developed (Fig. 7). Standard conditions for the assay involve a 38 °C pretreatment of 2.5 day-old seedlings, followed by 2 hr at 22 °C and then 2 hr at 45 °C.

Conclusions: *Under standard conditions the T-DNA insertion alleles of AtHSP101 and AtHSP92.7 show no difference in thermotolerance compared to wildtype (Fig. 7). However, variations of this and other thermotolerance assays (see below) have a good probability of uncovering a phenotype in these mutants.*

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SUMMARY - continued

➤ *AtHSP101* antisense plants have been generated (not shown). Absolute levels of *AtHSP101* expression in the best lines (lines 32/2-4 and 32/43-1) show variation between 1 and 30% of wildtype levels. Preliminary data indicate that these plants are defective in the development of thermotolerance (not shown). (Lindquist lab).

Conclusions: *AtHSP101 is required for the development of thermotolerance. It is important to confirm this preliminary result and assess other phenotypes of these plants.*

➤ Plants that constitutively express *AtHSP101* have been generated (not shown). (Lindquist lab).

Conclusions: *Preliminary data suggest these plants may show increased thermotolerance.*

➤ We have established multiple assays for plant thermotolerance. These include the seedling assay described in Fig. 8, the hypocotyl assay described above (Fig. 7), an assay based on in vivo activity of firefly luciferase (See Figs. 11 and 12), and other assays described.

Conclusions: *These assays provide multiple ways in which to assess the thermotolerance of HSP100 mutant plants. The decline and recovery of luciferase activity in intact plants affords a particularly sensitive and quantitative assay of thermotolerance. The luciferase plants are being crossed to the HSP100 mutants.*

➤ Antibodies that recognize *AtHSP101* were developed. These antibodies do not detect *AtHSP92.7* or other members of the HSP100 family that have highly similar ATP-binding domains (compare blots reacted with *AtHSP101* antibodies to those reacted with yeast *Hsp104* peptide antibodies (in Figs. 11 and 12).

Conclusions: *These antibodies can be used to detect specifically AtHSP101 in the background of other HSP100 proteins. In combination with the antibodies against a conserved peptide in the ATP-binding domain of yeast Hsp104, we should now be able to detect all members of the HSP100 family.*

➤ We have no evidence that *AtHSP101* is expressed in response to ozone stress, UV stress, amino acid starvation, NaCl stress, PEG stress, ABA application or pathogen attack (not shown). However, data indicate the protein is expressed in response to ethanol and to the amino acid analog canavanine (See Fig. 6), which are well known to induce other HSPs. Preliminary data also suggest the protein is induced in roots of plants subjected to drought stress (not shown).

Conclusions: *AtHSP101 is not a general stress protein, but may be required only under stress conditions that cause accumulation of denatured, aggregated proteins.*

➤ *AtHSP101*, which is expressed only during stress in vegetative tissues, is also expressed in embryos during seed maturation and is present in mature, dry seeds (Fig. 11). It declines during germination, consistent with its absence from mature leaves (Fig. 12). In the seed development mutants *fus3*, *lec1-2* and *abi3-6* *AtHSP101* expression is relatively unaffected (not shown). We have found no other instances of expression in the absence of stress - the protein is not detectable in mature pollen (not shown), and neither *AtHSP101* nor *AtHSP92.7* sequences have been reported as ESTs.

Conclusions: *The expression pattern in seeds implies AtHSP101 has a role in normal seed development and/or germination. AtHSP101 may act to protect or reactivate proteins in the dehydrated state. In contrast to small HSPs, which appear to be regulated by Abi3, AtHSP101 expression does not appear to be directly regulated in by the transcriptional activators encoded by Abi3, Fus3 or Lec1. Because all of these mutants are desiccation intolerant, AtHSP101 is either not necessary or not sufficient for desiccation tolerance. Absence from the ESTs suggests these HSP100 proteins are otherwise primarily restricted to stressed tissues or are of low abundance.*

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➤ A yeast strain has been constructed to initiate a genetic screen for suppressors of dominant negative mutants of yeast Hsp104. (Lindquist lab).

Conclusions: *This screen should identify proteins that interact with the HSP. Ultimately corresponding proteins could be isolated from Arabidopsis.*

DETAILED EXPERIMENTAL RESULTS:

Further details of the data presented above are given below according to each specific aim of the original grant.

AIM 1 - Characterization of HSP100 genes and their expression during stress and development.

This work was carried out in the P.I.'s laboratory. Data concerning expression of AtHSP101 and 92.7 are in preparation for publication.

a) Isolation of the *AtHSP101* gene: Promoter sequence and intron site. A genomic library from *Arabidopsis* (*Landsberg erecta*), was obtained from the ABRC Stock Center and multiple phage that hybridized with the *AtHSP101* cDNA at high stringency were identified. Restriction digestion of the isolated phage indicated all represented the same genomic locus. The 5' region of one of the *AtHSP101* genomic clones was subcloned and 1127 bp of sequence 5' to the ATG of the *AtHSP101* cDNA was determined (Fig. 1). Comparison of the genomic sequence and cDNA sequence revealed an intron in the 5' non-coding region. PCR amplification of the entire *AtHSP101* coding region from the genomic clone indicates there are likely additional introns in this gene. The presence of multiple introns in the *AtHSP101* gene was surprising, as mRNA splicing has been reported to be impaired during heat stress. We have no evidence that heat stress impairs *AtHSP101* mRNA splicing.

b) Identification and expression of an *AtHSP101* homolog - *AtHSP92.7*. *AtHSP92.7*, which is located on chromosome 4, was sequenced by the Arabidopsis genome project. The encoded protein is 71% identical and 78% similar to *AtHSP101* and is even less identical at the nucleotide level, consistent with our failure to identify this gene by low stringency hybridization with *AtHSP101*. *AtHSP92.7* shows much reduced similarity to other HSP100 family proteins (41% and 39% identity with Arabidopsis ERD1 and ClpC, respectively) and is clearly a member of subfamily B of the HSP100 proteins (Fig. 2). As we found for *AtHSP101*, the gene is interrupted by several introns (Fig. 4). Using a gene specific probe for Northern analysis, we found that accumulation of the corresponding mRNA is heat-regulated in seedling tissues, as seen for *AtHSP101* (Fig. 3). However, compared to *AtHSP101*, the level of mRNA accumulation is quite low, judging by relative signal intensities on Northern blots with comparable specific activity probes. The apparent low level of expression is consistent with the fact that this gene was not isolated in a differential screen for heat-induced mRNAs, while 53 independent cDNAs for *AtHSP101* were isolated.

c) Generation of *AtHSP101* specific antibodies. We had originally detected the *AtHSP101* protein with antibodies generated against a peptide corresponding to a conserved sequence within the first ATP-binding domain of *Saccharomyces cerevisiae* Hsp104 (see Fig. 2 for peptide). This antibody detects additional proteins in the 100 kDa range that likely represent *AtHSP92.7* as well as the organelle-localized ClpC and ERD1 proteins. To obtain an antibody specific to *AtHSP101*, we generated an His-tagged antigen from the N-terminal 148 aa acids of *AtHSP101*. This region of the gene shows little similarity to other members of the HSP100/ClpB family of proteins (Fig. 2), or to any other sequence in GENBANK. The antibodies generated against this antigen proved to be highly specific for *AtHSP101* as predicted (see Figs. 11 & 12).

d) *AtHSP101* expression during growth and development. We had previously established that *AtHSP101* is not expressed during normal vegetative growth of plants, although it is very strongly induced during heat shock. To determine if *AtHSP101* was expressed during development as had been

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DETAILED EXPERIMENTAL RESULTS: - continued

observed for HSP70 and certain small HSPs, both the AtHSP101 specific antiserum, and the yeast Hsp104 peptide antiserum were used to probe western blots of proteins isolated from developing seeds (Fig. 11). With the specific antiserum, protein was seen to accumulate starting at 10 DAF, reaching maximum levels in the dry seed. Some protein was also detected in the 4 DAF sample. A similar pattern of AtHSP101 accumulation was detected with the peptide antiserum, with two differences: this antiserum is less sensitive - AtHSP101 was not clearly detectable until 18 DAF, and the antiserum reacts with a major ~95 kDa polypeptide that is constitutive in leaves and abundant in immature pods, but declines during seed maturation. We hypothesize that this 95 kDa band represents chloroplast, and possibly mitochondrial-localized Clp homologs, although we cannot rule out that this protein is AtHSP92.7. In the proposed research we will perform experiments to clarify the identity of these different HSP100-related polypeptides.

The pattern of AtHSP101 expression was also assessed in germinating seeds using both antisera (Fig. 12). AtHSP101 declined during germination and was undetectable 6 days following imbibition, as expected since it is absent in mature plants. The ~95 kDa protein increased rapidly during imbibition, consistent with its presence in mature plants and with our hypothesis that this band represents organelle-localized Clp homologs.

To determine if it was likely that AtHSP101 expression is controlled by genetic loci that regulate late seed maturation, *lec1-2*, *abi3-6*, or *fus3*, we examined AtHSP101 levels during seed maturation in these mutants (not shown). AtHSP101 accumulated normally in *lec1-2* and *fus3* and was somewhat reduced in *abi3-6*. ABI3 and LEC1 are both transcriptional activators, and these alleles are nulls (J. Harada personal communication). Therefore, it is unlikely that the developmental regulation of AtHSP101 is controlled directly by either of these proteins. This is in contrast to the small HSPs, which may be directly controlled by the ABI3 protein. The fact that all three of these mutants are desiccation intolerant, but still have close to wild-type levels of AtHSP101, indicates that AtHSP101 is either not involved in desiccation tolerance, or is not sufficient for desiccation tolerance of seeds.

We also tested for the presence of AtHSP101 in mature pollen. By western analysis AtHSP101 was not detectable (not shown).

e) Expression during other stresses: AtHSP101 levels were examined by western blotting in Arabidopsis leaf tissues after treatment with different stresses. Samples from plants exposed to ozone, amino acid starvation, pathogen inoculation, or UVB treatment were kindly supplied by Dr. Rob Last's laboratory (Cornell University). No significant accumulation of AtHSP101 was observed by western analysis in any of these samples. AtHSP101 and 92.7 expression was also not observed on Northern analysis of mRNA prepared from plants after NaCl, PEG, 5°C and ABA treatment (samples from Dr. Z.-K. Zhu, University of Arizona). Preliminary studies suggest that AtHSP101 is induced in roots of plants subjected to drought stress by withholding water. Jordano and colleagues have reported that certain small HSPs are also expressed in roots of drought stressed plants. We will follow up on this observation and also extend our investigation to include other stresses. Other common inducers of the heat stress response, ethanol and the arginine analog canavanine, induce AtHSP101. These results are discussed further below.

It is also of interest to note that no *AtHSP101* or *AtHSP92.7* sequences have been identified in the Arabidopsis EST sequencing projects. For *AtHSP101* this is somewhat surprising given the expression observed in developing seeds. However, the absence from the EST database suggests that *AtHSP101* and 92.7 mRNA expression is indeed primarily restricted to stressed tissues, or at least is of low abundance in other tissues.

f) *AtHSP101* promoter-GUS reporter constructs. We expended considerable effort in generating plants expressing *AtHSP101* promoter-GUS fusions. Although all transgenics generated showed heat-regulated expression of the construct in leaves, they showed alarming heterogeneity in GUS

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DETAILED EXPERIMENTAL RESULTS: - continued

activity during seed development. We have therefore been reticent to continue analyses with these plants, and no further experiments with them are proposed. Rather we will focus on protein localization using immunocytochemistry.

g) Isolation of an HSP101 homologue from the moss, *Funaria hygrometrica*. In other studies in my laboratory we have investigated the heat shock response in basal land plants to understand what kinds of evolutionary processes may have driven the extensive development of HSP gene families in plants. In these studies we have recently sequenced a full-length cDNA from *Funaria* encoding a homologue of AtHSP101. The *Funaria* amino acid sequence is much more similar to AtHSP101 (83%) than to AtHSP92.7 (71%) and provides information on those sequences conserved over 500 million years of plant evolution.

AIM 2 - Requirement of HSP101 for thermotolerance

This work was carried out in collaboration with Dr. Lindquist's laboratory. My laboratory established conditions for thermotolerance tests and isolated T-DNA insertion mutants, while a student in Dr. Lindquist's laboratory generated the transgenic plants.

a) Development of thermotolerance assays. As described in the background section, all organisms develop resistance to normally lethal temperatures when pretreated at an elevated, but non-lethal temperature. We have now established several different thermotolerance assays: 1) using 7 day old seedlings and examining viability after stress (as in Fig. 8); 2) in germinating seeds, 1.5 days following imbibition, where radical elongation and cotyledon expansion can be observed; 3) root growth in 7 day-old seedlings; 4) hypocotyl elongation in dark grown seedlings; and 5) luciferase activity in transgenic Arabidopsis plants. In all assays a pretreatment at 38 °C for 90 min followed by 2 hr at 22 °C has been found to be sufficient to increase thermotolerance 3-4 °C over wild-type controls. Western analysis confirms that this pretreatment induces AtHSP101. Because the latter two assays are amenable to quantification, we have recently focused on these and they are described further below.

Thermotolerance of hypocotyl growth is easily measured in dark grown seedlings. Seeds are germinated and grown in the dark on plates maintained in a vertical orientation for 2.5 days and then subjected to heat stress at 45 °C for 2 hr either with a pretreatment or without a pretreatment at 38 °C as described above. After an additional 2.5 days in the dark hypocotyl length is measured. This allows excellent quantitative analysis of thermotolerance (Fig. 7).

Arabidopsis plants expressing cytosolically targeted, firefly luciferase were obtained from Dr. J. Callis (U.C. Davis). Luciferase is normally targeted to peroxisomes by a three amino acid C-terminal signal; this signal was removed in the constructs used to generate these plants. Luciferase is a highly heat-labile enzyme, showing complete inactivation in vitro when heated at 42 °C for 15 min. To determine if luciferase activity would exhibit thermotolerance, plants were preadapted at 38 °C for 90 min followed by 2 hr at 22 °C and then subjected to a 42 °C heat stress for either 15 or 30 min. The adapted plants were compared to plants heat-stressed at 42 °C without any prior adaptation treatment. Luciferase activity was assessed by direct imaging of in situ luminescence generated in the cotyledons after spraying with the substrate luciferin. As is clear from the data in Figs. 9 and 10, the level of luciferase activity in preadapted plants is unaffected by the 42 °C heat stress. In contrast, the 42 °C heat stress severely reduced luciferase activity in non-adapted plants, and activity had not recovered even one hour after heat stress. The luciferase expressing line will be crossed to the *AtHSP101* and *AtHSP92.7* mutants in order to test how the absence of these genes affects luciferase thermotolerance.

b) Duration of thermotolerance. We were interested to examine how long the induced thermotolerance was maintained. This was tested by examining increasing times between the 38 °C pretreatment (0 to 48 hours) and the lethal stress in 7 day old seedlings grown on plates. Almost

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complete thermotolerance was maintained for 24 hrs following the pretreatment, but plants were no longer thermotolerant 48 hrs after the pretreatment. We performed western analysis to examine AtHSP101 levels in these plants, and preliminary data suggest that the levels of AtHSP101 are well-correlated with treatments conferring thermotolerance, but that thermotolerance decays more rapidly than the AtHSP101 protein. These results are consistent with the idea that heat-induced factors in addition to AtHSP101 are required for thermotolerance.

c) Characterization of *AtHSP101* and *AtHSP92.7* T-DNA insertion lines.

From 15,000 T-DNA lines screened a single T-DNA insertion in both the *AtHSP101* and *AtHSP92.7* genes have been isolated. The site of the insertions were verified by sequencing and are shown in Figs. 1 and 3. In *AtHSP101* the T-DNA insertion occurred only 143 base pairs from the 5' end of the cDNA, which is likely close to the transcriptional start site. Plants homozygous for each insertion have been identified and confirmed by PCR and Southern analysis (Fig. 5). The homozygous mutant plants show no visible abnormalities in growth or fertility.

The *AtHSP101* mutant has been examined for protein expression under several conditions (Fig. 6). During development, or under heat, ethanol or canavanine stress the mutant shows about a 50% reduction in AtHSP101 levels. The mutants show a similarly reduced level of mRNA during heat shock (not shown). Because a gene specific probe for *AtHSP101* was used for the Northern analysis, we can rule out that expression of *AtHSP92.7* is contributing to the signal observed. We conclude that 143 bp of the 5' region of this gene is sufficient to control stress and developmental expression, and that the several putative heat shock elements within this region are likely to be the critical heat shock regulatory sequences.

Thermotolerance using the hypocotyl assay of both mutants is shown in Fig. 7. There is no apparent difference between the mutants and wildtype plants. Other assays, including tests of the duration of thermotolerance may well reveal a thermotolerance phenotype.

d) Generation of *AtHSP101* antisense plants. Two antisense gene vectors were constructed, one in which the antisense *AtHSP101* cDNA was driven by the CaMV 35S promoter, and a second utilizing the *AtHSP18.2* promoter. These constructs were transformed into Arabidopsis plants by standard tissue culture transformation methods in experiments performed by Dr. Lindquist's student, Christine Queitsch. Characterization of the transgenic plants has been delayed due to a series of unfortunate personal circumstances for Ms. Queitsch.

For the plants with the *HSP18.2* driven antisense gene, no plants exhibiting reduced AtHSP101 were identified. For the CaMV 35S-driven construct, 14 independent lines showing from 1 to 70% of wildtype AtHSP101 levels after heat stress were generated. The absolute levels of AtHSP101 expression in the best antisense lines (lines 32/2-4 and 32/34-1) show variation between 1 and 30% of wildtype levels (not shown). These lines have now been backcrossed twice to insure that any observed phenotypes are not due to additional mutations introduced during the tissue culture transformation procedure.

e) Preliminary phenotypic analysis of antisense plants. Antisense lines 32/2-4 and 32/34-1 were assayed for development of thermotolerance using conditions established as described above. The *AtHSP101* antisense plants were unable to survive the 45 °C treatment even when pretreated at 38 °C, which normally induces thermotolerance in wildtype seedlings (not shown). These preliminary data strongly suggest that wildtype levels of AtHSP101 are essential for the effective development of thermotolerance.

AIM 3 - Thermotolerance of plants over-expressing HSP100

This work was carried out in collaboration with Dr. Lindquist's laboratory.

Constitutive expression of *AtHSP101*. Transgenic Arabidopsis lines that constitutively express *AtHSP101* under the control of the CaMV 35S promoter were generated as described above for the

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antisense lines. The line with the highest expression of AtHSP101 (5/16) has constitutive levels similar to that seen in wildtype leaves after 90 min at 38° C (Fig. 9). Multiple other lines tested showed no significant accumulation of AtHSP101, nor evidence of cosuppression. Preliminary data suggest that these lines are somewhat more thermotolerant than wildtype in the absence of a pretreatment (Fig. 10, Part C). The 5/16 line has now been backcrossed to eliminate any phenotypes introduced by the transformation procedure.

AIM 4 - Identifying interacting proteins

This work was carried out in Dr. Lindquist's laboratory. A major goal of this aim was to initiate a screen for suppressors of dominant *hsp104* alleles in *Saccharomyces cerevisiae* as an approach to identifying proteins that functionally interact with Hsp104. Efforts were first directed toward purifying the dominant negative Hsp104 proteins and characterizing them biochemically. This work is still in progress. Simultaneously a pilot screen for dominant negative suppressors was initiated using a strain in which the dominant negative allele (double mutant G217S + T499I) had been introduced into wildtype yeast under GAL control. However, all of the suppressor mutants obtained in this screen appeared to be regulatory mutations affecting the expression of the Hsp104 mutant gene; they had either lost GAL induction or acquired stop codons in the mutant Hsp104 reading frame. To circumvent this problem, a new strain was constructed that carries two integrated copies of the GAL regulated Hsp104 dominant mutant. The suppressor screen will be reinitiated with this strain in work continuing in Dr. Lindquist's laboratory. In the long term my laboratory will exploit the findings of this screen by identifying the Arabidopsis homologs of any yeast Hsp104 interacting proteins, but this is beyond the scope of the current proposal.

Figure 1. Genomic structure of the AtHSP101 promoter region and site of the T-DNA insertion. Functional motifs are indicated by different colors. The T-DNA is inserted 143 bp upstream from the matching point of the cDNA and 432 upstream from the translation initiation site as confirmed by DNA sequencing as shown. The exact size of the insert is unknown.

Figure 2. Amino acid sequence comparison of HSP100 family members from *Arabidopsis thaliana*, AtHSP101, AtHSP92.7, ClpC and ERD1. Residues shown in upper case correspond to identical or conservative replacements in three or more sequences. The bottom line shows the consensus identity for all sequences. The two large conserved domains (as defined by Gottesman et al. 1990) encompassing the Walker ATP-binding motifs are highlighted in bold in the consensus identity line. Residues comprising the Walker motifs are underlined within the consensus. The short peptide within the first conserved domain that corresponds to the peptide from *S. cerevisiae* Hsp104 that was used to develop a peptide antibody is underlined. The amino terminal segment of AtHSP101 used to produce a fusion-protein antigen as described in the Progress Report is also underlined.

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Figure 2. Amino acid Sequence comparison of HSP100 proteins from Arabidopsis - see further details on previous page.

	1	101	201	301	401	501	600
AtHsp101	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~
AtHsp92.7	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~
AtClpC	mamatrvlaq	stppslacyq	rnvpsrgsgr	srrsvkmmci	ifnvwlpmqg	fmglrgnnal	dtlgksrqdf
AtERD1	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~
Consensus	-----	-----	-----	-----	-----	-----	-----
AtHsp101	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~
AtHsp92.7	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~
AtClpC	kaikvimlaq	eearrlghNf	vgteqillgl	Ige.....	.GtGiaa.kv	LksmginlkD	arvevekiIg
AtERD1	rairaiifsq	keakslgkdm	vytqhlllgl	IAedrdpqgf	lGsGitidka	reavwsiwde	ansdskqeea
Consensus	-----	-----	-----	-----	-----	-----	-----
AtHsp101	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~
AtHsp92.7	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~
AtClpC	lghnyigseh	lllgllrege	gvaarvlenL	Gadpsnirtq	vi.rmvge..		
AtERD1	mdcqyiapah	iavglftvdd	gsaGrvlkrL	Ganmllltaa	altrlkgeia	kdGrepssss	kgsfesppsg
Consensus	-----	-----	-----	-----	-----	-----	-----
AtHsp101	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~
AtHsp92.7	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~
AtClpC	KLDPVIGRde	EiRrVvrILs	RRTKNNPvLI	GEPGVGKTAv	VEGLAQRIvk	GDVPnsLtdv	rlISLDmGaL
AtHsp92.7	KLDPVIGRhr	EiRrVievLs	RRTKNNPvLI	GEPGVGKTAv	VEGLAQRIlk	GDVPinLtgv	klISLefGam
AtClpC	KLDPVvGRqp	qIeRvVqILg	RRTKNNPcLI	GEPGVGKTAI	aEGLAQRIas	GDVPetiegk	kvItLDmGll
AtERD1	liDPVIGRek	EvqRviqILc	RRTKNNPiLl	GEaGVGKTAI	aEGLAISiae	asaPgflLtk	rimSLDiGlL
Consensus	KLDPVIGR--	EI-RV--IL-	RRTKNNP-LI	GEPGVGKTA-	-EGLAQRI--	GDVP--L---	--ISLD-G-L
AtHsp101	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~
AtHsp92.7	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~
AtClpC	FIDEvHtliG	AGaa.....	GaiDAANiLK	PaLARGElQc	IGATTldEYR	khiEKDpAlE	RRFQpVkvPe
AtERD1	FIDEvHtliG	sGtvgrgnkg	sglDiANLLK	PsLgRGeLQc	IasTTLdEfr	sqfEKDkAla	RRFQpVline
Consensus	FIDE-H---G	AG-----	G--DAANLLK	P-LARGE-L-C	IGATTI-EYR	---EKD-A-E	RRFQ-V-V-E
AtHsp101	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~
AtHsp92.7	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~
AtClpC	inAAQLSaRY	ItgRhLPDKA	IDLvDEAcAn	VRvqldsqPE	EidnLErkrm	gleielhale	rek.dkaska
AtHsp92.7	vlsAQLSeRY	ItgRrLPDKA	IDLvDEscAh	VkaqlldiqPE	EidsLErkvm	gleieihale	kekddkasea
AtClpC	vaAAQLSyqY	IadRfLPDKA	IDLiDEAgSr	VRlrhaqvPE	EvreLE....		
AtERD1	daAvyLSsRY	IadRfLPDKA	IDLiDEAgSr	aRieafrkkk	Edaici....		
Consensus	--AAQLS-RY	I--R-LPDKA	IDL-DEA---	VR-----PE	E---LE----	-----	-----

Figure 1. - Continued

	601				650						700
AtHsp101	deirrlKqkr	eelmfsLqea	eRryDlaraa	dlrygAIQEv	esaiqlEgt	sseEnvMlte	nVGPEhIAeV	VSrWTGIPVt	rlgqnEkeRL	igLaDrLHkR	
AtHsp92.7	netrrlKqnr	ddlmiaLqea	eRqhDvpkaa	vlkygAIQEv	esaiakLE.k	sakdnvMlte	tVGPEhIAeV	VSrWTGIPVt	rldqnEkkRL	isLaDkLHeR	
AtClpC	tkekneavrg	qdfekagtlr	dReielraev	s....AIQak	gkemskaEse	tgeEgpM...	.VtesdIqhi	VSsWTGIPVe	kvstdEsdRL	lkmeetLHkR	
AtERD1	dywqeiktvq	amhevvlssr	qkqdDgdais	d....esgEl	veesslppaa	gddEpil...	.VGPddIAaV	aSvWsGIPVq	qitadEimlL	msLeDqLrgR	
Consensus	-----K---	-----L---	-R--D-----	-----AIQE-	-----E--	---E--M---	-VGP--IA-V	VS-WTGIPV-	-----E--RL	--L-D-LH-R	
	701				750						800
AtHsp101	VVGQnqAVnA	vSeAIlRSRa	GLgraaqPtg	SFLFlGPTGV	GKTELAKALA	eqlFddEnll	vRlDMSEYME	qHsVSrLiGa	PPG.YVGhEE	GGQLTEAVRR	
AtHsp92.7	VVGQDEAVkA	vaaAIlRSRV	GLgrPqqPsg	SFLFlGPTGV	GKTELAKALA	eqlFdSEnll	vRLDMSEYnd	kfsVnkLiGa	PPGyYigHEE	GGQLTEpVRR	
AtClpC	iiGQDEAVkA	iSrAIrRaRV	GLknPnrPia	SFiFsGPTGV	GKsELAKALA	ayyFgSEeam	iRLDMSEfME	rHtVStLiGs	lPG.YVGyte	GGQLTEAVRR	
AtERD1	VVGQDEAVaA	iSrAvkRSRV	GLkdPdrPia	amLFcGPTGV	GKTELtKALA	anyFgSEesm	lRLDMSEYME	rHtVSkLiGs	PPG.YVGfEE	GGmLTEAiRR	
Consensus	VVGQDEAV-A	-S-AI-RSRV	GL--P--P--	SFLF-GPTGV	GKTELAKALA	---F-SE---	-RLDMSEYME	-H-VS-LIG-	PPG-YVG-EE	GGQLTEAVRR	
	801				850						900
AtHsp101	RPYcViLFDE	vEKAHvaVFN	tLLQvldDGr	LTDgqGRTVD	FrNsvIIMTS	NlGaehllaG	ltGkvtmeva	rD.....	...cVmrEvr	khFRPELLNR	
AtHsp92.7	RPYcVVLfDE	vEKtHvtVFN	tLLQ.....	hGRTVD	FKNtvIIMTS	NlGadhivsG	ltGemtmqva	rD.....	...namkdaK	khFRPELLNR	
AtClpC	RPYtVVLfDE	iEKAHpdVFN	mmLQileDGr	LTDskGRTVD	FKNtllIMTS	NvGssvieK	gr.rigfdld	yDekdssynr	ikslVteElK	qyFRPEfLNR	
AtERD1	RPftVVLfDE	iEKAHpdIFN	iLLQlfeDGh	LTDsqGRrVs	FKNallIMTS	NvGslaiakG	rhGsigfilu	dDeeaasytg	mkaLVveElK	nyFRPELLNR	
Consensus	RPY-VVLfDE	-EKAH--VFN	-LLQ---DG-	LTD--GRTVD	FKN--IIMTS	N-G-----G	--G-----	-D-----	----V--E-K	--FRPELLNR	
	901				950						1000
AtHsp101	LDEIVvFdpL	shdqrlrkvar	LqmKdVavRL	aergvaLaVt	daaldyilae	sYdPvYGARP	iRRwmeKKVv	telSkmvvre	EIdenstVyi	Dagag..dlV	
AtHsp92.7	LDEIVmFhpL	shehlakIvq	LqvKnVv...		P	iRRwlerkVv	tdiSmmivre	EIgddsiVci	DvneaktdlV		
AtClpC	LDEmivFrqL	tklevkeIad	illKeVfeRL	kkkeieLqVt	erfkervvde	gYnPsYGARP	lRRaimrlle	dsmaekmlar	EIkegdsviv	DvdaegnvtV	
AtERD1	iDEIViFrqL	ekaqmmeIlN	LmlqdlksRL	valgvgleVs	epvkelickq	gYdPaYGARP	lRRtvteiVe	dplSeafIag	sfkpgdtafv	vlddtgnpsV	
Consensus	LDEIV-F--L	-----I--	L--K-V--RL	-----L-V-	-----	-Y-P-YGARP	-RR-----V-	---S-----	EI-----V--	D-----V	
	1001				1059						
AtHsp101	yrvesgglvd	astgkksdvl	ihiangpkrs	daaqav....	kkmrieeied	ddneemied					
AtHsp92.7	yridknvfvk	ie..qtldv	ihsgnkrgrs	ndedhivtlt	kkiknevvi	d~~~~~					
AtClpC	lngsgtptt	sleeqedsIp	va~~~~~	~~~~~	~~~~~	~~~~~					
AtERD1	rtkpdssstir	vtdktsia~	~~~~~	~~~~~	~~~~~	~~~~~					
Consensus	-----	-----	-----	-----	-----	-----

Figure 3. Comparison of *AtHSP101* and *AtHSP92.7* mRNA expression during heat stress. Total RNA was isolated from leaves of rosette stage plants either maintained at control temperature or heat stressed at 38 °C for 90 min. Northern blots were hybridized with gene specific probes for either gene.

Figure 4. Genomic structure of *AtHSP92.7* and location of the inserted T-DNA. Exons are indicated by open boxes. The exact size of the insert is unknown. The primers that were used for screening the T-DNA pools are represented by arrows.

A. AtHSP101

B. AtHSP92.7

Figure 5. Identification of plants homozygous for T-DNA insertions in *AtHSP101* and *AtHSP92.7*. (A) *AtHSP101*- Left: PCR analysis of wildtype (WT) and T-DNA insertion mutant plants (M). The band at 1 kb corresponds to the gene without the insertion. The mutant shows only a band at ~4.0 kb. Right: Southern blot analysis of genomic DNA. DNA was digested with HindIII and probed with a fragment of *AtHSP101* that spans the T-DNA insertion site. The predicted HindIII fragment of 1.2 kb seen in wildtype(WT) is completely absent from the mutant(M). The origin of the minor band (*) in both lanes is unclear, but may represent non-digested DNA. (B) *AtHSP92.7* - Left: PCR analysis of wildtype (WT) and T-DNA insertion mutant plants (M). The band at ~2 kb corresponds to the gene without the insertion. No band is seen in the mutant because of the large size of the inserted T-DNA. Right: Southern analysis of genomic DNA. DNA was digested with EcoRI and probed with a fragment of *AtHSP92.7* that spans the T-DNA insertion site.

Figure 6. Comparison of AtHSP101 expression between the wildtype and AtHSP101 T-DNA mutant plants during seed germinating and in response to stress such as heat, EtOH or canavanine. Proteins from germination seeds and leaves treated with heat, EtOH or canavanine were separated by SDS-PAGE and probed with AtHSP101 specific antibodies (AtHSP101) or Arabidopsis HSP17.6 antibodies (sHSP). (A) 7 day old wildtype (WT) and T-DNA insertion mutants (M) on the plates were sampled 2 hr after heat treatment at 38 °C for 90 min. (B) Proteins from germinating seed of wildtypes (WS-O) and T-DNA mutant plants were sampled at the indicated days after imbibition (DAI). (C) 7 day old plants on plates were sampled after incubation in 10 ml of different concentrations of different ethanol concentrations (1, 2.5, 5 and 10 %) for 1 day. (D) 7 day old plants on the plates were sampled after incubation in 10 ml of different concentrations of the amino acid analogue, canavanine (5, 10, 20 and 40 µg/ml) for 1 day.

Figure 7. Hypocotyl elongation assay of thermotolerance: assay of wildtype and mutant plants. Seedlings were grown on plates vertically in the dark at 22 °C and then subjected to a 2 hr 45 °C treatment either with (adapted) or without (nonadapted) a pretreatment of 38 °C for 90 min followed by 2 hr at 22 °C. The hypocotyl length of the etiolated seedlings was measured 2.5 days after the 45 °C treatment. For the control, one plate was maintained at 22 °C for 5 days (normal) and another plate was treated at the same time point at 38 °C for 90 min (pretreated).

Figure 8. Development of thermotolerance in *Arabidopsis* seedlings. *Arabidopsis* seedlings grown on plates for 7 days were treated at 45 °C for 2 hours and photographed 5 days later. Seedlings on the left were pretreated at 38°C for 90 min before the 45 °C stress, while the seedlings at right received no pretreatment. Seedlings at the left developed thermotolerance.

Figure 9. Thermotolerance of luciferase activity imaged in situ. 10 day old seedlings grown on agar plates were either left untreated or pretreated at 38 °C for 90 min followed by 2 hr at 22 °C. Preadapted and nonadapted seedlings were then transferred to the same plates, sprayed with luciferin (1mM in 0.01% Triton X-100) and imaged using a high performance CCD camera (courtesy of Dr. J-K. Zhu, Plant Science Dept., Univ. of Arizona). Duplicate plates containing both preadapted and nonadapted seedlings were then heat treated at 42 °C for either 15 or 30 min. Seedlings were then imaged just after the 42 °C heat stress or 1 hr after the heat stress.

Figure 10. Luciferase activity during heat shock and recovery in intact seedlings. 10 day old seedlings grown on agar plates were either left untreated (nonadapted) or pretreated (adapted) at 38 °C for 90 min followed by 2 hr at 22 °C. Preadapted and nonadapted seedlings were then heat stressed at 42 °C for 50 min. These seedlings were sprayed with luciferin (1mM in 0.01% Triton X-100) and the luciferase activities were measured in situ at various time points by use of the winview program (courtesy of Dr. J-K. Zhu, Plant Science Dept., Univ. of Arizona). The different heat treatment conditions are indicated by different colors. The heat treatment changing points are shown by arrows.

A HSP 101

B Yeast HSP 104

Figure 11. Accumulation of HSP100 proteins during seed development. Equal quantities of seed proteins were separated by SDS-PAGE and probed with either the AtHSP101 antibody (A) or the yeast Hsp104 peptide antibody (B). The * indicates the 95 kDa protein that most likely represents one or more of the chloroplast-localized homologs (ClpC and ERD1). Control (C) and heat shock (HS) leaf samples are shown for comparison.

A HSP 101

B Yeast HSP 104

Figure 12. HSP100 protein levels during seed germination and early seedling growth. Proteins from germinating seeds were isolated at the indicated days after imbibition (DAI). Equal quantities of protein were separated by SDS-PAGE and probed with either the AtHSP101 antibody (A) or the yeast Hsp104 peptide antibody (B). Control (C) and heat shock (HS) leaf samples are shown for comparison.

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