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An Arabidopsis Heat Shock Protein Complements a Thermotolerance Defect in Yeast

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The heat shock protein Hsp104 of the yeast *Saccharomyces cerevisiae* plays a key role in promoting survival at extreme temperatures. We found that when diverse higher plant species are exposed to high temperatures they accumulate proteins that are antigenically related to Hsp104. We isolated a cDNA corresponding to one of these proteins from Arabidopsis. The protein, AtHSP101, is 43% identical to yeast Hsp104. DNA gel blot analysis indicated that AtHSP101 is encoded by a single- or low-copy number gene. *AtHsp101* mRNA was undetectable in the absence of stress but accumulated to high levels during exposure to high temperatures. When AtHSP101 was expressed in yeast, it complemented the thermotolerance defect caused by a deletion of the *HSP104* gene. The ability of AtHSP101 to protect yeast from severe heat stress strongly suggests that this HSP plays an important role in thermotolerance in higher plants.

INTRODUCTION

All organisms, including higher plants, synthesize heat shock proteins (HSPs) in response to high temperature stress (Vierling, 1991; Parsell and Lindquist, 1993). Many HSPs are also expressed at optimal growth temperatures and play essential roles in normal growth, acting as molecular chaperones to assist the folding, assembly, and transport of other proteins (Gething and Sambrook, 1992; Parsell and Lindquist, 1993). Genetic analysis in the yeast *Saccharomyces cerevisiae* and in *Escherichia coli* demonstrates that increased synthesis of HSPs is required to maintain growth at the upper end of an organism's normal temperature range. A large number of experiments suggest that the chaperone activities of HSPs help to prevent protein aggregation at high temperatures, thereby limiting heat-induced cell damage (Craig et al., 1994; Frydman and Hartl, 1994; Parsell and Lindquist, 1994).

The induction of HSPs also plays a key role in acquired thermotolerance, the ability of an organism to withstand a short period at an otherwise lethal temperature if it is first conditioned by a treatment at an intermediate temperature (Lindquist and Craig, 1988). In at least some cases, the HSPs that are required for short-term survival at extreme temperatures are distinct from those that are required for growth at elevated temperatures. For example, deletion of certain *Hsp70* genes in yeast severely reduces growth at high temperatures but has no detectable effect on acquired thermotolerance (Werner-Washburne et al., 1987). On the other hand, deletion of the major *Hsp100* gene of yeast, *HSP104*, has no effect on growth at high temperatures but severely reduces acquired thermotolerance (Sanchez and Lindquist, 1990).

Yeast Hsp104 belongs to a large family of highly conserved proteins known as the HSP100 or Clp protein family (Squires and Squires, 1992). Members of the HSP100/Clp family are found in both prokaryotes and eukaryotes, where they are present in the cytosolic/nuclear compartment and in organelles. Some members are induced by heat; others are not. The HSP100/Clp proteins share two large blocks of sequence homology (~200 amino acids) centered around two ATP binding consensus elements. The ATP binding domains are flanked by less conserved N-terminal, spacer, and tail domains (Gottesman et al., 1990; Squires and Squires, 1992). The size of the spacer domain has been used to define three subfamilies, ClpA, ClpB, and ClpC, with short, long, and intermediate spacers, respectively. Hsp104 belongs to the ClpB subfamily. The ClpB homolog of *E. coli* is also induced by heat and plays a role in heat tolerance in that organism (Squires et al., 1991).

HSP100/Clp proteins are poorly characterized in higher eukaryotes. Only two vertebrate members have been identified, and little is known of their functions or patterns of expression (C.A. Vandenberg, personal communication). In several plant species, heat-induced proteins in the 100-kD size range have been observed by in vivo labeling of proteins during heat stress (Vierling, 1991; Medina and Cardemil, 1993), and recently antibodies against yeast Hsp104 were found to cross-react with

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a heat-induced protein in rice (Lata Singla and Grover, 1993). Genes encoding two members of the HSP100/Clp family of proteins have been isolated from higher plants. *ClpC* genes have been identified in pea (Moore and Keegstra, 1993), tomato (Gottesman et al., 1990), and Arabidopsis (Squires and Squires, 1992). The ClpC proteins encoded by these plant genes contain an N-terminal transit peptide that acts as a chloroplast targeting signal (Moore and Keegstra, 1993). No information is available on the regulation of *ClpC* expression in response to stress. A second gene in the HSP100/Clp family, named *Erd1*, was cloned from Arabidopsis as a gene responsive to dehydration stress (Kiyosue et al., 1993). ERD1 also has an N-terminal targeting sequence, but whether it is localized to chloroplasts or mitochondria is not known. *Erd1* mRNA levels do not increase in response to heat stress or cold stress. Thus, an HSP100/Clp homolog encoding a cytosolic protein that accumulates in response to heat stress (as does yeast Hsp104) has not yet been isolated from plants.

We demonstrate that a wide range of plant species synthesize both constitutive and heat-inducible proteins of ~100 kD; these proteins have antigenic similarity with Hsp104. To characterize the heat-inducible species further, a full-length cDNA was isolated from Arabidopsis. It encodes a protein with 43% identity with Hsp104. The Arabidopsis protein, AtHSP101, can partially substitute for the function of Hsp104 in yeast, restoring induced thermotolerance in strains carrying a deletion of the *HSP104* gene. Thus, AtHSP101 represents a novel heat-inducible, cytosolic member of the HSP100 gene family from a higher eukaryote, and it is the only plant protein for which a function in thermotolerance is genetically implicated.

RESULTS

Detection of Heat-Induced HSP100 Proteins

To determine if the induction of proteins related to yeast Hsp104 is a common feature of the plant heat shock response, diverse species were subjected to heat stress and total leaf proteins were isolated. Equal quantities of protein from the heat-stressed and control samples were compared by SDS-PAGE and protein gel blotting using an antiserum generated against a conserved peptide from the first ATP binding domain of Hsp104 (antibody 2-3; Parsell et al., 1991). In all samples, the anti–Hsp104 peptide antibodies detected one to three polypeptides in the 100-kD size range (Figure 1). In maize and pea, a faster migrating band also reacted with the antibody. Similar cross-reacting bands are consistently observed in certain other organisms, and may represent smaller members of the HSP100 family rather than proteolytic degradation products (M. Feder, E.C. Schirmer, D.A. Parsell, and S. Lindquist, unpublished data).

Additional proteins in the 100-kD range were induced in all species, except maize, following heat shock (Figure 1). In all cases, these were the highest molecular mass forms detected.

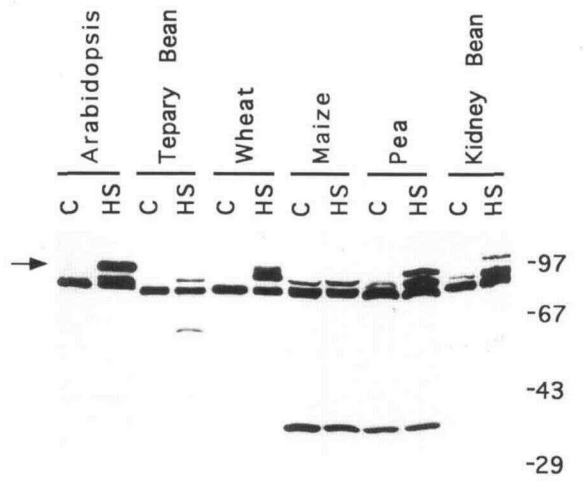

Figure 1. Diverse Plant Species Accumulate Proteins during Heat Stress That Cross-React with Antibodies Generated against a Conserved Peptide from Yeast Hsp104.

Plants indicated were either heat treated (HS) or maintained at normal growing temperatures (C). Equal quantities of leaf proteins (120 μg per lane) were subjected to SDS-PAGE and transferred to nitrocellulose membranes. The membranes were incubated with the antiHsp104 antiserum 2-3, which was generated against a peptide from the highly conserved first ATP binding domain underlined in Figure 2. Antibody binding was visualized by fluorography with a chemiluminescence detection system. The arrow at left indicates the position of the heat-induced proteins of ~100 kD.

In maize, the higher molecular mass form seen in the control samples increased somewhat following heat stress, but this induction was apparent only on shorter exposures than that shown in Figure 1. In all other species, the heat-induced polypeptides showed a very strong induction. These results indicate that a broad array of higher plants synthesize proteins related to Hsp104 and that induction of one or more of these proteins is characteristic of the heat shock response in plants.

Cloning and Sequence Analysis of Arabidopsis *Hsp101*

Based on the strong induction of the 100-kD protein in response to heat and observations of induction of mRNA encoding a protein of ~100 kD as seen by in vitro translations (E. Vierling, unpublished observations), a differential hybridization strategy was used to screen for the corresponding gene in Arabidopsis. A cDNA library constructed using heat shock poly(A) RNA from Arabidopsis (Helm and Vierling, 1989) was hybridized with ^{32}P-labeled cDNA prepared from the high molecular mass fraction of control or heat shock poly(A) RNA (see Methods). Approximately 50 cDNA clones were purified that showed (1) no hybridization with the control probe, (2) strong hybridization with the heat shock probe, and (3) no hybridization with previously isolated HSP cDNAs. The longest clones

in this group were ~3 kb and had similar restriction fragment patterns. One of these clones (1-1S) was chosen for sequence analysis.

The complete nucleotide sequence and derived amino acid sequence of the 1-1S cDNA clone have a GenBank accession number of U13949. The cDNA is 3124 bp long, including a poly(A) tract of 20 residues. The largest open reading frame is 911 amino acids and encodes a protein of 101,278 D, leaving 5′ and 3′ noncoding regions of 162 and 229 bp, respectively. Based on the calculated molecular mass of the open reading frame, we designated this gene as *AtHsp101*.

Comparison of the *AtHsp101* coding sequence with the GenBank and EMBL data bases revealed high homology with the HSP100/Clp family of proteins (Figure 2). As is characteristic of other members of this family, the highest sequence conservation is found in the two ATP binding domains (boxed in

Figure 2. Amino Acid Sequence Alignments of AtHSP101 with Other Members of the HSP100/Clp Family of Proteins.

(A) Comparison to ClpB proteins. AtHSP101 is aligned with yeast Hsp104 (ScHsp104) and *E. coli* ClpB (EcClpB). Residues identical to the AtHSP101 sequence are replaced with dots, and adjustments to the alignment are indicated by dashes. Nucleotide binding domains are boxed, and highly conserved regions outside the binding folds are highlighted in black. The peptide sequences used to generate the 2-3 and 8-1 antibodies used in this study are underlined in the Hsp104 protein sequence.

(B) AtHSP101 is distinct from the ClpC class of HSP100 proteins. Amino acid sequence alignments of ClpC family members from plants, algae, and bacteria with the three identified Arabidopsis HSP100/Clp proteins, AtClpC, ERD1, and AtHSP101, are shown. The alignment is shown as far as the sequence is known for AtClpC, and only a portion of the highly variable N-terminal organelle targeting sequence is shown, with the sequence truncation marked with dashes at the N terminus. In the alignment, residues identical to AtClpC are indicated by dots, and gaps are shown as dashes.

Figure 2A). The N-terminal, spacer, and C-terminal regions display much lower levels of homology. The HSP100/Clp family has been divided into three subfamilies, based upon the size of the spacer region between the two ATP binding domains (Gottesman et al., 1990; Squires and Squires, 1992). ClpA members have little or no spacer, ClpB members have a long spacer (~120 residues), and ClpC members have a spacer of intermediate length (~65 amino acids). The length of the spacer in AtHSP101 is ~120 residues marking it for inclusion in the ClpB subfamily, which also includes yeast Hsp104. Short but clear regions of homology between AtHSP101, Hsp104, and *E. coli* ClpB proteins are observed in the spacer and C-terminal regions (boxed in black in Figure 2A), which are only partially shared with the other Clp proteins (data not shown). Overall, AtHSP101 is 47.5% identical to *E. coli* ClpB and 43.3% identical to Hsp104. It shares only 39.1% identity with the tomato ClpC protein and 33.4% identity with the *E. coli* ClpA protein.

The lack of any apparent N-terminal targeting sequence suggests that the AtHSP101 protein is primarily cytosolic, as is the yeast Hsp104 protein. The two other members of the HSP100/Clp family cloned to date from Arabidopsis, *AtClpC* and *Erd1*, have an N-terminal extension that presumably targets them to organelles. The chloroplast-localized plant ClpC proteins, the plastid-encoded ClpC protein from a chromophytic alga (*Heterosigma carterae*), as well as the bacterial ClpC proteins share considerable identity (Figure 2B). The ERD1 protein is less similar but shares tracts of homology with the ClpC proteins, whereas AtHSP101 is clearly distinct. Thus, AtHSP101 shows greater similarity with the yeast Hsp104 protein than it does to either of the previously cloned Arabidopsis HSP100/Clp family members.

AtHsp101 Is a Single- or Low-Copy Number Gene

To estimate the number of *AtHsp101* genes in the Arabidopsis genome, total genomic DNA was digested with a variety of enzymes and analyzed by DNA gel blot hybridization (Figure 3). Using a probe derived from the 3′ end of the *AtHsp101* cDNA and enzymes that do not cut the cDNA in the probe region (BamHI, EcoRI, XhoI, HindIII), a single band of hybridization was obtained at high stringency (see Methods). Using an enzyme that cuts both the 3′ probe and the cDNA once (ClaI), two bands of hybridization were detected. Similar results were obtained at the same stringency with another probe, an internal HindIII fragment that includes all of the first conserved ATP binding domain plus an additional ~200 bp of the nonconserved spacer (data not shown). Finally, the internal HindIII fragment was also used for hybridization at reduced stringency (see Methods), and no additional hybridizing bands were detected (data not shown). We concluded that the AtHSP101 protein is most likely encoded by a unique gene in the Arabidopsis genome. However, we cannot rule out the presence of another gene, derived from a recent duplication event, that has the same restriction sites for the enzymes tested here.

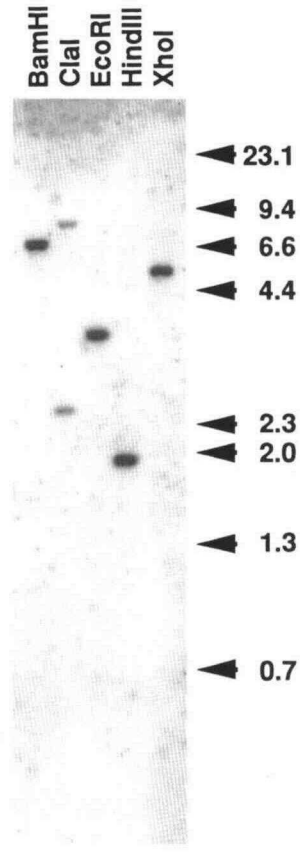

Figure 3. *AtHsp101* Is a Single- or Low-Copy Number Gene.

Total Arabidopsis genomic DNA was digested with the indicated enzymes (1.5 μg per sample) and processed for DNA gel blot analysis. The nitrocellulose filter was hybridized with a 753-bp probe derived from the 3′ end of the *AtHsp101* cDNA (from the SmaI site at nucleotide 2380 to the 3′ EcoRI cloning site) at high stringency (see Methods). Molecular mass markers (in kilodaltons) are indicated at right.

Isolation of genomic clones for *AtHsp101* will be needed to resolve this question definitively.

AtHsp101 and the Chloroplast *AtClpC* Gene Respond Differently to Heat Stress

Because the *AtHsp101* cDNA was isolated by differential hybridization, we expected that *AtHsp101* mRNA would accumulate to high levels in response to heat stress. RNA gel blot analysis was performed to confirm this prediction and to determine whether the chloroplast-localized homolog *AtClpC* (Squires and Squires, 1992) is similarly regulated. *Erd1*, the other HSP100/Clp family member identified in Arabidopsis, is not heat inducible (Kiyosue et al., 1993). Poly(A) RNA was isolated from Arabidopsis leaves treated for 90 min at 22°C (control) or 37°C (heat shock). As predicted, *AtHsp101* mRNA was

undetectable in the control sample and very abundant in the heat-shocked sample (Figure 4). In contrast, the *AtClpC* transcript was present in the control and actually declined during heat stress. Thus, *AtHsp101* is the only identified gene in the Arabidopsis HSP100/Clp family that is heat regulated.

Expression of AtHSP101 in Yeast

To investigate the function of AtHSP101, we sought to determine whether it could provide thermotolerance in yeast cells that were deficient in thermotolerance due to the deletion of their own *Hsp100* gene, *HSP104*. To this end, the *AtHsp101* coding sequence was cloned into a galactose-regulated yeast expression vector. Surprisingly, initial attempts to obtain such clones failed repeatedly, whereas similar attempts to clone the yeast *HSP104* coding sequence into the same vector were successful. When the region surrounding the AUG translation initiation codon was mutated to prevent fortuitous expression in *E. coli*, the desired *AtHsp101* expression constructs were readily obtained (see Methods). These observations suggest that expression of the Arabidopsis but not the yeast HSP100 protein is toxic in *E. coli*.

To examine the effects of AtHSP101 expression in yeast, strains containing a deletion of the *HSP104* gene were transformed with the galactose-regulated *AtHsp101* expression plasmid. Control strains, carrying the yeast *HSP104* coding sequence under the control of the same promoter or the vector alone, were also established. Finally, isogenic strains that had lost the plasmid were derived from each of the transformants by growing them in nonselective medium for several generations.

Proteins produced by these strains during growth on raffinose or galactose medium were analyzed on protein gel blots by using two antisera, one that recognizes both the Arabidopsis and the yeast protein (antiserum 2-3) and another that recognizes only the yeast protein (antiserum 8-1). The antisera were derived from the yeast peptides underlined in Figure 2. Little or no expression of AtHSP101 or Hsp104 was detected during growth on raffinose (data not shown). During growth on galactose, the initial transformants carrying the *HSP104* or *AtHsp101* plasmids produced proteins of the expected size and specificity, whereas strains that had lost the plasmids produced no cross-reacting proteins (Figure 5A). Some degradation products were also observed in the galactose-induced sample, but a substantial quantity of full-length protein accumulated. The actual quantity of full-length Hsp104 and AtHSP101 in the corresponding expression strains was estimated to be similar based on Coomassie blue staining (data not shown). In addition, when strains containing the *AtHsp101*, *HSP104*, or vector plasmids were grown on galactose-containing agar medium, the sizes of the colonies produced were indistinguishable. Thus, expression of AtHSP101 is not toxic to growth in yeast, as it appears to be in *E. coli*.

To confirm the identity of the Arabidopsis protein, proteins expressed in yeast transformants were analyzed on the same gels as proteins from control and heat-stressed leaves (Figure 5B). The protein produced by the *AtHsp101* expression plasmid in yeast cells comigrated with the larger immunoreactive leaf protein, which was strongly induced by heat. We conclude that the larger immunoreactive Arabidopsis leaf protein represents the *AtHsp101* gene product.

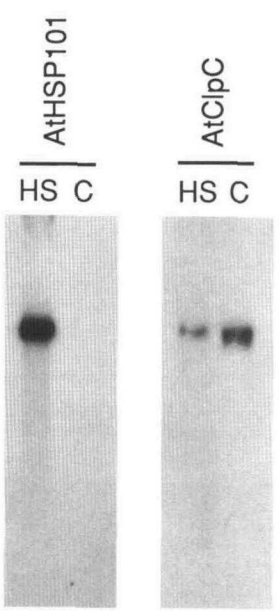

Figure 4. Expression of *AtHsp101* and *AtClpC* mRNA during Heat Stress.

Poly(A) RNA isolated from control (C) or heat-stressed (HS) leaves was analyzed on RNA gel blots probed with the entire coding region of either *AtHsp101* or *AtClpC*.

Thermotolerance of Yeast Expressing AtHSP101

To determine whether AtHSP101 can complement the function of Hsp104 in acquired thermotolerance, strains expressing AtHSP101 or Hsp104 were compared in thermotolerance assays. The plasmid-encoded proteins were induced by growth on galactose media for 7 hr. Cells were then given a conditioning pretreatment at 37°C and subjected to a 50°C heat stress for 10, 20, or 30 min. Cultures were serially diluted to determine levels of survival (Figures 6 and 7).

When yeast Hsp104 was expressed from the galactose-regulated plasmid in cells carrying a deletion of *HSP104*, it provided the same level of thermotolerance as the genome-encoded Hsp104 of wild-type cells (Figure 6). Remarkably, the Arabidopsis protein also provided a substantial level of thermotolerance in these cells. In fact, it increased thermotolerance ~100-fold over the vector alone (Figures 6 and 7). Similar results were obtained with the *AtHsp101* transformants analyzed in

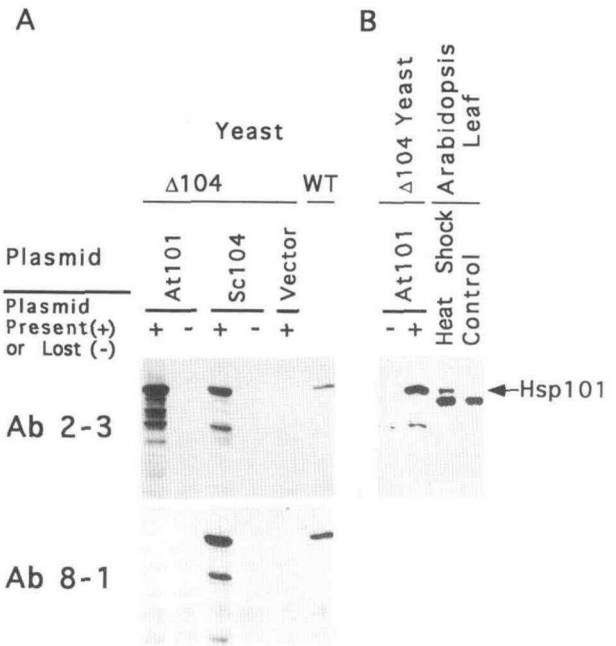

Figure 5. AtHSP101 Expression in Yeast.

(A) Strains carrying either the *AtHsp101* (At101), *HSP104* (Sc104), or vector plasmid (Vector) were examined by protein gel blot analysis. Proteins from 2.5 × 10^6 cells grown for 7 hr in galactose were separated by SDS-PAGE, transferred to membranes, and incubated either with antiserum 2-3 (which reacts with both Hsp104 and AtHSP101) (Ab 2-3) or with antiserum 8-1 (which reacts with Hsp104, but not AtHSP101) (Ab 8-1). Immunoreactive bands were visualized as described in Figure 1. As previously reported, in wild-type (WT) cells, Hsp104 is constitutively expressed at a low level in galactose medium. No Hsp104 or AtHSP101 was expressed in the *hsp104* deletion mutants (Δ104), except in cells carrying the appropriate expression vector.

(B) Proteins extracted from yeast cells expressing AtHSP101 (shown in [**A**]) were analyzed on the same gel as leaf proteins from Arabidopsis plants maintained at normal temperature (Control) or heat stressed as described in Methods (Heat Shock). The AtHSP101 protein expressed in yeast corresponds to the large, heat-inducible HSP100 protein from Arabidopsis leaves (indicated by arrow at right).

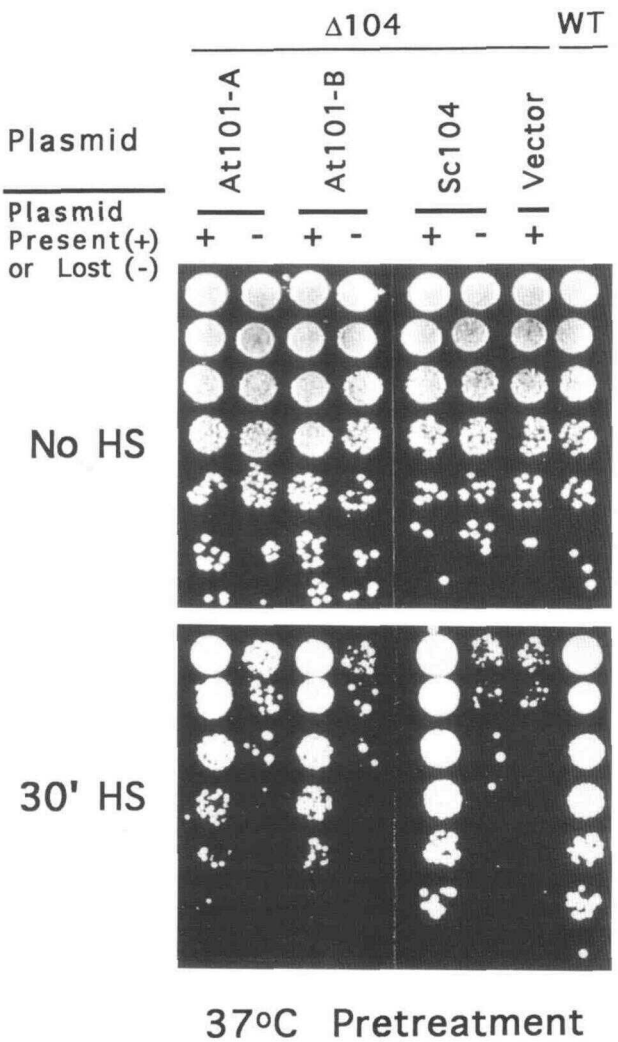

Figure 6. AtHSP101 Expression Rescues the Thermotolerance Defect of a Yeast *hsp104* Deletion Mutant.

Cells carrying a deletion of the *HSP104* gene (Δ104) were transformed with the galactose-regulated expression plasmids for AtHSP101 (At101-A or -B), yeast Hsp104 (Sc104), or the vector alone (Vector) and maintained on selective medium (+) or allowed to lose the plasmid on nonselective medium (−). Log-phase cells were transferred to galactose for 7 hr to induce the plasmid-borne genes, and a portion of the culture was removed. These cells were serially diluted (fivefold at each step), and 5 μL of each dilution was spotted onto agar medium (undiluted cells at the top) to assess the starting cell density (No HS, top). The remaining cells were given a conditioning pretreatment at 37°C, subjected to a 50°C severe heat stress for 30 min, and then serially diluted to assess survival (30′ HS, bottom). For comparison, wild-type (WT) cells are shown at right.

these experiments and with each of several independent transformants in other experiments.

In control experiments, cells were maintained in noninducing, raffinose medium prior to heat shock. In this case, strains carrying the expression plasmids or the vector alone exhibited similar low levels of thermotolerance (data not shown). The increased thermotolerance observed in galactose medium for At101 and Sc104 was lost from these strains when the plasmid was lost through growth on nonselective medium (Figure 6). Thus, increased thermotolerance in strains carrying the *AtHsp101* galactose-driven expression plasmid was due to expression of AtHSP101 and not to any background mutations induced by transformation.

DISCUSSION

We isolated a heat-inducible *Hsp100* gene from Arabidopsis, *AtHsp101*, that is able to provide thermotolerance to yeast cells that are missing their own *Hsp100* gene, *HSP104*. The AtHSP101 protein is 43% identical to the yeast Hsp104 protein; this result, together with the complementation data, demonstrates that the Arabidopsis protein is both a structural and functional homolog of the yeast HSP. We found that, in addition to Arabidopsis, other plant species including both monocots and dicots accumulated proteins of ~100 kD during heat stress that cross-react with antiserum generated against a conserved peptide from yeast Hsp104. These results indicate that synthesis of proteins in the HSP100/Clp family is a basic characteristic of the heat shock response in higher plants and suggest that these proteins play a major role in plant thermotolerance.

Analysis of the amino acid sequence indicates that, similar to other members of the HSP100/Clp family, AtHSP101 contains two highly conserved ATP binding domains and more variable N-terminal, spacer, and C-terminal regions. Several features support the inclusion of AtHSP101 in the ClpB subfamily of the HSP100/Clp proteins. The length of the spacer region between the ATP binding domains, small regions of homology within this spacer, and the absence of an organellar targeting sequence are all typical of the ClpB subfamily.

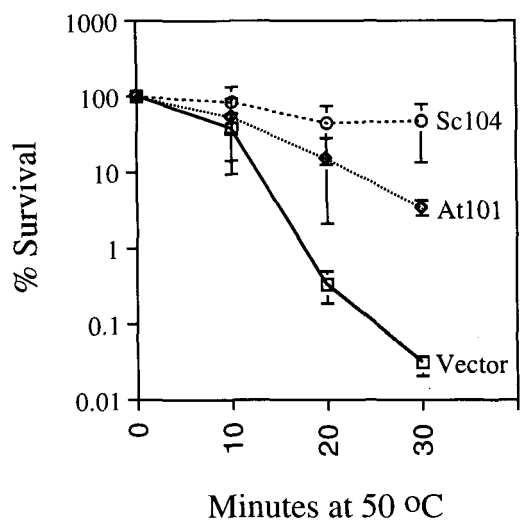

Figure 7. Survival versus Time at 50°C.

Three independent yeast transformants for each of the three vectors (as shown in Figure 6) were cultured as described in Figure 6, except that they were exposed to 50°C for 10, 20, or 30 min. Mean survival and standard deviations are expressed as a percentage of the survival obtained in cultures that were pretreated at 37°C but not exposed to 50°C. Squares, vector alone; diamonds, cells transformed with the *AtHsp101* expression plasmid (At101); circles, cells transformed with the yeast *Hsp104* expression plasmid (Sc104).

Furthermore, all ClpB proteins that have been tested are strongly heat regulated (Squires and Squires, 1992; Parsell and Lindquist, 1993) as is AtHSP101. Thus, AtHSP101 is clearly functionally distinct from the previously identified Arabidopsis *Erd1* and *AtClpC* genes, which also encode members of the HSP100/Clp protein family. Both of these proteins have organelle targeting sequences and as shown here and by Kiyosue et al. (1993) they are not heat regulated. The ERD1 and AtClpC proteins may be among those proteins present constitutively in Arabidopsis that also reacted with the anti-Hsp104 antiserum. The HSP100/Clp family of proteins in higher plants is clearly complex, including multiple proteins with different intracellular targets and modes of regulation, as is the case for other HSP protein families (Vierling, 1991).

A recently isolated soybean homolog of yeast Hsp104 shares 86% identity with AtHSP101. As we found for *AtHsp101*, the soybean gene is also heat regulated, and the encoded protein provides partial complementation of the thermotolerance defect of an *hsp104* mutant (Lee et al., 1994). It is difficult to compare directly the degree of thermoprotection provided by the plant proteins relative to yeast Hsp104, because it is not possible to determine the absolute level of functional Arabidopsis or soybean protein produced in yeast. However, the fact that we found similar levels of Hsp104 and AtHSP101 polypeptide in our expression strains suggests that AtHSP101 is not as effective in interacting with other yeast cell components that participate in thermotolerance as is the endogenous Hsp104. Considering the differences between the plant and yeast cellular milieu, it is significant that AtHSP101 can enhance survival 100-fold in the *hsp104* mutant background. We intend to investigate whether the organelle-targeted AtClpC and ERD1 proteins, engineered to remove their targeting signals, can also act in the yeast cytosol to provide thermotolerance.

It has long been known that plants are capable of developing thermotolerance (Yarwood, 1965). The ability to survive normally lethal temperatures can be induced by a variety of pretreatments, and the synthesis of HSPs is induced by pretreatments that lead to thermotolerance (Altschuler and Mascarenhas, 1982; Lin et al., 1984; Neumann et al., 1989; Hsieh et al., 1992). HSPs are also found in dry seed (Abernethy et al., 1989; Almoguera and Jordano, 1992; DeRocher and Vierling, 1994), which is a plant life stage that is typically heat resistant. However, there is no direct evidence that HSPs play a role in acquired thermotolerance in plants or that they are responsible for the heat resistance of seed or for differences in thermotolerance observed between genotypes/cultivars within the same species (for review, see Vierling, 1991). Our results indicate that it will be of interest to reevaluate HSP synthesis in plants, focusing on the relationship between HSP100 expression and temperature tolerance. The fact that *Erd1* is regulated by abscisic acid and desiccation stress (Kiyosue et al., 1993) also suggests the HSP100/Clp proteins play a role in a broad range of stress tolerances. This would be consistent with the fact that Hsp104 also protects yeast cells from ethanol exposure and cold storage and is involved in the natural

thermotolerance of stationary-phase cells (Sanchez et al., 1992). We do not yet know whether *AtHsp101* is induced by other stress treatments or whether it will complement other phenotypes of the yeast *HSP104* deletion strain.

Recent work suggests that Hsp104 in yeast functions in thermotolerance by promoting the reactivation of aggregated, heat-damaged proteins after high temperature stress (Parsell et al., 1994; J.L. Vogel, D.A. Parsell, and S. Lindquist, unpublished data). Our results indicate that there is a remarkable conservation of function between the yeast and plant HSP100 proteins. Thus, continued study of Hsp104 in yeast should provide further insight into mechanisms of thermotolerance in plants. Furthermore, increased expression of Hsp104 can confer thermotolerance to yeast cells in the absence of synthesis of other HSPs, and overexpression of Hsp104 is not toxic to yeast cells (G. Kim, E.C. Schirmer, and S. Lindquist, unpublished data). Therefore, it may be possible to manipulate expression of HSP100 proteins to engineer plants with greater stress tolerance.

METHODS

Plant Growth and Heat Stress Treatments

Arabidopsis thaliana (Columbia ecotype) was grown in soil in growth chambers or on lighted shelves (125 to 250 μmol m^{-2} sec^{-1} of photosynthetically active radiation) under a 16-hr day length for 21 to 28 days. Other species were grown under similar conditions for the indicated number of days: maize (*Zea mays* FunkF G4343), 14 days; wheat (*Triticum aestivum* Durum cv Mexicali), 7 days; cowpea (*Vigna unguiculata* TVU 4552), 14 days; kidney bean (*Phaseolus vulgaris* cv Commodore), 14 days; pea (*Pisum sativum* cv Little Marvel), 10 days; tepary bean (*Phaseolus acutifolius*), 14 days.

Arabidopsis protein samples were prepared from plants stressed as follows. Intact 21- to 28-day-old plants were placed in a growth chamber and subjected to a gradual increase in temperature (4°C per hour) as described previously (Chen et al., 1990) until the chamber temperature had reached a maximum temperature of 38 or 40°C. After 4 hr at the maximum temperature, the temperature was gradually returned to 22°C, and tissue was harvested for analysis.

Heat treatments of other plant species were also performed using the gradual stress regime, with a maximum temperature of 40°C. Approximately 1.0 g of leaf tissue was excised and placed in Petri dishes lined with moistened filter paper. The tissue was then incubated in the lighted growth chamber throughout the stress period until the chamber temperature had returned to 22°C, at which time samples were harvested and processed.

For heat treatments prior to RNA isolation, Arabidopsis plants (~21 days old) were removed from soil and the roots were thoroughly rinsed. The entire mass of tissue was then placed in beakers in H_2O and incubated either at room temperature (21 to 22°C) or at 37 to 38°C in a shaking water bath for 90 min.

Protein Sample Preparation and Protein Gel Blot Analysis

Total proteins were prepared from leaf tissues by homogenizing tissue directly in SDS gel sample buffer (60 mM Tris-HCl, pH 8.0, 60 mM DTT, 2% SDS, 5.0 mM ε-aminocaproic acid, 1.0 mM benzamidine, 15% sucrose) using a ground glass homogenizer. The tissue-to-buffer ratio used for homogenization is as follows: 0.4 g fresh weight of tissue per milliliter for Arabidopsis samples and for all other species 0.2 g 1.0 mL^{-1} buffer. Samples were boiled for 3 to 5 min, and insoluble debris was removed by centrifugation at 12,000*g* for 1 to 5 min. Protein concentrations were estimated using the Coomassie blue binding assay of Ghosh et al. (1988) or with the BCA reagent (Pierce Chemical Co., Rockford, IL) after acetone precipitation. Proteins (120 μg per lane in Figure 1; ~20 μg per lane in Figure 5) were separated on 7.5% SDS–polyacrylamide gels and transferred to nitrocellulose. Equal protein loading was confirmed by Coomassie Brilliant Blue R 250 staining of gels loaded with the same samples.

For preparation of *Saccharomyces cerevisiae* proteins, cells were transferred to synthetic galactose medium for 7 hr in the log phase of growth. A portion of the culture was used for thermotolerance experiments, and the remaining cells were collected by centrifugation and resuspended in ethanol containing 2 mM phenymethylsulfonyl fluoride and ~400 mg of 425- to 600-μm glass beads for the preparation of protein samples. Cells were disrupted by vigorous agitation on a vortex mixer for 5 min at 4°C. Proteins were dried under vacuum and resuspended in 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 65 mM Tris-HCl, pH 6.8, bromophenol blue. Proteins from 2.5×10^6 cells were analyzed per lane on 7.5% SDS–polyacrylamide gels and transferred to Immobilon-P membranes (Millipore Corp., Bedford, MA). Equal sample loading was confirmed by Coomassie blue staining of membranes.

Antisera used to detect HSP100 proteins were generated in rabbits (Berkeley Antibody Company, Richmond, CA) using two different Hsp104 peptides. Antiserum 2-3 was produced against a 16–amino acid peptide from the glycine-rich loop of the first ATP binding site and shows broad cross-species reactivity with HSP100 proteins (Parsell at al., 1991). Antibody 8-1 was generated against the C-terminal 15 amino acids of Hsp104 and has more limited cross-species reactivity. Peptides 2-3 and 8-1 are underlined in Figure 2. Antibody 2-3 was used at a dilution of 1:600, and antibody 8-1 was used at a 1:7500 dilution. Blots were blocked in 10% reconstituted milk powder in PBS, incubated with the antibodies diluted into 10% fetal bovine serum in PBS for 1 hr, washed, incubated with protein A–peroxidase (Boehringer Mannheim) for 30 min, washed, and visualized with the ECL detection kit and Hyperfilm-MP (Amersham Corp.).

RNA Isolation and Gel Blot Analysis

RNA was isolated from Arabidopsis at the end of the heat treatments previously detailed. Excess water was removed from the tissue by blotting, and the tissue was homogenized directly in RNA extraction buffer as previously described (Vierling and Key, 1985). Poly(A) RNA was prepared by oligo(dT)-cellulose chromatography and quantified by absorbance at 260 nm.

RNA (0.5 μg per sample) for RNA gel blot analysis was denatured in formamide–formaldehyde and separated on 1.2% formaldehyde–agarose gels. RNA was transferred to nitrocellulose, and filters were hybridized as previously described (Vierling and Key, 1985) with ^{32}P-dATP–labeled probes prepared by random primer labeling (Feinberg and Vogelstein, 1983). For detection of the *AtHsp101* or *AtClpC* transcripts, the entire plasmid containing the full-length coding region was labeled.

cDNA Library Screening

A cDNA library was prepared from the Arabidopsis heat shock RNA in λ ZAP (Stratagene) (Helm and Vierling, 1989). Control and heat shock

poly(A) RNAs to be used for the synthesis of single-stranded cDNA probes were fractionated on sucrose gradients, and RNA from each fraction was analyzed by in vitro translation as described by Vierling (1987). Fractions that produced translation products of 70 kD or greater were pooled and ethanol precipitated. This high molecular mass RNA (an estimated 0.1 to 0.5 μg based on counts per minute incorporated by in vitro translation) was reverse transcribed for 30 min at 42°C in a 20-μL reaction mixture composed of 50 mM Tris-HCl; pH 8.3 (at 42°C); 10 mM $MgCl_2$; 10 mM DTT; 4 mM sodium pyrophosphate; 400 μM dGTP, dCTP, and dTTP; 50 μM dATP; 100 μg mL^{-1} oligo(dT); 100 μCi ^{32}P-dATP (ICN Radiochemicals, Irvine, CA; 3000 Ci/mmol); and ~50 units of avian myeloblastosis virus reverse transcriptase (Stratagene). Duplicate filter lifts prepared from each plate of the heat shock cDNA λ ZAP phage (~30,000 plaques screened) were hybridized with ~1.0 × 10^7 cpm of either the control or heat shock probe. Positive plaques were rescreened twice with these probes. To eliminate phage corresponding to previously isolated heat shock cDNAs, phage were also hybridized with probes prepared by random primer labeling of cDNAs corresponding to Arabidopsis HSP70 and HSP90 (E. Vierling, unpublished data), HSP21 (Osteryoung et al., 1993), HSP22 (Helm et al., 1994), or HSP17.6 (Helm and Vierling, 1989). Plaques that hybridized only to the single-stranded heat shock cDNA probe and not to the other probes were further characterized. Plasmids were rescued from these phage according to instructions of the supplier (Stratagene) and analyzed by restriction digestion.

DNA Sequence Analysis

Both strands of the *AtHsp101* cDNA were completely sequenced using primers generated during sequencing. Sequencing was performed using a *Taq* DyeDeoxy terminator cycle sequencing kit (Applied Biosystems, Foster City, CA). Reaction products were analyzed on a model 370A Automated Sequencer (Applied Biosystems). Regions that were ambiguous were sequenced by traditional ^{33}P-dATP sequencing using the Sequenase 2.0 kit (U.S. Biochemical Corp.).

The AtHSP101 amino acid sequence was aligned to other HSP100 family members using the EMBL homology-derived secondary structure of proteins, Version 1.0 1991 program (Sander and Schneider, 1991). Assessments of identity were derived by dividing the number of identical residues in the EMBL alignment by the total number of residues in the AtHSP101 protein (911). For sequence comparisons with the Arabidopsis *ClpC* gene, the partial AtClpC amino acid sequence was derived from DNA sequence data communicated to us by N. Hoffman (Carnegie Institute, Stanford, CA) and additional sequence derived in our laboratory. For the other proteins in the alignment in Figure 2, sequences were obtained using the following accession numbers: *S. cerevisiae*, M67479, *Escherichia coli*, M29364, *Bacillus subtilis*, X75930; *Mycobacterium leprae*, X53488; *Sirpulina hyodysenteriae*, X73140; *Heterosigma carterae*, Z25810; *Brassica napus*, S37557; *Lycopersicon esculentum* (tomato), M32603; *Pisum sativum* (pea), L09547; ERD1, D17582.

DNA Gel Blot Analysis

Arabidopsis genomic DNA, which had been isolated by standard phenol extraction and CsCl banding (Murray and Thompson, 1980), was a gift from J. Celenza (Whitehead Institute for Biomedical Research, Cambridge, MA). Arabidopsis DNA (1.5 μg per sample) was digested with restriction enzymes for 2 hr and separated on an 0.8% agarose Tris–borate–EDTA gel and blotted to nitrocellulose. The membrane was hybridized in aqueous buffer according to Church and Gilbert (1984), and membranes were washed in 0.1 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate), 0.1% SDS. For high stringency, membranes were hybridized and washed at 65°C. For reduced stringency, membranes were hybridized and washed at 50°C. Either of two probes was used under both conditions: (1) 753 bp of the 3′ end of the *AtHsp101* cDNA from the SmaI site at nucleotide 2380 to the 3′ EcoRI cloning site or (2) an internal HindIII fragment corresponding to nucleotides 650 to 1528 of the cDNA that includes all of the first conserved ATP binding domain plus an additional ~200 bp.

Construction of Yeast Strains Expressing AtHSP101

Repeated attempts to clone the *AtHsp101* coding sequence into yeast expression vectors behind the *HSP104* or *GAL1* promoters failed, whereas parallel transformations with the yeast *HSP104* coding sequence succeeded. We reasoned that expression of the Arabidopsis protein in *E. coli* might be toxic, thereby preventing recovery of the plasmid. To modify the sequence around the initiating AUG to a context unfavorable for expression in *E. coli*, site-directed mutagenesis was used to add a BamHI site followed by three guanine nucleotides directly in front of the initiating AUG of both the yeast *HSP104* and the Arabidopsis *AtHsp101* genes. The modified genes were subcloned into a pRS313-based vector carrying *HIS3* as the selectable marker (Sikorski and Hieter, 1989) and containing the *GAL1-10* promoter in the polylinker. Using these modified clones, the expression plasmids pGALSc104 and pGALAt101 were readily obtained.

The two plasmids and the vector alone were introduced into *S. cerevisiae* SL304A containing a *LEU2* insertion in the *HSP104* gene and a deletion of *HSP104* codons 1 to 321. The genotype is *leu2-3,112, trp1-1, ura3-1, ade2-1, his3-11,15 lys2D, can1-100, hsp104::LEU2, MATa* (Sanchez et al., 1993). Transformants containing pGALSc104 (Sc104) and pGALAt101 (At101) were maintained on noninducing, histidine-deficient synthetic medium (2% dextrose, 0.5% ammonium sulfate, 0.17% nitrogen base without amino acids, 10 mg L^{-1} adenine, 50 mg L^{-1} arginine, 50 mg L^{-1} lysine, 20 mg L^{-1} methionine, 50 mg L^{-1} phenylalanine, 100 mg L^{-1} threonine, 50 mg L^{-1} tyrosine, 70 mg L^{-1} aspartic acid, 20 mg L^{-1} uracil, 50 mg L^{-1} tryptophan, 100 mg L^{-1} leucine). To obtain isogenic derivatives that had lost the plasmid, the Sc104 and At101 strains were passaged on medium supplemented with histidine at 20 mg L^{-1} and replica plated to selective plates to determine whether they had lost the plasmid.

Thermotolerance Experiments

Transformants were grown to mid-log phase in synthetic medium, with raffinose substituting for dextrose and with histidine supplementation as required. Cells were collected by centrifugation and resuspended at a density of 1 × 10^6 cells mL^{-1} in synthetic medium with galactose substituting for dextrose. After 7 hr of induction in galactose, cells were recounted and densities were adjusted to 1 × 10^7 cells mL^{-1}. Cells were distributed to 10- × 75-mm glass tubes (0.1 mL per tube) and preincubated in a 37°C water bath for 30 min before transfer to a 50°C water bath for the times indicated in Figures 6 and 7. Samples were cooled on ice for 2 min and then diluted 1:5 serially eight times. Five microliters of each dilution was spotted onto YPDA plates (1% yeast extract, 2% bactopeptone, 2% glucose, 40 mg L^{-1} adenine sulfate, 3% agar). For control experiments, strains were maintained in noninducing medium (synthetic raffinose). Plates were incubated at 25°C for 3 days and photographed with a Nikon F3 35-mm camera using an AF Micro Nikkor 55-mm lens (Nikon Corporation, Tokyo, Japan). Experiments were repeated three times with the strains shown in the text and several times with strains isolated from multiple independent transformations. Similar results were obtained in all cases.

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