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A Soybean 101-kD Heat Shock Protein Complements a Yeast *HSP104* Deletion Mutant in Acquiring Thermotolerance

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A cDNA clone encoding a 101-kD heat shock protein (HSP101) of soybean was isolated and sequenced. Genomic DNA gel blot analysis indicated that the corresponding gene is a member of a multigene family. The mRNA for HSP101 was not detected in 2-day-old etiolated soybean seedlings grown at 28°C but was induced by elevated temperatures. DNA sequence comparison has shown that the corresponding gene belongs to the Clp (caseinolytic protease) (or Hsp100) gene family, which is evolutionarily conserved and found in both prokaryotes and eukaryotes. On the basis of the spacer length between the two conserved ATP binding regions, this gene has been identified as a member of the ClpB subfamily. Unlike other Clp genes previously isolated from higher plants, the expression of this soybean *Hsp101* gene is heat inducible, and it does not have an N-terminal signal peptide for targeting to chloroplasts. Transformation of the soybean *Hsp101* gene into a yeast *HSP104* deletion mutant complemented restoration of acquired thermotolerance, a process in which cells survive an otherwise lethal heat stress after they are given a permissive heat treatment.

INTRODUCTION

A set of proteins referred to as heat shock proteins (HSPs) is synthesized by cells in response to an increasing growth temperature and has been found in almost every organism studied to date (see Lindquist and Craig, 1988; Nover, 1991). The HSPs are classified into several families according to their molecular masses, and the HSPs with similar molecular masses among organisms usually share significant sequence identity. Not only is the synthesis of HSPs an immediate response to heat stress, but constitutively expressed homologs of HSPs are also essential for growth and metabolism at normal growth temperatures and during various stages of development (see Hightower and Nover, 1991; Vierling, 1991), suggesting that HSPs have fundamental and essential biological functions in cells.

The functions of HSPs have been extensively studied; some biochemical functions attributable to the HSP90, HSP70, and HSP60 proteins relate to their role as molecular chaperones (see Georgopoulos and Welch, 1993; Hendrick and Hartl, 1993). Whether HSPs play a role in protecting cells from heat damage and many other stresses has been a long-standing question. Cells or organisms can survive an otherwise lethal heat stress if they are given a permissive heat treatment prior to the severe heat stress. This phenomenon is referred to as acquired thermotolerance (Gerner and Scheider, 1975). Although some contradictory data exist (see Nagao et al., 1986; Lindquist and Craig, 1988), a large body of accumulated circumstantial evidence shows that synthesis of HSPs is strongly correlated with the acquisition of thermotolerance. It is commonly assumed that HSPs participate in the maintenance of cellular structures during the stress period and in the repair of structural damage, which allows cells to recover normal functions quickly after stress (Lin et al., 1984; Nagao et al., 1986; Lindquist and Craig, 1988). The fact that several HSPs function as molecular chaperones to prevent the aggregation and/or promote the proper folding of heat-denatured proteins helps to explain their role in heat stress (Jinn et al., 1989; Beckmann et al., 1992). In addition, because some HSPs have proteolytic activities and others serve as auxiliary components in proteolysis, HSPs may promote degradation of heat-damaged proteins during heat stress (see Parsell and Lindquist, 1993).

In yeast cells, Hsp104 is not detectable at normal growth temperatures but becomes a major product of protein synthesis shortly after a shift to high temperatures (Sanchez and Lindquist, 1990). By genetic deletion analysis, Sanchez and Lindquist (1990) showed that the yeast *HSP104* gene is not an essential gene under normal growth conditions. When given a permissive heat treatment, however, yeast *HSP104* deletion mutant cells (Δ*hsp104*) do not acquire tolerance to an otherwise lethal heat treatment. Complementation of the Δ*hsp104* cells with the *HSP104* gene restores the ability to acquire thermotolerance (Sanchez and Lindquist, 1990). These results demonstrate that Hsp104 plays a critical role in cell survival at extreme growth temperatures.

Soybean seedlings also synthesize HSPs with molecular masses of 100 kD and higher during heat treatment (Nagao et al., 1986). In this study, we report the complete sequence of an *Hsp101* cDNA isolated from soybean and examine its

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expression and function in response to heat treatment and the acquisition of thermotolerance.

RESULTS

Isolation of Soybean cDNA Clones Encoding HSP101

A genomic fragment of the yeast *HSP104* gene (EcoRI-SacI fragment, 2.6 kb, spanning the coding region of the gene) was used to screen a soybean cDNA library constructed from enriched high molecular mass poly(A)$^+$ RNAs extracted from etiolated soybean seedlings incubated at 40°C for 1 hr (see Methods). Three independent cDNA clones hybridized to the yeast *HSP104* gene. None of these cDNA clones contained a complete reading frame. One of these cDNA clones, p101-1, whose sequence was more similar to the yeast *HSP104* gene than the others (Y.-R.J. Lee, R.T. Nagao, and J.L. Key, unpublished data), was selected for further analysis; results from these studies are reported here.

The cDNA clone p101-1, which is shown in Figure 1, contained a 1.2-kb insert. The open reading frame derived from p101-1 aligned with approximately one-third of the C-terminal portion of the yeast *HSP104* coding region. Primer-directed extension reactions were conducted to synthesize the full-length cDNA with a complete open reading frame. Primer-directed extension was performed with a 24-base oligonucleotide designed to be specific to p101-1 and tested to be noncomplementary to the other two cDNA clones. A cDNA clone, designated p101-2, was obtained in the first primer-directed extension reaction. The open reading frame of the combination of p101-1 and p101-2 (Figure 1) aligned with ~90% of the yeast *HSP104* coding region. Another cDNA clone, designated p101-3, was obtained in a subsequent primer-directed extension reaction; it provided the N-terminal end of the open reading frame (Figure 1). The soybean cDNA clone *GmHsp101* was then created by ligation of p101-1, p101-2, and p101-3 (Figure 1). The *GmHsp101* cDNA is 3049 bp long and contains an open reading frame encoding a putative peptide of 911 amino acids. The peptide, soybean HSP101, has a calculated molecular mass of 101 kD and a predicted pI of 6.1.

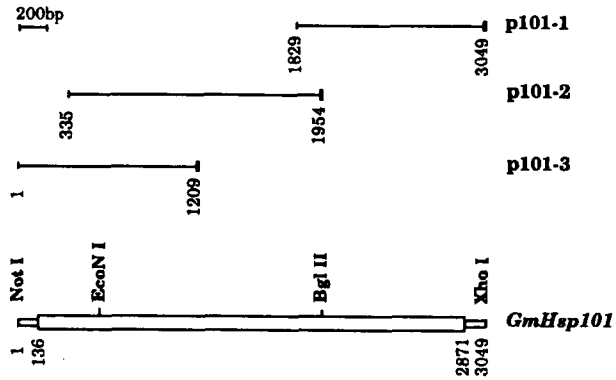

Figure 1. Cloning of a *GmHsp101*.

The clone p101-1 was isolated by plaque hybridization from a soybean cDNA library with a yeast *HSP104* genomic fragment. The clones p101-2 and p101-3 were obtained by primer-directed extension. The primers were a 24-base oligonucleotide and an 18-base oligonucleotide complementary to nucleotides 1931 to 1954 (5′-GACCAAGGAATAGGAATGAACCAG-3′) and 1192 to 1209 (5′-TCTCTCTTTCAAGCCACG-3′), respectively. The complete soybean *Hsp101* cDNA was assembled by the ligation of the NotI-EcoNI fragment of p101-3, the EcoNI-BglII fragment of p101-2, and the BglII-XhoI fragment of p101-1 into pCR-Script at the NotI and XhoI sites; it was named *GmHsp101*. The thicker box represents the open reading frame; the thinner flanking boxes represent the 5′ and the 3′ untranslated regions; numbers indicate positions of nucleotides on *GmHsp101*.

Characterization of *GmHsp101*

To identify the peptide product of the *GmHsp101* cDNA, the poly(A)$^+$ RNA that was hybrid selected by *GmHsp101* was translated in vitro and resolved on a two-dimensional polyacrylamide gel. The hybrid-selected mRNAs translated into one major and one minor peptide, shown in Figure 2A. The minor peptide (97 kD) was also present in the vector control (data not shown) and therefore is considered nonspecific. Compared with the standard markers on the gel, the major peptide showed a molecular mass of 112 kD and a pI of 6.1; these values are close to those calculated from the deduced peptide sequence of *GmHsp101* (Figure 2A).

The deduced amino acid sequence of *GmHsp101* is similar to those of members of the conserved Clp (caseinolytic protease; Katayama et al., 1988) (or Hsp100) gene family found in both prokaryotes and eukaryotes (see the following discussion). The size of the soybean Hsp100 gene family was estimated by DNA gel blot analysis. Soybean genomic DNA was digested with BstXI and EcoRI, and the DNA gel blot was hybridized to *GmHsp101*. In each case, mutiple bands were detected (Figure 2B), suggesting that the soybean Hsp100 gene family is composed of multiple copies of genes. Clp proteins are assigned into three subfamilies according to the length of the spacer between two highly conserved ATP binding regions (Gottesman et al., 1990; Squires and Squires, 1992). A probe representing the spacer region of *GmHsp101* was also used to hybridize the soybean genomic DNA gel blot. A single band was detected in each case (Figure 2B), indicating that the spacer region–specific probe does not cross-hybridize with other Hsp100 genes. These data suggest that the corresponding subfamily of *GmHsp101* may be composed of a single copy or of multiple copies having the same genomic organization in the spacer region.

Accumulation of *Hsp101* mRNA Is Induced by Heat Treatments

Heat-induced accumulation of *Hsp101* transcripts was examined by incubating 2-day-old etiolated soybean seedlings at

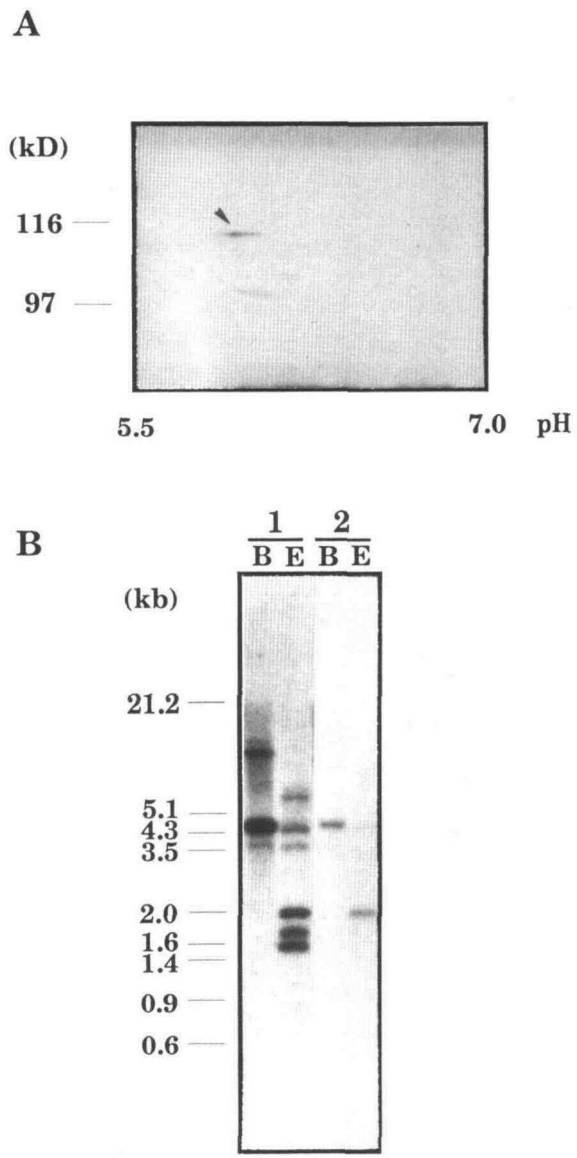

Figure 2. Characterization of *GmHsp101*.

(A) Hybrid selection and in vitro translation. The peptide (indicated by an arrow) produced by hybrid selection and in vitro translation of transcripts hybridized to *GmHsp101* was separated on a two-dimensional polyacryamide gel. The 97-kD peptide was considered a nonspecific signal because it also appeared in the hybrid selection with the vector only. Molecular mass markers of 116 and 97 kD are indicated.

(B) DNA gel blot analysis. Soybean genomic DNA was digested with BstXI (B) or EcoRI (E); the DNA gel blots were hybridized to *GmHsp101* and a probe representing the spacer region of *GmHsp101* (a HindIII-HincII fragment, nucleotides 1501 to 1718), respectively. Molecular length markers are given at left in kilobases. Lane 1, the blot hybridized to *GmHsp101*; lane 2, the blot hybridized to the spacer-specific probe.

the normal growth temperature of 28°C and at various elevated temperatures. Soybean seedlings were incubated at 28, 35, 40, and 42.5°C for 1 hr, and poly(A)$^+$ RNAs isolated from the seedlings were hybridized with *GmHsp101*. *GmHsp101* hybridized to a 3-kb band on RNA gel blots, which is consistent with the length of the cDNA clone (3049 bp) and suggests that this cDNA is full length. Transcripts hybridizing to *GmHsp101* were induced by evaluated temperatures but were not detectable in the mRNA isolated from soybean seedlings incubated at 28°C. The highest accumulation of *Hsp101* transcripts was detected at 40 to 42.5°C, although there was substantial induction at 35°C. The results are shown in Figure 3A.

The accumulation of soybean *Hsp101* transcripts was rapid, being readily detected after 30 min of heat treatment at 40°C (Figure 3B). During continuous heat treatment at 40°C, the steady state level of *Hsp101* mRNA accumulation increased to a maximum level by 1 hr; the steady state level of *Hsp101* mRNA declined significantly by 2 hr at 40°C (Figure 3B). This rapid decline between 1 and 2 hr contrasts with the case seen in other Hsp gene families. For example, the steady state levels of *Hsp70* mRNA accumulation (hybridized to the cDNA

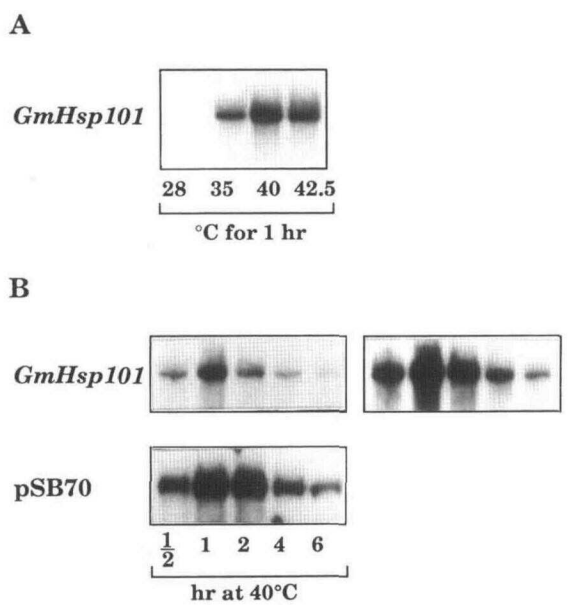

Figure 3. Accumulation of RNAs Hybridizing to *GmHsp101*.

Poly(A)$^+$ RNAs were isolated from 2-day-old etiolated soybean seedlings that had been incubated in buffer for various treatments as indicated. RNA gel blots with 5 μg of poly(A)$^+$ RNAs in each lane were hybridized with ^{32}P-labeled probes.

(A) Accumulation of *Hsp101* mRNA induced by heat treatments. The induction of *Hsp101* mRNA at temperatures from 28 to 42.5°C for 1 hr is shown.

(B) Accumulation of *Hsp101* mRNA during continuous heat treatment. The steady state accumulations of mRNA for *Hsp101* (*GmHsp101*) or *Hsp70* (pSB70) during continuous heat treatment at 40°C are shown. The autoradiographs at left were exposed for the same amount of time; a longer exposure of the RNA gel blot hybridized with *GmHsp101* is shown at right.

clone pSB70; Roberts and Key, 1991) was highest between 1 and 2 hr and then declined after 2 hr of 40°C treatment (Figure 3B).

Amino Acid Sequence Alignment of Soybean HSP101 and Other ClpB Proteins

The deduced amino acid sequence of the *GmHsp101* cDNA is similar to those of members of the conserved Clp (or Hsp100) gene family (Gottesman et al., 1990; Parsell et al., 1991). The Clp proteins possess two highly conserved regions of ~200 amino acid residues. Each of these regions contains a putative ATP binding site with the characteristic amino acid sequence $GX_2GXGGKT$ (X indicates an unspecified amino acid), which forms a glycine-rich flexible loop; this sequence is followed by another motif of at least four hydrophobic amino acids terminated by an aspartate ~60 residues downstream (Gottesman et al., 1990; Parsell et al., 1991). These two regions are separated by a variable spacer region and flanked by less conserved leader and trailer regions. Clp proteins are assigned to three subfamilies according to the length of the spacer region between the two ATP binding regions (Gottesman et al., 1990; Squires and Squires, 1992). The ClpA subfamily has the shortest spacer (five amino acid residues), and the ClpB subfamily has the longest (120 to 130 amino acid residues), with the ClpC subfamily being intermediate (60 to 70 amino acid residues). Soybean HSP101 has 122 amino acid residues between the two ATP binding regions and thus is assigned to the ClpB subfamily by these criteria.

Figure 4 shows the amino acid sequence alignment of soybean HSP101 with ClpB proteins from *Trypanosoma brucei* (Gottesman et al., 1990), yeast (Parsell et al., 1991), and *Escherichia coli* (Gottesman et al., 1990). Soybean HSP101 is most similar to *T. brucei* ClpB, with these two proteins having 54% identity (71% similarity). Soybean HSP101 is also highly related to yeast Hsp104, with 44% identity (65% similarity), and *E. coli* ClpB, with 53% identity (72% similarity). Noticeably, soybean HSP101 possesses a highly negatively charged C terminus (EEIDDDEMEE), which is also a characteristic of *T. brucei* ClpB (DEWE) and yeast Hsp104 (DTLGDDDNEDSNEIDDDLD), but is dissimilar to the *E. coli* ClpB protein (Figure 4).

Several Clp genes have been characterized from higher plants, including tomato (Gottesman et al., 1990), pea (Moore and Keegstra, 1993), and Arabidopsis (Kiyosue et al., 1993; Schirmer et al., 1994). Except for Arabidopsis HSP101, which is a ClpB homolog (see Schirmer et al., this issue), the rest of these proteins were classified as members of the ClpC subfamily owing to their spacer lengths in the range of 60 to 70 amino acids. These ClpC proteins also contain signal peptides for targeting to chloroplasts. ClpC in pea has been found localized in the chloroplasts (Moore and Keegstra, 1993). Although the cellular localization of soybean HSP101 remains to be determined, unlike the ClpC proteins found in higher plants to date, soybean HSP101 does not appear to have any of the characteristics of known N-terminal signal peptides for targeting to the chloroplast (Keegstra et al., 1989).

Functional Analysis of Soybean HSP101

Sanchez and Lindquist (1990) demonstrated that Hsp104 is required for the acquisition of thermotolerance in yeast cells. To test whether soybean HSP101 and yeast Hsp104 are functionally similar, Δ*hsp104* cells were transformed with the plasmid construct pYSSB101, which expresses soybean HSP101 under the control of the yeast *HSP104* promoter (see Methods), and tested for the acquisition of thermotolerance. The transformation of pYSSB101 into the Δ*hsp104* cells was checked by DNA gel blot hybridization, and the expression of soybean HSP101 in the transformed cells was verified by protein gel blot analysis with polyclonal antibodies against soybean HSP101 (data not shown).

To measure the ability of the cells to acquire thermotolerance, yeast cells were incubated at 37°C for 30 min prior to the heat treatment at 50°C. After heat treatment at 50°C, yeast cells were diluted and plated on agar plates to determine colony-forming ability (Sanchez and Lindquist, 1990). Soybean HSP101 provided thermotolerance as effectively as yeast Hsp104 in the Δ*hsp104* cells when the cells were treated at 50°C for 10 min. The protective effect of soybean HSP101 was less than that of yeast Hsp104 when the 50°C treatment was extended to 20 min, but the Δ*hsp104* cells transformed with pYSSB101 were about 20-fold more tolerant to treatment at 50°C for 20 min than the corresponding untransformed Δ*hsp104* cells. The results are shown in Figure 5. A control experiment in which Δ*hsp104* cells were transformed with the vector showed a killing curve similar to that of the Δ*hsp104* cells in the thermotolerance assay (data not shown), suggesting the plasmid-borne *Hsp101* is responsible for the induced thermotolerance.

DISCUSSION

Although low molecular mass HSPs (15 to 30 kD) are the most abundant HSPs synthesized in higher plants during heat stress, higher plants also accumulate substantial levels of several families of high molecular mass HSPs (60, 70, 90, and 100 kD), as do most other organisms (see Nagao et al., 1986; Vierling, 1991). A soybean cDNA clone that is homologous to the yeast *HSP104* gene was isolated. The genes encoding yeast Hsp104 and soybean HSP101 share significant sequence identities with members of the Clp (or Hsp100) gene family (Gottesman et al., 1990; Parsell et al., 1991; Squires and Squires, 1992). Based upon the length of the spacer between the two conserved ATP binding regions, both yeast Hsp104 and soybean HSP101 are classified as members of the ClpB subfamily.

The cDNA *GmHsp101* was assembled with an original cDNA clone isolated from library screening (p101-1 in Figure 1) and

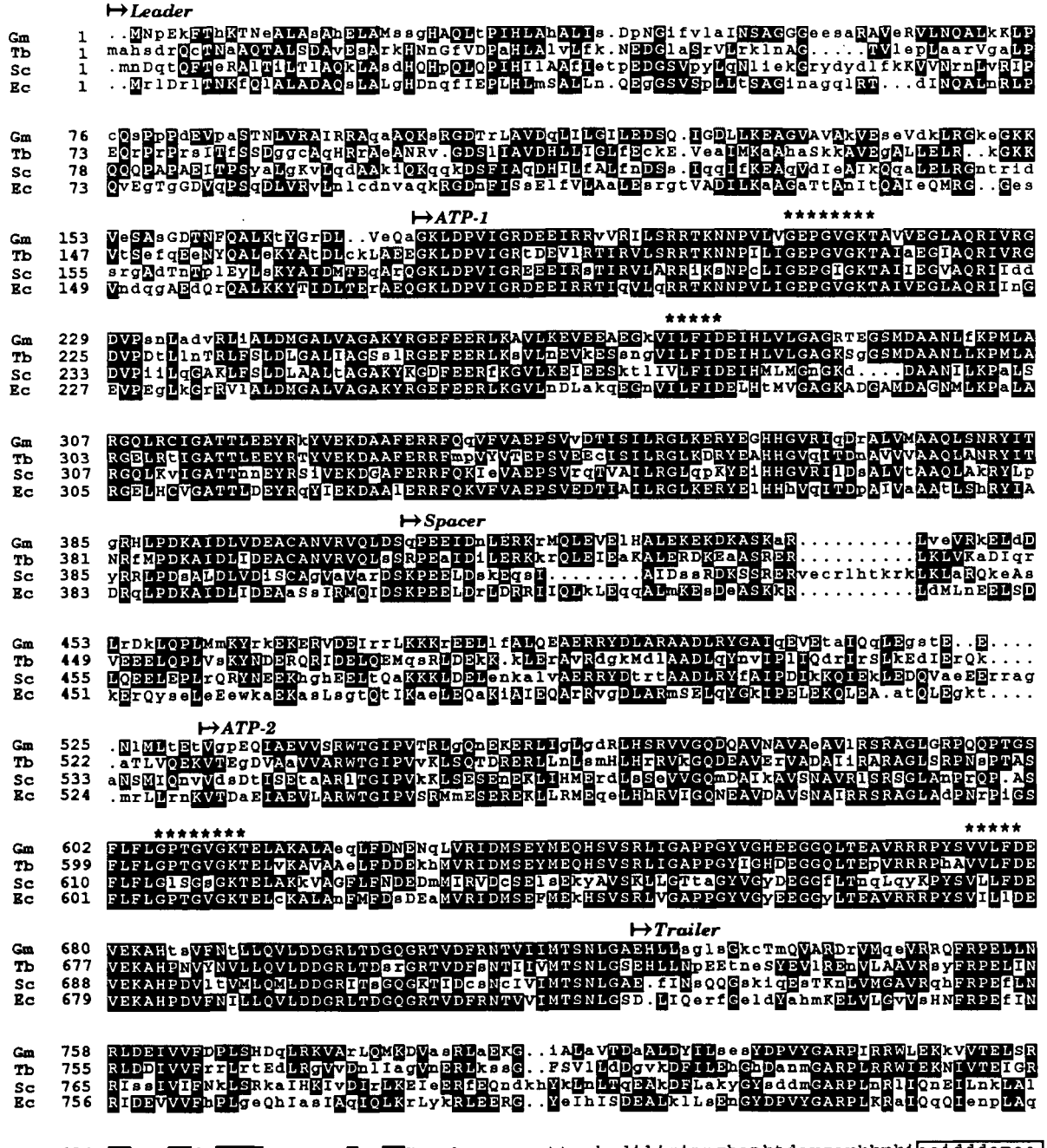

Figure 4. Amino Acid Sequence Comparison of Soybean HSP101 and ClpB Proteins.

Amino acid sequences of soybean HSP101 (Gm), *T. brucei* ClpB (Tb), yeast Hsp104 (Sc), and *E. coli* ClpB (Ec) were aligned using the PILEUP program of the UWGCG suite. A gap weight of 3.0 and a gap length weight of 0.1 were used. Dots were introduced to optimize the alignment. Identical residues are indicated by uppercase letters on a black background, and similar residues are indicated by uppercase letters on a white background. Residues that are neither identical nor similar to each other are represented by lowercase letters on a white background. ATP-1 and ATP-2 stand for the first and second ATP binding regions, respectively. Each of the ATP binding regions is composed of two conserved sequence motifs, which are indicated by asterisks. The negatively charged C-terminal ends are boxed.

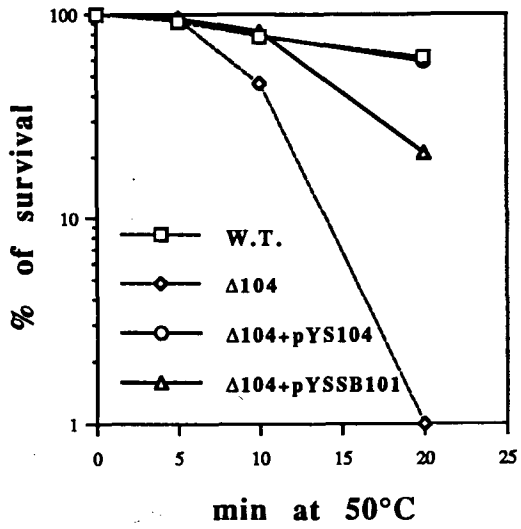

Figure 5. Thermotolerance Assay of the *HSP104* Deletion Mutant Transformed with pYSSB101.

Prior to the 50°C heat treatment, yeast cells were incubated at 37°C for 30 min to induce HSP synthesis. After heat treatment at 50°C for the period of time indicated, cells were diluted in ice-cold YPDA medium and immediately plated on YPDA plates. Colonies that survived the 50°C treatment were counted 3 days after the treatment. Values of relative survival percentage were averaged from three independent transformation experiments. W.T., the wild-type yeast cells; Δ*104*, the Δ*hsp104* cells; Δ*104* + pYS104, the Δ*hsp104* cells transformed with the yeast *HSP104* genomic clone pYS104; Δ*104* + pYSSB101, the Δ*hsp104* cells transformed with pYSSB101 that contains the *GmHsp101* coding sequence under the control of the yeast *HSP104* promoter.

two cDNA clones derived from subsequent primer-directed extension reactions (p101-2 and p101-3 in Figure 1). The primer used in the first extension reaction was chosen based upon its specificity for p101-1, which is not complementary to two other soybean Hsp100-homologous cDNA clones that were also obtained by library screening (Y.-R.J. Lee, R.T. Nagao, and J.L. Key, unpublished data). When designing the primer for the second primer-directed extension reaction, sufficient comparative sequence data were unavailable among the three soybean Hsp100 homologs to generate a gene-specific primer. However, the sequences of p101-2 and p101-3 are exactly matched in an overlapping region of 875 bp, suggesting the extension of the same gene. Although the possibility that *GmHsp101* represents a chimeric gene is not definitively excluded, the hybridization to a single band on the soybean genomic DNA gel blot with a spacer-specific probe (Figure 2B) indicates that cross-hybridization among different genes did not occur and suggests that cDNA clones p101-1, p101-2, and p101-3 are most likely derivatives of the same gene. Transcripts hybrid selected by this cDNA were translated in vitro, and analysis by two-dimensional gel electrophoresis yielded a peptide (Figure 2A) whose molecular mass and pI value are in good agreement with the predictions from the deduced amino acid sequence.

Like other Hsp gene families, the Clp gene family includes members that are induced by heat stress and members that are constitutively expressed. Heat-inducible Clp proteins characterized to date are members of the ClpB subfamily (Sanchez and Lindquist, 1990; Kitagawa et al., 1991; Squires et al., 1991; Squires and Squires, 1992; Leonhardt et al., 1993). The accumulation of the transcripts that hybridized to the *GmHsp101* cDNA was strictly heat inducible, as shown by RNA gel blot hybridization analyses (Figure 3). These data suggest a role for the ClpB proteins in heat stress.

The functions of several Clp proteins have been demonstrated. *E. coli* ClpA is the regulatory subunit of an ATP-dependent Clp protease (Hwang et al., 1988; Katayama et al., 1988); ClpA functions as an ATPase activating the proteolytic activity of the catalytic subunit (Katayama et al., 1988; Woo et al., 1989). Although it is possible that the general role for Clp proteins is to regulate cellular proteases, members of the Clp family may be involved in controlling some enzymatic activity other than proteolysis (Squires and Squires, 1992). An alternate hypothesis for the function of the Clp proteins is that they act as molecular chaperones, preventing the formation of insoluble protein aggregates, promoting their dissolution, or facilitating transport of precursor proteins across envelope membranes (Squires and Squires, 1992; Moore and Keegstra, 1993; Parsell and Lindquist, 1993).

E. coli ClpB and yeast *HSP104* genes are essential for cells to survive an otherwise lethal heat stress. A *ClpB* null mutation of *E. coli* led to reduced cell survival at 50°C (Squires et al., 1991). Yeast *HSP104* is required for cells to acquire thermotolerance (Sanchez and Lindquist, 1990). The function of soybean HSP101 was tested by complementation of a yeast *HSP104* deletion mutant (Δ*hsp104* cells). Transformation of the soybean *Hsp101* coding sequence into the Δ*hsp104* cells restored the defect of the mutant in the acquisition of thermotolerance 20-fold, although it was not as effective as the yeast *HSP104* gene (Figure 5). The production of soybean HSP101 in the transformed yeast cells was verified by protein gel blot analysis, but it was not possible to make a quantitative comparison of the amount of soybean HSP101 and yeast Hsp104 produced. Therefore, partial complementation could be due to insufficient production of protein or to the heterologous soybean HSP101 (44% identity, 65% similarity with yeast Hsp104) not completely fulfilling the function of the yeast protein. However, *GmHsp101* appears to perform a function similar to if not exactly the same as yeast *HSP104* in the acquisition of thermotolerance during heat stress. Schirmer et al. (1994) isolated a heat-inducible *Hsp101* cDNA clone from Arabidopsis; it was also able to complement partially the yeast Δ*hsp104* mutant in acquiring thermotolerance.

Clp proteins may be important to cells that must tolerate not only heat stress, but also other environmental stresses. Sanchez et al. (1992) showed that yeast Hsp104 is responsible for tolerance to heat, ethanol, arsenite, and long-term storage in the

cold; however, this protein has little or no importance in tolerance to copper and cadmium. The Clp-homologous cDNA clone (*Erd1*) from Arabidopsis was isolated based upon its dehydration-inducible property, suggesting that Clp proteins may have a function in protection from water stress (Kiyosue et al., 1993). Immunological analysis with antibodies against soybean HSP101 detected the corresponding proteins from seedlings incubated in buffer containing heavy metals (arsenite or cadmium) or an amino acid analog (azetidine-2-carboxylic acid) (Y.-R.J. Lee, R.T. Nagao, and J.L. Key, unpublished data). These preliminary data may also indicate a relatedness of synthesis of Clp proteins to several different physical/environmental stress conditions. How these Clp proteins serve to protect cells challenged by various environmental stresses remains to be clarified.

METHODS

Materials

Two-day-old etiolated seedlings of soybean (*Glycine max* cv Williams 82) were grown as described by Lin et al. (1984). Two strains of yeast (*Saccharomyces cerevisiae*), the wild-type W303a and the *HSP104* deletion mutant W303aΔ*hsp104*, were prepared as described by Sanchez and Lindquist (1990). The heat treatments were done in incubation buffer or culture medium equilibrated at the desired temperatures in shaking water baths as previously described for soybean seedlings (Lin et al., 1984) or for yeast cells (Sanchez et al., 1992).

Construction and Screening of a Soybean cDNA Library

Poly(A)$^+$ RNAs were isolated from 2-day-old etiolated soybean seedlings that had been heat treated at 40°C for 1 hr (Schöffl and Key, 1982). High molecular mass poly(A)$^+$ RNAs encoding proteins above 40 kD were enriched as described by Roberts and Key (1991) and used to construct a cDNA library in the phage vector Uni-Zap (Stratagene). The library was screened by plaque hybridization to radiolabled fragments of the yeast *HSP104* gene using standard techniques (Sambrook et al., 1989) and reduced stringency. Filters were prehybridized under aqueous conditions using 6 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate), 0.5% SDS, 5 × Denhardt's solution (1 × Denhardt's solution is 0.02% Ficoll, 0.02% PVP, 0.02% gelatin), and 100 μg/mL salmon sperm DNA at 60°C for 4 hr. ^{32}P-labeled probe was added to fresh hybridization buffer, and filters were hybridized for 24 hr at 60°C. Filters were then washed in 3 × SSC, 0.1% SDS three times, for 15 min each, at room temperature followed by two similar washes at 60°C.

Extension of Truncated cDNA Clones

Primers complementary to sequences deduced from initial cDNA partial clones (Figure 1) were used to synthesize first-strand cDNA by reverse transcriptase Superscript II (Boehringer Mannheim) as recommended by the manufacturer. Poly(A)$^+$ RNAs extracted from soybean seedlings incubated at 40°C for 1 hr were used as templates for the reverse transcription. The second-strand synthesis was performed with *Escherichia coli* DNA polymerase I (New England Biolabs, Beverly, MA), and the double-stranded product was blunt ended with T4 DNA polymerase (New England Biolabs). Blunt-ended cDNAs were ligated into the SurfI site of pCR-Script (Stratagene) and transformed into *E. coli* XL1-Blue cells using standard techniques (Sambrook et al., 1989).

Hybrid Selection and in Vitro Translation

Soybean *Hsp101* cDNA was bound onto nitrocellulose and hybridized to poly(A)$^+$ RNAs extracted from soybean seedlings that had been incubated at 40°C for 1 hr. The filters were washed, and the selected RNAs were eluted as described by Gantt and Key (1985). The RNA was translated in a wheat germ cell–free translation system (Promega), and the translation products were analyzed by two-dimensional electrophoresis (O'Farrell, 1975).

DNA and RNA Gel Blot Analyses

Genomic DNA was prepared from soybean seedlings (Nagao et al., 1981) and digested with restriction enzymes. The restriction fragments were separated by electrophoresis in 0.8% agarose gels for DNA gel blot hybridization. RNAs were separated in 1% agarose gels containing 6% formaldehyde and 10 mM Mops, pH 7.0, for RNA gel blot hybridization. The gels were blotted onto nitrocellulose filters, and hybridization at 42°C was performed using standard techniques (Sambrook et al., 1989). Probes for hybridization were prepared by random primer labeling of cDNA inserts with α-^{32}P-dATP (6000 Ci/mmol; Du Pont–New England Nuclear). All blots were prehybridized for 4 hr in hybridization buffer containing 50% formamide, 5 × SSC, 50 mM sodium phosphate, pH 7.0, 5 × Denhardt's solution, 100 μg/mL salmon sperm DNA, 100 μg/mL yeast tRNA, and 0.1% SDS. ^{32}P-labeled probe was added to fresh hybridization buffer, and blots were hybridized for 24 hr at 42°C. Blots were then washed three times (15 min each) with 2 × SSC, 0.1% SDS at room temperature, two times (15 min each) with 2 × SSC, 0.1% SDS at 60°C, and one time with 0.2 × SSC, 0.1% SDS at 60°C for 15 min.

Sequence Analysis

Nested deletions from each end of the cDNA clones were generated by ExoIII/mung bean nuclease (Stratagene). DNA sequencing was performed on an Applied Biosystems 374A (Foster City, CA) at the University of Georgia Molecular Genetics Instrumentation Facility, Athens, GA. The DNA sequence of soybean *GmHsp101* was submitted to GenBank (accession number L35272). DNA sequences were analyzed with the WUGCG computer program suite, which was developed by the Genetics Computer Group, University of Wisconsin, Madison (Devereux et al., 1984), and accessed through the BioSciences Computational Resource, University of Georgia. Alignment of sequences was done using the PILEUP program of the WUGCG suite with the default parameters (Devereux et al., 1984).

Oligonucleotide-Directed Mutagenesis and Construction of pYSSB101

To construct a plasmid that expressed soybean *Hsp101* under the control of the yeast *HSP104* promoter, the following strategy was applied. Two restriction sites, AflII and SphI, were introduced in front of and behind

the open reading frames of pYS104 (Sanchez and Lindquist, 1990) and *GmHsp101*, respectively, by oligonucleotide-directed mutagenesis (Perlak, 1990; Deng and Nickoloff, 1992). The plasmid pYS104 contains the entire yeast *HSP104* gene. The construct, designated pYSSB101, was obtained by substitution of the AflII-SphI fragment of pYS104 containing the coding region of the yeast *HSP104* gene with the AflII-SphI fragment of *GmHsp101* containing the coding region of the soybean *Hsp101* gene.

Thermotolerance Assay in Yeast Cells

The plasmid pYSSB101 was used to transform the yeast strain W303aΔ*hsp104* by the lithium acetate method (Ausubel et al., 1987). Induction and assay of thermotolerance in yeast cells were done as described by Sanchez and Lindquist (1990). The medium containing 10 g of yeast extract, 20 g of peptone, 20 g of glucose, and 40 mg of adenine per liter is referred to as YPDA medium. Yeast cells were grown at 25°C to mid-log phase (~6 × 10^6 cells per mL) in YPDA liquid medium (wild type and Δ*hsp104*) or in minimal medium (1.7 g of yeast nitrogen base minus amino acids and 20 g of glucose per liter) plus amino acids to maintain transformed plasmids (pYS104 or pYSSB101). Prior to the 50°C heat treatment, cells were incubated at 37°C for 30 min to induce heat shock protein synthesis. Following heat treatment at 50°C, cells were diluted in ice-cold YPDA medium and immediately plated on YPDA plates. Colonies that survived the 50°C treatment were counted 3 days after the treatment.

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