Maize HSP101 Plays Important Roles in Both Induced and Basal Thermotolerance and Primary Root Growth

Jorge Nieto-Sotelo, Luz María Martínez, Georgina Ponce, Gladys I. Cassab, Alejandro Alágón, Robert B. Meeley, Jean-Marcel Ribaut, and Runying Yang

Department of Plant Molecular Biology, Institute of Biotechnology, National Autonomous University of Mexico, 62250 Cuernavaca, Morelos, Mexico
Department of Molecular Recognition and Biostructure, Institute of Biotechnology, National Autonomous University of Mexico, 62250 Cuernavaca, Morelos, Mexico
Trait and Technology Development, Pioneer Hi-Bred International, Inc., Johnston, Iowa 50310
International Maize and Wheat Improvement Center, Lisboa 27, Apdo Postal 6-641, 06600 Mexico City, Distrito Federal, Mexico

HSP101 belongs to the ClpB protein subfamily whose members promote the renaturation of protein aggregates and are essential for the induction of thermotolerance. We found that maize HSP101 accumulated in mature kernels in the absence of heat stress. At optimal temperatures, HSP101 disappeared within the first 3 days after imbibition, although its levels increased in response to heat shock. In embryonic cells, HSP101 concentrated in the nucleus and in some nucleoli. Hsp101 maps near the umc132 and npi280 markers on chromosome 6. Five maize hsp101-m-::Mu1 alleles were isolated. Mutants were null for HSP101 and defective in both induced and basal thermotolerance. Moreover, during the first 3 days after imbibition, primary roots grew faster in the mutants at optimal temperature. Thus, HSP101 is a nucleus-localized protein that, in addition to its role in thermotolerance, negatively influences the growth rate of the primary root. HSP101 is dispensable for proper embryo and whole plant development in the absence of heat stress.

INTRODUCTION

Plants and most other living organisms are able to sense, respond to, and acclimate to mild high temperatures. This property eventually allows them to survive extreme temperature shocks that would be lethal under other circumstances (Yarwood, 1961). In addition to the within-species variation in heat tolerance, heat tolerance can be dramatically different during the course of the life cycle of a given plant. The difference in threshold temperatures for heat injury between the fully hydrated and dry states (i.e., seeds and pollen) can be $\sim$40 to 50°C, the dry state being the most tolerant (for review, see Levitt, 1980).

It is speculated that the observed differences in the levels of basal thermotolerance within the plant life cycle are the result of a developmental and/or a physiological state(s). High temperatures can damage several plant metabolic and physiological processes, such as photosystem II activity (Havaux et al., 1991), pollen and seed development (Cheikh and Jones, 1994), acetylene-reducing activity (Michiels et al., 1994), endoplasmic reticulum integrity (Grindstaff et al., 1996), and leaf growth (Beator et al., 1992).

The first studies of the protein factors involved in the acquisition of thermotolerance dealt with the characterization of a heat-shock protein of 104 kD, Hsp104, in the yeast Saccharomyces cerevisiae and its homolog, ClpB, in Escherichia coli (Sanchez and Lindquist, 1990; Squires et al., 1991). Hsp104 and ClpB belong to a large protein family named Hsp100/Cip (for review, see Schirmer et al., 1996). Its members perform a great variety of functions that range from being regulatory subunits of the bacterial Clp protease, to factors that control the inheritance of prions such as [PSI+] in yeast, to factors required for the tolerance of high salt in bacteria (Schirmer et al., 1996).

This family of proteins is divided into two large classes. Class I subfamilies (A to D) contain two nucleotide binding domains (NBDs) as well as conserved N-terminal, middle, and C-terminal regions. Class II proteins (M, N, X, and Y) are smaller and contain only one NBD. Members of the class I subfamilies are divided by the length of their middle region. The middle region in A, B, and C subfamily members is predicted to form a coiled-coil supersecondary structure (Celerin et al., 1998; Nieto-Sotelo et al., 1999). Both the NBD 1 To whom correspondence should be addressed. E-mail jorge@ibt.unam.mx; fax 52-7-313-9988.

Article, publication date, and citation information can be found at www.plantcell.org/cgi/doi/10.1105/tpc.010487.
HSP101 function relied on the use of *juncea* acid change in growth conditions, low levels of HSP101 could be sufficient function under normal temperatures. Recently, a new development at normal temperatures in studies clearly showed a role for HSP101 in acquired thermotolerance in lines in which the expression of HSP101 was leaky (Hong et al., 1994; Wells et al., 1998; Nieto-Sotelo et al., 1999; Keeler et al., 2000; Campbell et al., 2001). The genetic analysis of heat tolerance has begun to yield interesting results. Five independent mutants (*hot1, hot2, hot3, hot4*, and *AtTSO2*) with defects in the acquisition of thermotolerance have been described in Arabidopsis (Burke et al., 2000; Hong and Vierling, 2000). One of the mutant loci, *hot1*, has been identified as a mutation in the *HSP101* gene (Hong and Vierling, 2000). Thus, as in yeast and *E. coli*, HSP101 plays an important role in heat-induced thermotolerance in plants (Quetsch et al., 2000; Hong and Vierling, 2000).

In addition to the role played in heat stress, plant HSP101 has been proposed to function as a regulator of the translational activity of both *Tobacco mosaic virus* and ferredoxin mRNAs, as shown by in vitro studies and in a yeast heterologous system (Wells et al., 1998; Ling et al., 2000). A role in seed development has been proposed for HSP101 because it accumulates in mature seeds of wheat, maize, *Brassica juncea*, and rice (Singla et al., 1998).

The initial genetic approaches to the study of Arabidopsis HSP101 function relied on the use of *hot1-1* missense alleles or the use of antisense RNA—expressing transgenic lines in which the expression of HSP101 was leaky (Hong and Vierling, 2000; Quetsch et al., 2000). Although these studies clearly showed a role for HSP101 in acquired thermotolerance, no deficiencies were observed in growth and development at normal temperatures in *hsp101* plants.

These reports left open the possibility that, under optimal growth conditions, low levels of HSP101 could be sufficient to fulfill its biological function and/or that the single amino acid change in *hot1-1* was not sufficient to provoke a loss of function under normal temperatures. Recently, a new *hsp101* mutant allele (*hot1-3*), which is null for the HSP101 protein, was described in Arabidopsis (Hong and Vierling, 2001). In addition to its known role in both induced and basal thermotolerance, it was shown that germination and development in *hot1-3* plants grown under optimal conditions follow a normal course.

Here, we characterized the expression of maize HSP101, in mature and dry kernels, during the course of germination and in response to heat shock in young seedlings. We also studied the subcellular localization of HSP101 in germinating kernels. *hsp101* was mapped to chromosome 6, and we discuss its potential functions with regard to quantitative trait loci in maize. We followed a reverse genetics approach to obtain *hsp101* mutants and obtained four null alleles.

As shown recently (Hong and Vierling, 2001), we found that HSP101 is required for both induced thermotolerance and maintenance of the high basal thermostolerant state typical of germinating kernels. Both in the field and in controlled laboratory conditions, HSP101 was not essential for kernel or whole-plant development or for germination at optimal or mild heat-shock temperatures. However, we found that HSP101 expression negatively influenced primary root growth. We discuss this latter finding in terms of maize evolution.

### RESULTS

#### Accumulation of HSP101 in Response to Heat Shock and during Development

To study the expression of HSP101 in response to heat shock and throughout the development of the caryopsis, we raised and purified, by affinity chromatography, an antibody (HSP101-P15C) directed against the C-terminal 15 amino acids of maize HSP101 (see Methods). HSP101-P15C antibody recognized a single protein band of 101 kD in protein blots made with total protein extracts from young seedlings that were heat shocked at 40°C for 1 h (Figure 1A). The intensity of this protein band was very low, or not detectable in tissues from control seedlings kept at 28°C (Figures 1A and 1B).

To study the time course of expression of HSP101 in response to heat shock, 2.5-day-old seedlings were heat shocked at 40°C for up to 8 h. Total protein extracts were prepared from both shoots and primary roots. HSP101 levels increased after 1 h of heat shock, and maximum accumulation occurred after 4 h in both organs (Figure 1B). As in most experiments with 2.5-day-old seedlings grown at 28°C, both the time 0 and 8-h controls showed low levels of HSP101 protein.

To determine if these low levels of HSP101 resulted from the accumulation of the protein during kernel development, we estimated the HSP101 levels in dry and mature embryos during germination and during seedling growth at 28°C. Shoots and primary roots were dissected carefully at each time point. In dry embryos (total extract that includes shoots and roots), HSP101 levels were high and disappeared gradually 3 days after imbibition in shoots and 2 days after imbibition in primary roots (Figure 1C).

Because HSP101 accumulation in the embryo could be attributable to heat-induced expression during embryo development in the field, we grew maize plants in a greenhouse with temperature and humidity controls. The temperature in the greenhouse during the full growth cycle never increased to >30°C. Control of the maximum temperature is...
important because accumulation of small amounts of Hsp101 mRNA is observed at 30°C in plants acclimated at 28°C (Nieto-Sotelo et al., 1999).

Total protein extracts of fully mature and dry embryos were prepared from several pools of five kernels each. A comparison of a dilution series of the total protein, extracted from greenhouse- and field-grown embryos, indicated that under both growth conditions plants accumulated HSP101 to similar levels (Figure 1D). Therefore, HSP101 accumulation in mature embryos is independent of heat-stress induction and most likely is dependent on developmental or dehydration stress–related signals that occur during embryo development and/or maturation.

**Expression of HSP101 in the Embryo of Germinating Kernels**

Because of the high specificity shown by the HSP101-P15C antibody, we performed experiments to detect HSP101 in germinating kernels (after 16 h of imbibition) by indirect immunocytochemistry using an alkaline phosphatase (AP)–coupled secondary antibody and bright-field optics in longitudinal sections of the whole caryopsis. In addition to the full immunochromic reaction, several controls were made to assess the specificity of the signal: without primary antibody, without secondary antibody, and without primary and secondary antibodies.

A very strong signal was observed in the embryo and the aleurone layer in all thin sections made with a complete reaction (Figures 2A to 2C). Signal in all controls was very faint in the embryo but strong in the aleurone (data not shown). Distribution of HSP101 was homogeneous in the whole embryo. Cells of the primary root, coleorhiza, scutellar node, first internode, coleoptile, plumule, stem, leaves, scutellum, and the glandular layer of the scutellum stained intensely (Figure 2A). Because coleoptiles showed a yellow-brown appearance before AP development, we considered the apparent high signal observed in this region to be an artifact. This coloration was attributable perhaps to the presence of pigment precursors that accumulate in the germinating kernel.

At high magnification, it was observed that, in all embryonic cells, HSP101 is found predominantly in the nuclei and at low levels in the cytoplasm (Figures 2B and 2C). To refine these observations, we performed confocal laser microscopy with a double-staining protocol that uses a secondary antibody coupled to Alexa 594 and Sytox green as probes for the detection of HSP101 and DNA, respectively. Controls similar to those used in AP immunocytochemistry were made. Optical sectioning of cells from the embryo revealed that HSP101 levels were highly accumulated inside the nuclei. In some cells, the nucleoli did not stain (Figures 2D to 2G). Moreover, confocal microscopy confirmed the lack of specificity of the HSP101 signal in both the aleurone and the pericarp and its absence in the endosperm.

**Mapping of Hsp101 and Screening of**

**hsp101-m::Mu** **Mutants**

Hsp101 was mapped using two segregating populations, developed at the International Maize and Wheat Improvement
Center for quantitative trait loci (QTL) characterization, that resulted from the cross Ac7643\textasciitilde H11003\textasciitilde Ac7729\textasciitilde TZSRW (Ribaut et al., 1996) and from the cross CML9\textasciitilde H11003\textasciitilde A632Ht (J.M. Ribaut et al., unpublished data). Hsp101 mapped to the long arm of chromosome 6 near markers umc132 and npi280 (Figure 3).

To learn more about the function of HSP101 in maize during kernel development and germination and in response to heat stress, we isolated hsp101 mutants using a reverse genetics approach. The TUSC procedure (Meeley and Briggs, 1995; Chuck et al., 1998) was used to screen maize plants containing insertions of the Mutator (Mu) transposable element within the Hsp101 gene, giving five independent hsp101-m::Mu alleles. The precise site of each Mu insertion and the identity of each resident Mu element were defined (see Methods). Two insertions were found in exon I, two in exon III, and one in intron I (Figures 4A and 4B).

Analysis of the terminal inverted repeat sequences in each insertion indicated that all insertions can be attributed to Mu elements. We refer to these maize lines and their alleles as L1 (hsp101-m1::Mu1), L4 (hsp101-m2::Mu1), L5 (hsp101-m3::Mu1), L7 (hsp101-m4::Mu1), and L10 (hsp101-m5::Mu1). After one backcross to B73 and two consecutive self-pollinations, homozygous hsp101-m::Mu1 individuals and their wild-type siblings were isolated from only four lines (L4, L5, L7, and L10).

We assessed the levels of HSP101 using the HSP101-P15C antibody in both dry embryos and primary roots of both mutant and wild-type seedlings that had been heat shocked at 40°C. As shown in Figures 5A and 5B, homozygous mutants for hsp101-m::Mu1 were completely null for HSP101, because no detectable amounts of HSP101 were found. The expression of HSP70 in embryos, and the heat induction of HSP70, HSP17.8, and HSP17.6, were completely normal in the mutant lines relative to their wild-type siblings.

We found no correlation between germination rate and HSP101 activity (data not shown). To test the phenotype of the mutants under field conditions at mild temperatures (not exceeding 30°C), two locations were chosen for planting (see Methods). No major phenotypic differences between mutant hsp101-m::Mu1 plants and their corresponding wild-type siblings (in L4, L5, L7, and L10) were found in field-grown adult plants. The following features were analyzed: number, color, and width of leaves; number of ears; morphology of the tassel; and fertility (data not shown).

Figure 2. Immunolocalization of HSP101 in Germinating Kernels.

Whole kernels were allowed to imbibe in 0.1 mM CaCl$_2$ at 28°C for 16 h and processed for immunocytochemistry as described in Methods. Tissue sections were stained with HSP101-P15C primary antibody followed by an alkaline phosphatase–conjugated secondary antibody [A] to [C] or by an Alexa 594–conjugated secondary antibody [D] to [G]. Images [A] to [C] were obtained with a bright-field microscope. Images [D] to [F] represent internal optical sections, 1 μm thick, obtained by confocal microscopy.

(A) Whole vertical section of a germinating maize kernel that shows the aleurone layer (a), endosperm (en), coleoptile (co), scutellum (s), scutellar node (sn), primary root (pr), and pericarp (pe). The presence of HSP101 is revealed as a purple precipitate.

(B) Detail of the primary root and coleorhiza (cr). The presence of HSP101 is revealed as a blue precipitate.

(C) Detail of the scutellar node (sn). The presence of HSP101 is revealed as a purple precipitate.

(D) to (G) The same pair of scutellar cells visualized under different conditions.

(E) Combined images in (D) and (F).

(F) Sytox green staining to visualize nucleic acids.

(G) Phase-contrast image of the same pair of scutellar cells.
HSP101 Is Necessary for Acclimation to Extreme Temperatures

To study the effects of the disruption of Hsp101 in maize subjected to heat-stress conditions, 2.5-day-old L4, L7, and L10 seedlings grown at 28°C were used. As an estimate of growth, we measured the increase in length of both primary roots and shoots during the 4 days of recovery at 28°C after the temperature treatments indicated in Figure 6. In the controls, which were kept at a continuous temperature of 28°C for 6.5 days, or after a heat shock at 40°C, shoot growth in the three mutant lines was similar to that of their wild-type siblings (Figures 6A and 6E). On the contrary, primary root growth at 28°C was faster in L10 mutants than in their corresponding wild-type siblings (Figure 6C).

Similar results were obtained with mutant L4 (data not shown). By contrast, primary root growth at 28°C in L7 was similar between the mutant and the wild type (Figure 6G). Four days after a single heat-shock treatment at 40°C for 1 h, the primary roots of L10 mutants grew faster than wild-type roots (Figure 6C). In one experiment, the primary root of L7 mutants showed a growth rate similar to that of the wild type after the 40°C treatment (Figure 6G), but in a second experiment, primary roots of L7 mutants grew faster (data not shown).

Because of differences in heat tolerance between lines, a lethal heat shock of 48°C was given to L7 plants and heat shocks of 50°C were imposed on L4 and L10 plants. Without previous acclimation, all wild-type and mutant seedlings died after a single lethal heat shock for 1 h (Figures 6 and 7). In one experiment, the primary root of L7 mutants showed a growth rate similar to that of the wild type after the 40°C treatment (Figure 6G), but in a second experiment, primary roots of L7 mutants grew faster (data not shown).

Because of differences in heat tolerance between lines, a lethal heat shock of 48°C was given to L7 plants and heat shocks of 50°C were imposed on L4 and L10 plants. Without previous acclimation, all wild-type and mutant seedlings died after a single lethal heat shock for 1 h (Figures 6 and 7). However, when L4, L7, or L10 plants were acclimated at 40°C before the lethal heat shock treatment for 1 h, all of the mutants showed a very small increase in growth, but most of them eventually died. On the contrary, both primary roots and shoots of their wild-type siblings were able to grow after the lethal heat shock, and their viability was close to 100%
The levels of HSP101 were high in dry and mature embryos but diminished gradually during germination and early seedling growth (Figure 1C). We studied the possible role of HSP101 in establishing the known high basal thermotolerance displayed by most plants at these stages of development. As seen in Table 1, at 1 h after imbibition, both mutants and their wild-type siblings showed full basal thermotolerance. At 24 h after imbibition, growth of the mutants was reduced to ~50% relative to that of the wild type. The viability of the primary roots was reduced to 30% and that of shoots was reduced to <50% in the mutants relative to the wild type. At 48 and 72 h after imbibition, both populations were highly sensitive to the lethal heat shock.

At optimal temperature, the mutant seedlings showed a different pattern of primary root growth than their wild-type siblings. Growth of the primary roots of the mutant was faster between 1 h after imbibition and day 7, between day 1 and day 7, and between day 2 and day 7. However, growth was not significantly different than in the wild type in the third time frame (Table 2). Between day 3 and day 7, primary root growth was similar between the wild type and the mutant. These observations suggest that HSP101 has a negative effect on the growth of the primary root during the first 2 days after kernel imbibition. Shoot growth was significantly slower in the mutant seedlings only between day 2 and day 7 but not at other time intervals (Table 2).

To evaluate more precisely the effect of HSP101 on growth at optimal temperatures, we focused our observations on the first 3 days after imbibition, because the protein was present in wild-type seedlings during this period (Figure 1C). Twenty-four hours after imbibition in L10 kernels at 28°C, mutants showed a similar germination rate to the wild type (57% ± 18% and 42% ± 19%, respectively; not significantly different at P = 0.05). Furthermore, the growth of the primary root was not different between wild-type and mutant kernels at 24 h (Table 3).

At 48 h, both mutant and wild-type kernels had reached a 100% germination rate, and the length of the primary root was significantly greater in the mutants. At 72 h, primary root length was still greater in the mutants. Although the value of the mean of shoot length was higher in the mutants, it was not significantly different from that in the wild type at 48 and 72 h. The slower growth of the primary root in the wild type correlates with the presence of HSP101 in this organ at 28°C during this period (Figure 1C). Thus, HSP101 acts as a negative regulator of plant growth under nonlethal temperatures during the first 3 days after imbibition of the kernels. Moreover, HSP101 protects, in addition to other factors, the germinating kernel from the damage caused by lethal heat shocks.
HSP101 Roles in Thermotolerance and Growth

Blanket that HSP101 encompasses more cellular roles than originally thought. At 16 h after imbibition, cells of the maize embryo have entered S-phase (Baiza et al., 1989) and are engaged in respiration and protein and RNA synthesis (Bewley and Black, 1994). HSP101 may serve to protect some of these functions. In any case, the nuclear and cytoplasmic functions of HSP101 become essential only upon severe heat stress and not during optimal growth conditions, as shown in the analyses of growth of the mutants.

Figure 6. Quantitative Thermotolerance Assays of Lines Homozygous for hsp101-m4::Mu1 (L7), hsp101-m5::Mu1 (L10), and Their Corresponding Wild-Type Siblings.

Seedlings (2.5 days old) grown in the dark at 28°C were kept at 28°C (28°C), given a 1-h heat shock at 40°C (40°C), given a 1-h heat shock at 48 or 50°C (50°C), or given a 1-h heat shock at 40°C followed by a 1-h incubation at 28°C and a second heat shock for 1 h at 48 or 50°C (T1). Subsequently, seedlings were incubated in the dark at 28°C for 4 days. Data shown for L7 correspond to one of two independent experiments. Data for L10 are averages of three independent experiments. Both growth and viability were assessed after 4 days of recovery at 28°C after the indicated temperature treatments. Bars show means and SD values of the populations analyzed. Differences in the growth of primary roots at 28°C or after a mild heat shock at 40°C between mutant and wild-type seedlings in L10 (C) were statistically significant according to a two-tailed t test (P < 0.001 and P < 0.05, respectively). Differences in shoot growth of mutant and wild-type seedlings after a 48°C heat shock in L7 (E) also were statistically significant (P < 0.05). Differences in both shoot and root growth in L7 grown at 28°C or after a 1-h heat shock at 40°C were not statistically significant (P < 0.05) (E) and (G)]. Differences in both shoot and root growth between the wild type and mutants after induction of thermotolerance (T1) were statistically significant in lines L10 and L7 (P < 0.001) (A], [C], [E], and [G]). Open bars show data for wild-type seedlings, and hatched bars indicate mutant seedlings.

Figure 7. Photographs of L10 and L7 Seedlings Obtained at the End of a Typical Thermotolerance Assay Similar to the One Described for Figure 6.

(A) and (B) Wild-type and hsp101-m5::Mu1 homozygous individuals, respectively, from L10.
(C) and (D) Wild-type and hsp101-m4::Mu1 homozygous individuals, respectively, from L7.
Temperature treatments indicated at left were the same as those described for Figure 6. Seedlings were photographed with shoots oriented upward and roots oriented downward. T1, thermotolerance; wt, wild type.
Our immunochemical analyses were made in germinating kernels from a commercial harvest incubated at optimal temperature. We cannot exclude the possibility that upon kernel development, high temperatures induced the nuclear localization of HSP101. It remains to be shown if heat shock is required for the nuclear localization of HSP101. Maize HSP101 contains consensus sequences for nuclear localization. One includes a four-residue pattern of basic amino acids (RRRP, at positions 669 to 672). A second one is a bipartite sequence (RKEKERIDEIRKLKQRR, at positions 466 to 482) that is similar to the nucleoplasmin nuclear localization signal (NLS) (Robbins et al., 1991). Both types of sequences are found in maize HSP101 and are conserved in all plant homologs. Recently, in yeast, it was shown that Hsp104 accumulates in the nuclei upon heat shock (Kawai et al., 1999) even though it does not contain a consensus NLS. Further studies are needed to analyze the functional significance of the putative NLS sequences in plant HSP101 proteins.

The mapping of Hsp101 to chromosome 6, combined with information available on QTL associated with stress responses and other biological processes, is useful for the identification of other potential functions of this gene. We found that Hsp101 is closely linked to two genetic markers, umc132 and npi280. The chromosomal region linked to umc132 is involved in determining drought sensitivity and Table 1. Basal Thermotolerance of Line L10 Individuals

<table>
<thead>
<tr>
<th>Beginning of 50°C Shock (h)</th>
<th>Window of Growth (h)</th>
<th>Organ</th>
<th>Wild Type Increase in Length (cm)</th>
<th>Wild Type Viability (%)</th>
<th>Mutant Increase in Length (cm)</th>
<th>Mutant Viability (%)</th>
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<tbody>
<tr>
<td>1</td>
<td>1 to 168</td>
<td>Shoots</td>
<td>6.6 ± 2.1</td>
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<td>6.4 ± 2.8</td>
<td>94</td>
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<td></td>
<td></td>
<td>Roots</td>
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<td>Shoots</td>
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<td>Roots</td>
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Both wild-type and homozygous hsp101-m5::Mu1 kernels (L10) were allowed to imbibe for 1, 24, 48, or 72 h at 28°C in 0.1 mM CaCl$_2$ and heat shocked for 1 h at 50°C. After heat-shock treatment, kernels or seedlings were returned to 28°C for recovery. Increase in length and percent viability after heat-shock treatment of both shoot and roots were estimated until day 7 (168 h) after imbibition. Data for growth are means ± SD.

<table>
<thead>
<tr>
<th>Time Span (h)</th>
<th>Organ</th>
<th>Wild Type Increase in Length (cm)</th>
<th>Wild Type Viability (%)</th>
<th>Mutant Increase in Length (cm)</th>
<th>Mutant Viability (%)</th>
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<tr>
<td>1 to 168</td>
<td>Shoots</td>
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<td></td>
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Both wild-type and homozygous hsp101-m5::Mu1 kernels (L10) were allowed to imbibe for 1, 24, 48, or 72 h at 28°C in 0.1 mM CaCl$_2$. After imbibition, kernels or seedlings were further incubated at 28°C until day 7 (168 h). Growth of both shoot and roots between the end of imbibition and day 7 was estimated as increase in length and percent viability. Data for growth are means ± SD.

$^a$Significantly different according to two-tailed t test (P < 0.005). All other pairs are not significantly different (P = 0.05). Combined data from three experiments.

$^b$Significantly different according to two-tailed t test (P < 0.002). All other pairs are not significantly different (P = 0.05). Combined data from three experiments.

$^c$Significantly different according to two-tailed t test (P < 0.001). All other pairs are not significantly different (P = 0.05). Combined data from three experiments.
both leaf and xylem abscisic acid concentration (Lebreton et al., 1995; Tuberosa et al., 1998; Sanguineti et al., 1999). The chromosomal region linked to npi280 is involved in grain yield, ear length, kernel depth, plant height, and growth degree-days to 50% anthesis (Veldboom and Lee, 1996; Austin et al., 2001).

Under water-limited conditions imposed before and during flowering time, a QTL for plant and ear height has been identified in two different crosses, and for both QTLs the closest marker to the QTL peak was umc132 (J.-M. Ribaut, personal communication). Because of the observed significant difference in the growth of the primary roots of young seedlings in hsp101-m::Mu1 mutants and the involvement of a region of the maize genome near the Hsp101 gene in plant height determination (Austin et al., 2001; J.-M. Ribaut, personal communication), a comparison of growth between the wild type and hsp101-m::Mu1 mutants should be made under different stress conditions (i.e., high temperatures, water deficit, etc.) and at different stages of the maize life cycle.

To evaluate the biological function of HSP101 during plant development under optimal and heat-stress conditions, we obtained hsp101 mutants using a reverse genetics approach. The absence of HSP101 did not reduce their germination rate or their further growth and development at optimal temperatures. Thus, HSP101 activity is not important for seed development in the absence of heat stress.

We have two alternative hypothesis to explain these observations. (1) The presence of HSP101 and its accumulation during seed desiccation could play a preventive role if very high temperatures are encountered during embryogenesis, germination, or early seedling growth. (2) Alternatively, HSP101 could play active roles in these processes, but a redundant gene function (i.e., other heat-shock proteins, late-embryogenesis-abundant proteins, etc.) might complement the absence of HSP101 during normal growth and development in the mutants. Thus, HSP101 is not a limiting factor for embryogenesis or for the protection of the kernel from desiccation stress.

The decrease in basal thermotolerance in L10 mutant kernels at 24 h after imbibition, at which time substantial amounts of HSP101 are present in both shoots and primary roots, underscores the preventive role of HSP101 during germination. We suggest that other factors must determine the level of basal thermotolerance in addition to HSP101, because basal thermotolerance was not 0% in the mutants at 24 h after imbibition. In addition to other heat-shock proteins, these other factors could be encoded by maize homologs of HOT2, HOT3, HOT4, and AtTSO2, which are known to be involved in induced thermotolerance (Burke et al., 2000; Hong and Vierling, 2000). Further work remains to be done to test this hypothesis.

To date, we have studied the phenotypes of hsp101-m::Mu1 mutants grown in the field under very mild temperatures (i.e., maximal temperature in the field seldom exceeded 30°C during the growth season). It is of primary importance to test the performance of the mutants in natural or artificial conditions in which the daily maximum temperature is ~40°C. Under these conditions, a strong expression of HSP101 in wild-type plants is expected; thus, the real impact of HSP101 on growth and other physiological processes could be tested by performing comparative studies with the hsp101 mutants. hsp101 null mutants also can be used to test the role of HSP101 in response to other abiotic (heavy metals, salt, ethanol, cold, etc.) or biotic (viral, bacterial, or fungal pathogens) stress conditions.

Previous studies have shown that the reduction of expression of HSP101 by antisense RNA expression caused a decrease in induced thermotolerance (Queitsch et al., 2000). Moreover, the analysis of thermotolerant mutants has identified HSP101 as a factor necessary for the establishment of the induced thermotolerant state (Hong and Vierling, 2000). A recent study in Arabidopsis, which analyzed hot1-3 null mutants for HSP101, showed that HSP101 is dispensable for development, germination, and growth when cultivated under optimal conditions but is required for both induced and basal thermotolerance (Hong and Vierling, 2001).

Our work validates these findings in an important crop plant and extends some of these observations to field conditions. Nonetheless, in Arabidopsis hot1-3 mutants, hypocotyl elongation was similar to that in the wild type (Hong and Vierling, 2001). Because the growth of the primary root was not examined, a comparison with our observations cannot be made.

The growth of the primary root at 28°C was significantly faster in the mutant lines L4, L7, and L10 during the first 3 days after imbibition. It is too early to speculate on how maize HSP101 affects the growth of the primary root and shoot at optimal and mild heat-shock temperatures (40°C). At 28°C, no difference in the germination rate of the kernels was observed after 24 h between the mutant and the wild type. Similarly, no difference in the elongation of the radicle

### Table 3. Growth of L10 Seedlings at 28°C during the First 3 Days after Imbibition

<table>
<thead>
<tr>
<th>Time Span (h)</th>
<th>Shoots</th>
<th>Primary Roots</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wild Type</td>
<td>Mutant</td>
</tr>
<tr>
<td>0 to 24</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0 to 48</td>
<td>0.57 ± 0.36</td>
<td>0.68 ± 0.36</td>
</tr>
<tr>
<td>0 to 72</td>
<td>1.63 ± 0.74</td>
<td>1.89 ± 0.74</td>
</tr>
</tbody>
</table>

Total length (in cm), achieved by primary roots or shoots of L10 seedlings after kernel imbibition for 24, 48, or 72 h at 28°C. Data are means ± SD.

*Significantly different according to two-tailed t test (P < 0.005). All other pairs are not significantly different (P = 0.05). Combined data from three experiments.

**Significantly different according to two-tailed t test (P < 0.001). All other pairs are not significantly different (P = 0.05). Combined data from three experiments.
was noticed at 24 h. The negative effect on growth was more pronounced in the primary roots than in the shoots during seedling growth at 24 to 72 h after imbibition. This effect could represent an adaptation to ensure a proper balance between growth (i.e., cell division and/or elongation) and the maintenance of a high heat-tolerant state during the first days of seedling growth.

Maize was domesticated from teosinte in the Balsas region of the Mexican tropical zone (Wang et al., 1999) and evolved in areas with a higher probability of encountering increased temperatures at the beginning of the growth cycle. The negative regulation of growth by HSP101 during highly stress-sensitive processes such as cell division could help to “scout the weather” before a faster growth rate is established. Maize seedlings increase their growth rate substantially at 72 h after imbibition (Deltour et al., 1989). The increase in growth rate coincides with the disappearance of HSP101 in wild-type primary roots (data not shown), further supporting the idea that HSP101 plays a role in the negative regulation of primary root growth. Further studies are needed to determine if HSP101 acts negatively upon the growth of the primary root at the cell elongation and/or cell division level(s).

METHODS

Plant Material, Seed Germination, and Seedling Growth

In all experiments dealing with the analysis of HSP101 expression in wild-type maize (Zea mays) by immunoblotting or by immunocytochemistry, kernels from a commercial hybrid were used (Merit N’ yellow su1; Asgrow Seed Company, Kalamazoo, MI). Maize lines used for the mapping of Hsp101 in the maize chromosomes were obtained from the International Maize and Wheat Improvement Center and are described in Results. Maize lines containing hsp101-m-:: Mu1 alleles are part of the Pioneer Hi-Bred International TUSC collection and are described in Results. For growth in the laboratory, maize kernels were surface-sterilized in 7% Clorox and rinsed in sterile water several times.

Kernel germination and seedling growth were performed under aseptic conditions on enamel trays containing paper towels saturated with 0.1 mM CaCl₂ and wrapped in aluminum foil. Kernels were incubated in the dark at 28°C in growth chambers. Growth of hsp101-m-::Mu1 lines was conducted in Mexico at two experimental stations from Híbridos Pioneer de México: for the spring-summer cycle, lines were grown at Tlajomulco, Jalisco; for the autumn-winter cycle, they were grown at Tapachula, Nayarit. These locations were chosen to avoid heat stress during the full growth cycle. Maximum temperatures during the indicated growth cycles seldom reached >30°C.

HSP101-P15C Antibody Generation and Purification

To obtain specific antibodies against HSP101, a peptide (D645) with sequence NH₂-CKMRIMEEDEDGMDEE-COOH was synthesized by Biosynthesis (Lewisville, TX). D645 is 16 amino acids in length corresponding to a 15–amino-acid segment from the C-terminal end of the published maize HSP101 sequence (residues 898 to 912) (Nieto-Sotelo et al., 1999) and an extra cysteinyl residue added to the N-terminal end for coupling purposes. A D645-BSA conjugate was prepared via intermolecular disulfide bridge formation as described in Cumber et al. (1985). The conjugate, containing 4.5 mol of D645 per mol of BSA, was used to immunize two New Zealand White rabbits. To purify specific antibodies from total sera, we passed 3-mL aliquots of immune sera through a thiopropyl-Sepharose 6B (Pharmacia) affinity column (1.25 mL) coupled to D645 (330 nmol) according to Alagon and King (1980).

Protein Extraction and Immunoblotting

To prepare total protein extracts, dissected tissues were ground under liquid nitrogen and resuspended in 2 × Laemmli buffer (4% SDS, 20% glycerol, 10% β-mercaptoethanol, and 2 M Tris-HCl, pH 6.8) (Laemmli, 1970). Samples were boiled immediately at 95°C with shaking at 700 rpm for 10 min in a Thermomixer (Eppendorf). The extracts were spun twice at 14,000 rpm for 5 min and twice for 30 min at room temperature in an Eppendorf microcentrifuge. Supernatants were stored at −20°C until further use.

Protein quantitation was performed by a modified Lowry procedure (Schleif and Wensink, 1981). Separation of proteins was performed by SDS-PAGE on 10% polyacrylamide gels for the detection of HSP101 and HSP70 or on 15% polyacrylamide gels to detect HSP17.8 and HSP17.6. Each lane was loaded with 20 μg of total protein. After electrophoresis, proteins were transferred to Hybond-C membrane (Amersham) and processed according to standard protocols (Gallagher et al., 1993).

Detection of HSP101 was performed with HSP101-P15C primary antibody at a dilution of 1:250. To detect HSP70, an anti-HSP70 antibody at a dilution of 1:1000 was used. For HSP17.8 and HSP17.6
Detection, primary antibodies at a dilution of 1:500 were used. A secondary goat anti-rabbit IgG antibody conjugated with alkaline phosphatase (AP) was used at a dilution of 1:6000. Detection of AP activity was made in a solution containing 5% nitroblue tetrazolium and 5% 5-bromo-4-chloro-3-indolyl phosphate in buffer A (0.1 M NaCl, 0.1 M Tris, pH 9.5, and 5 mM MgCl$_2$).

Immunocytochemistry

Kernels were allowed to imbibite for 16 h in distilled water at 28°C in the dark and then fixed in 0.5% glutaraldehyde and 2.5% paraformaldehyde in buffer A (10 mM phosphate buffer, pH 7) overnight at 4°C. Kernels then were dehydrated in a graded ethanol/H$_2$O series followed by an ethanol/xylene series and a xylene/paraffin series. Sections were mounted for microscopic observation. Controls without primary antibody, without secondary antibody, and without both primary and secondary antibodies were analyzed.

To perform immunocytochemistry by means of confocal microscopy, a goat anti-rabbit IgG (H+L) antibody conjugated to Alexa 594 (Molecular Probes, Eugene, OR) was used as a secondary antibody. Confocal microscopy observations were made with a Bio-Rad MRC600 system coupled to a Carl Zeiss Axioskop microscope (Jena, Germany). For nucleus localization, immunostained sections were treated further with RNase A to remove RNA and then stained with Sytox green (Molecular Probes) at a final concentration of 10 nM. Controls without primary antibody, without secondary antibody, and without both primary and secondary antibodies were analyzed.

Candidate Gene Mapping

To identify the position of Hsp101 in the maize molecular map, five linkage maps constructed at the International Maize and Wheat Improvement Center were selected based on the quality of the map and the remaining amount of DNA available. A DNA fragment containing the Hsp101 open reading frame was labeled with digoxigenin-dUTP by PCR and hybridized to a new set of small membranes containing DNA from the nine parental lines of the five crosses (one parental line was common to two crosses) prepared as described above. Because only two bands of ~6 and 6 kb presented the highest signal intensity, and therefore the highest sequence identity, three of the five populations tested presented a clear polymorphism, and Hsp101 was mapped in two of them.

For the crosses presenting a polymorphism, the Hsp101 DNA probe was hybridized on a medium membrane with DNA from 56 genotypes from the same cross. The allelic segregation of those 56 genotypes was incorporated in the linkage map constructed previously using the “append F2” option of Mapmaker. The new locus was incorporated into the existing map based on the recombination frequency among loci of the 56 genotypes. Because the two maps derived for chromosome 6 have few restriction fragment length polymorphism markers in common, published information from “reference” maize maps was used to locate the umc38 and umc132 markers on the map derived from the CML9 x A632Ht cross. This procedure was simple to perform, because only a few centimorgan separate umc38 from umc138 and umc132 from umc36 (Anonymous, 1998).

Screening of hsp101-m::Mu Maize Lines

Approximately 40,000 F1 plants from an inbred x Mu cross were used. DNA pools from this population were screened using the TUSC procedure from Pioneer Hi-Bred International (Meeley and Briggs, 1995; Chuck et al., 1998). Pool screening was performed with Hsp101-specific primers 27,236 (5'-AGAGACCGCTGATCCGGCAACAATCTAC-3'), 27,237 (5'-TTGAGCGCTTTGGAAGTTC-3'), 27,238 (5'-AGGCCGAGGGAGGATTTGTC-3'), 27,239 (5'-GGACTGAGGACCGAGCAAGTC-3'), and 27,240 (5'-GAGTGGCTCGGAACCTCGAT-3'). Each in combination with the Mutator terminal inverted repeat-specific primer 9242 (5'-AGAGAAGGCAATCGAATCGTCT-3'). To confirm the identification of the products with Hsp101, PCR products were analyzed by DNA gel blot hybridization. Products were hybridized to the Hsp101 cDNA insert from clone pJN31 (Nieto-Sotelo et al., 1999). This primary screen identified 10 lines with putative Mu insertions in Hsp101.

F2 plants from the same lines were rescreened to confirm insertions. Pools of DNA from five kernels from each line were used as templates in the PCR. Only five F2 lines remains positive. Fine mapping of the five heritable Mu insertions was accomplished by sequencing each hsp101-m::Mu PCR product. Each sequence also was trimmed down to the 39 bp of unique TIR sequence flanking Mu
primer 9242, and BLAST searches (Altschul et al., 1997) were performed against a database created from all published Mu element sequences. BLAST results were interpreted to establish a proper Mu element identity for each mutation.

To obtain homozygous lines for each mutant allele, kernels from each of the five F2 lines were grown in the field for self-pollination. Positive lines were backcrossed once to B73. The resulting plants were self-pollinated twice in additional cycles. Screening of both heterozygous and homozygous mutant or wild-type lines was performed by PCR using some of the oligonucleotide combinations mentioned above in addition to primers P6 (5’-TTGAGCAGGTAT-TCCA-3’), P17 (5’-CGGCACCTGCTGACAAAGCCATAGAC-3’), and P18 (5’-CCGCACTTITTAATTCTGTCATGCO-3’). For each line, siblings wild type for HSP101 were maintained as controls.

Accession Number
The GenBank accession number for maize Hsp101 is AF077337.

ACKNOWLEDGMENTS
We thank José Carlos Rosas for performing the preliminary experiments to study Hsp101 expression. Paul Gaytán and Eugenio López helped with the synthesis of oligonucleotides, and Rene Hernández and Maricela Olvera helped with the sequencing of all DNA samples. We thank Yoloxochitl Guevara for kindly helping us with Adobe Photoshop. María Jesús Sánchez offered technical assistance, and Xochitl Alvarado kindly helped with confocal microscopy analyses. We thank Roberto Stock for providing us with Sytox green stain and Elizabeth Vierling for providing us with anti-HSP70, anti-HSP17.8, and anti-HSP17.6 antibodies. Heriberto Torres, Baltazar Baltazar, José de Jesús Figueroa, Roberto Camberos Aguas, and Benjamin Ramos from Híbridos Pioneer de México gave field support for the growth of all maize mutant lines. Oscar Rodríguez from Centro de Investigación sobre Fijación del Nitrógeno of the Universidad Nacional Autónoma de México offered greenhouse space to grow maize under temperature control. We thank Joseph Dubrovsky for his valuable comments on the manuscript. This project was made possible, in part, through the use of technology developed by Pioneer Hi-Bred International. R.Y. was the recipient of a postdoctoral fellowship from UNAM. This work received full financial support from grants DGAPA-UNAM IN202795, DGAPA-UNAM IN209599, CONACYT 3461-N9310, and CONACYT 25303-N to J.N.-S. and grant DGAPA-UNAM IN-208999 to G.I.C.

Received November 7, 2001; accepted April 25, 2002.

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HSP101 Roles in Thermotolerance and Growth


Maize HSP101 Plays Important Roles in Both Induced and Basal Thermotolerance and Primary Root Growth

Jorge Nieto-Sotelo, Luz María Martínez, Georgina Ponce, Gladys I. Cassab, Alejandro Alagón, Robert B. Meeley, Jean-Marcel Ribaut and Runying Yang

PLANT CELL 2002;14;1621-1633
DOI: 10.1105/tpc.010487

This information is current as of July 11, 2010

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