Genetic Analysis Reveals Domain Interactions of Arabidopsis Hsp100/ClpB and Cooperation with the Small Heat Shock Protein Chaperone System [W]

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We have defined amino acids important for function of the *Arabidopsis thaliana* Hsp100/ClpB chaperone (*At*Hsp101) in acquired thermotolerance by isolating recessive, loss-of-function mutations and a novel semidominant, gain-of-function allele [*hot1-4* (A499T)]. The *hot1-4* allele is unusual in that it not only fails to develop thermotolerance to 45°C after acclimation at 38°C, but also is sensitive to 38°C, which is a permissive temperature for wild-type and loss-of-function mutants. *hot1-4* lies between nucleotide binding domain 1 (NBD1) and NBD2 in a coiled-coil domain that is characteristic of the Hsp100/ClpB proteins. We then isolated two classes of intragenic suppressor mutations of *hot1-4*: loss-of-function mutations (Class 1) that eliminated the 38°C sensitivity, but did not restore thermotolerance function to *hot1-4*, and Class 2 suppressors that restored acquired thermotolerance function to *hot1-4*. Location of the *hot1-4* Class 2 suppressors supports a functional link between the coiled-coil domain and both NBD1 and the axial channel of the Hsp100/ClpB hexamer. In addition, the strongest Class 2 suppressors restored solubility of aggregated small heat shock proteins (sHsps) after heat stress, revealing genetic interaction of the Hsp100/ClpB and sHsp chaperone systems. These results also demonstrate that quantitative phenotypes can be used for in vivo genetic dissection of protein mechanism in Arabidopsis.

INTRODUCTION

Hsp100/ClpB chaperones are hexameric members of the AAA+ family of proteins, which are ATPases that couple ATP binding and hydrolysis to a variety of protein-remodeling activities (Neuwald et al., 1999; Vale, 2000; Ogura and Wilkinson, 2001; Lupas and Martin, 2002). Hsp100/ClpB proteins have been shown to be essential for the development of thermotolerance to high temperature in bacteria (Goloubinoff et al., 1999), yeast (Glover and Lindquist, 1998), some parasitic protozoa, and higher plants (Hong and Vierling, 2000, 2001), which are the only higher eukaryotes known to express the Hsp100/ClpB subgroup of chaperones. The demonstration that the highly heat-induced Hsp100/ClpB protein in *Arabidopsis thaliana*, *At*Hsp101, was essential for acquisition of tolerance to high temperature also represented a direct link of a heat shock protein to heat tolerance in plants. Both in vivo and in vitro evidence indicates that the protective function of these chaperones is a result of their ability to resolubilize protein aggregates in cooperation with the Hsp70/DnaK chaperone system (Goloubinoff

et al., 1999; Zolkiewiski, 1999). It remains unknown, however, whether general removal of aggregated proteins or rescue of certain critical substrates defines the essential activity of Hsp100/ClpB in thermotolerance.

This unique subgroup of ATPases contains two AAA modules, each comprised of a nucleotide binding domain (NBD1 or 2) with conserved Walker A, Walker B, and sensor motifs and a smaller C-terminal domain. The AAA modules are flanked by additional N- and C-terminal domains and are separated by a large coiled-coil domain, which is actually an insertion into the C-terminal small domain of AAA module 1 (see Figure 1A). The length of the coiled-coil domain is a major feature that distinguishes Hsp100/ClpB proteins from other Clp proteins with two AAA modules (e.g., ClpA) (Schirmer et al., 1996; Celerin et al., 1998; Nieto-Sotelo et al., 1999). The recent 3.0-Å structure of a complete *Thermus thermophilus* ClpB subunit, and a cryoelectron microscopy reconstruction of the hexamer (21-Å resolution), has defined the structure of the different ClpB domains and how they are oriented relative to each other (Lee et al., 2003). In each AAA module, NBD1 and 2 have a RecA-like mononucleotide binding fold, and the C-terminal smaller domain has a mixed α/β structure. The two AAA modules are stacked on each other within a subunit such that the hexamer appears as two rings of six ATPase sites, surrounding a pore or axial channel on the order of ∼16 Å in diameter. The coiled-coil domain, which is inserted into the smaller domain of NBD1, is 85-Å long and resembles a two-bladed propeller ringing the outside of the hexamer. Based on the observation of three different conformations in the crystal structure, the N-terminal domain and coiled-coil domain are
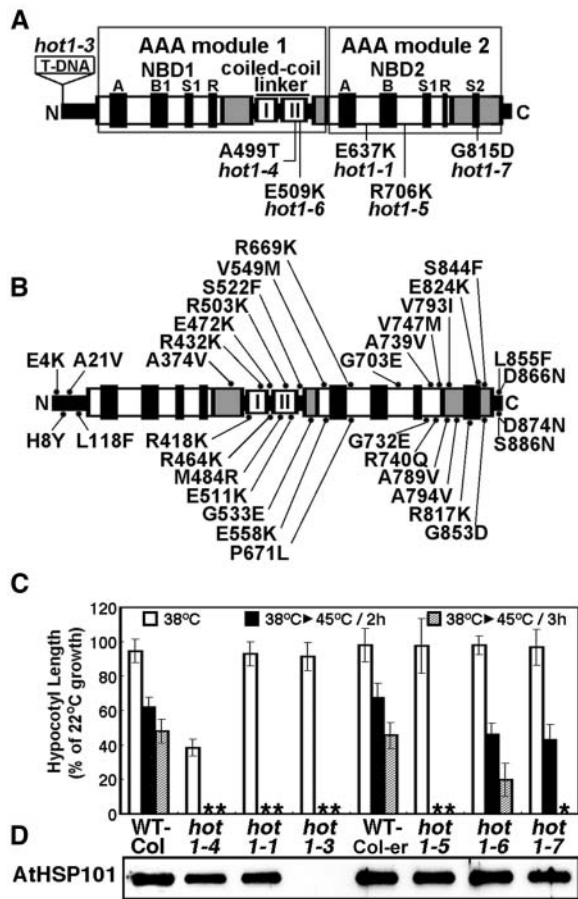
Figure 1. Location and Phenotype of Mutations in *At*Hsp101 (See Also Supplemental Figure 1 Online).

(A) Location of *hot1* mutations with a thermotolerance phenotype on a schematic diagram of the *At*Hsp101 protein. The conserved AAA modules consist of two domains, a nucleotide binding domain (NBD1 or 2), containing conserved motifs [Walker A, Walker B, Sensor 1(S1), and Arg finger (R)], and a C-terminal small domain (gray boxes), containing Sensor 2 (S2) in AAA2. The coiled-coil domain contains two signature motifs (Lee et al., 2003), with *hot1-4* located in signature motif II.
(B) Location of missense mutations that are wild type for thermotolerance, which were obtained by Tilling analysis (see Methods).
(C) Quantitative assessment of thermotolerance in *hot1* mutant seedlings compared with their corresponding wild type (Columbia for *hot1-1* and *hot1-4*, Columbia *erecta* for all others). After growth for 2.5 d in the dark at 22°C, seedlings were pretreated at 38°C for 90 min and returned to 22°C for 2.5 d (38°C samples), or pretreatment was followed by 2 h at 22°C then 2 or 3 h at 45°C before 2.5 d of recovery (38°C > 45°C samples). *hot1-4* seedlings are more sensitive to 38°C treatment than wild-type or null alleles. Asterisks indicate values equal to zero. Mean and standard deviations were derived by measurement from three independent experiments performed with 60 or more seedlings per mutant and values plotted as a percentage of the 22°C value.
(D) Protein gel blot analysis of *At*Hsp101 protein levels in wild-type and *hot1* seedlings after treatment at 38°C.

proposed to be mobile. This is consistent with the fact that deletion of either domain does not disrupt the hexamer (Mogk et al., 2003b).

Biochemical studies using both in vitro assays for ATP hydrolysis and protein disaggregation (chaperone activity), along with in vivo assays for thermotolerance, have also begun to dissect functional domains of Hsp100/ClpB (Smith et al., 1999; Barnett et al., 2000; Clarke and Eriksson, 2000; Schirmer et al., 2001; Beinker et al., 2002; Cashikar et al., 2002; Mogk et al., 2003b; Strub et al., 2003). However, details of the Hsp100/ClpB reaction cycle are unknown. The ATPase activity of both NBDs, as well as an intact coiled-coil domain, are essential for chaperone activity and in vivo thermotolerance (Kim et al., 2000a; Schirmer et al., 2001; Hattendorf and Lindquist, 2002b; Mogk et al., 2003b). A majority of data indicate that deletion of the N-terminal domain does not impair measured activities, and its function remains unknown (Clarke and Eriksson, 2000; Beinker et al., 2002). The C-terminal domain is required for activity, but because deletion of this domain prevents hexamerization, which is required for ATPase activity, its direct role is not revealed by such experiments. Studies with model substrates and effectors of the ATPase activity have suggested that the C-terminal domain is a substrate binding site (Smith et al., 1999), but others conclude this domain modulates ATPase activity only (Strub et al., 2003). The N-terminal domain, NBD1, and the coiled-coil domain have all also been proposed to be involved in substrate interactions (Dougan et al., 2002; Liu et al., 2002; Weibezahn et al., 2003). However, the only data on binding of a natural substrate (TrfA to *Escherichia coli* ClpB) reveals an interaction only with the surface of NBD1 near the axial pore (Schlieker et al., 2004). Lindquist and coworkers (Cashikar et al., 2002; Hattendorf and Lindquist, 2002a) proposed that in yeast Hsp104, stimulation of NBD2 activity drives a conformational change in the coiled-coil domain, which in turn stimulates ATPase activity in NBD1. Cross-linking studies with ClpB further suggest that movement of the coiled-coil domain is necessary for chaperone activity in vitro (Lee et al., 2003). How these motions are connected to protein disaggregation remains to be defined. It has been suggested that ClpB has a crowbar action in protein disaggregation, perhaps mediated by the coiled-coil domain (Vale, 2000; Maurizi and Xia, 2004; Weibezahn et al., 2004). Alternatively, or in addition, substrate unfolding may be coupled to threading through the axial channel of the ClpB hexamer in a mechanism related to that used by ClpA (Ishikawa et al., 2001) and ClpX (Kim et al., 2000b) for protein unfolding and transfer to the ClpP protease. Recent experiments indicate mutations that affect protein loops in the axial channel eliminate yeast Hsp104 function in protein disaggregation, perhaps because of disruption of protein threading through the channel (Lum et al., 2004).

We have taken a genetic approach to investigating the function and mechanism of Hsp100/ClpB in Arabidopsis. In previous work we showed that *At*Hsp101, encoded by the *HOT1* gene, is a major component controlling acquired thermotolerance, based on the phenotypes of missense and protein null alleles, *hot1-1* (E637K; in NBD2) and *hot1-3* (a T-DNA insertion in exon 1), respectively (Hong and Vierling, 2000, 2001). Loss of *At*Hsp101 function, however, does not appear to have major effects on plant growth and development under optimal temperature

conditions. To dissect further the mechanism of Hsp101 action in thermotolerance, we sought additional mutant alleles either by direct screening for mutants in a seedling thermotolerance assay (Hong and Vierling, 2000) or by analysis of point mutations in *At*Hsp101 obtained through the Arabidopsis Tilling Resource (http://tilling.fhcrc.org:9366). We describe four new mutant alleles of Hsp101 that provide insight into structure and function of this class of chaperone ATPase. One mutant allele, *hot1-4*, which is a missense mutation in the coiled-coil domain (A499T) provides direct in vivo evidence for an essential function of this unique domain. Using this allele, we then screened for suppressor mutations and defined 13 intragenic suppressors. The location of these suppressor mutations defines features essential for in vivo activity of Hsp101 and provides in vivo evidence for a novel mechanistic link between the coiled-coil domain and the axial channel of Hsp100/ClpB proteins. Furthermore, certain suppressors restore the solubility of small heat shock proteins (sHsps) after heat stress in the *hot1-4* mutant, providing genetic evidence for interaction of the Hsp100/ClpB and sHsp chaperone systems in higher plants.

RESULTS

*At*Hsp101 Mutants with Defects in Thermotolerance

Arabidopsis seedlings will continue to grow after a 45°C, 2-h heat treatment if first preconditioned at 38°C for 90 min followed by 1 to 2 h at 22°C. This ability to acclimate to 45°C is lost in the previously characterized missense (*hot1-1*; E637K) and protein null alleles of *At*Hsp101 (*hot1-3*; T-DNA insertion in exon 1) (Hong and Vierling, 2000, 2001). We used this assay to screen for additional *At*Hsp101 mutants in a population of mutagenized Arabidopsis seedlings and also to assay identified point mutations in *At*Hsp101 from the Arabidopsis Tilling Resource (http://tilling.fhcrc.org:9366) for loss of thermotolerance. From our screen for additional thermotolerance mutants (Hong et al., 2003), only one other mutant was recovered that failed to complement *hot1-1* in allelism tests and that mapped to the Hsp101 chromosomal region (data not shown). Sequencing of the *At*Hsp101 gene from this mutant identified a single missense mutation resulting in an exchange of Thr for Ala-499 in the coiled-coil domain (Figure 1A; see Supplemental Figure 1 online). Analysis of the AtHsp101 tilling mutants uncovered three additional *hot1* alleles that were defective in thermotolerance, R706K (*hot1-5*), E509K (*hot1-6*), and R815D (*hot1-7*), all of which lie in AAA module 2 (Figure 1A). The locations of an additional 35 missense mutations obtained by tilling showed no defect in thermotolerance and are indicated in Figure 1B.

The relative severity of each of the newly identified defective alleles (*hot1-4* to *hot1-7*) was compared with the wild type and the functionally null *hot1-1* allele (Hong and Vierling, 2001) in a quantitative assay for seedling thermotolerance (Figure 1C). The *hot1-4* and *hot1-5* alleles are the most severe, showing no ability to acclimate to treatment at 45°C for 2 h, whereas the *hot1-6* and *hot1-7* alleles have a less severe phenotype, with *hot1-6* showing the mildest phenotype. Differences in severity are not because of differences in protein levels as all the alleles accumulate *At*Hsp101 protein to wild-type levels (Figure 1D). There-

fore, we conclude that relative thermotolerance of the alleles reflects the degree to which each mutation disrupts *At*Hsp101 function in vivo and defines critical *At*Hsp101 residues in the coiled-coil domain and NBD2.

hot1-4 Is a Semidominant, Gain-of-Function *At*Hsp101 Allele

We noted that in contrast with all the other *hot1* alleles, *hot1-4* was not only defective in acquired thermotolerance to 45°C treatment, but also showed sensitivity to the 38°C acclimation pretreatment (Figure 1C), which is a permissive treatment for the other *hot1* alleles. To determine the extent of this sensitivity, elongation of *hot1-4* seedlings was compared with the wild type, protein null (*hot1-3*), and the missense *hot1-1* alleles after increasing time at 38°C. The *hot1-4* mutant displays dramatic sensitivity to 38°C treatment compared with the wild type and the other loss-of-function alleles (Figure 2). The fact that the phenotype of *hot1-4* at 38°C is worse than the absence of the protein (*hot1-3* phenotype, Figure 1D) defines *hot1-4* as a gain-of-function mutation and indicates that the *hot1-4* protein interferes with essential cell functions at 38°C. Furthermore, whereas the *hot1-5*, *hot1-6*, and *hot1-7* as well as the previously isolated *At*Hsp101 alleles are recessive, loss-of function mutants, the *hot1-4* phenotype proved to be semidominant (Table 1). Compared with wild-type and *hot1-4* homozygous seedlings, heterozygous *hot1-4* plants showed an intermediate hypocotyl length after a 45°C heat treatment with preconditioning. Despite the sensitivity of *hot1-4* to mild heat treatment, the *hot1-4* mutation had no obvious effect on growth and development in the absence of stress (data not shown).

To confirm that the *hot1-4* mutant protein causes the observed heat-sensitivity phenotypes, we cloned a *hot1-4* mutant genomic fragment, including its own 1.5-kb promoter region, and transformed wild-type Arabidopsis plants with the mutated gene. As
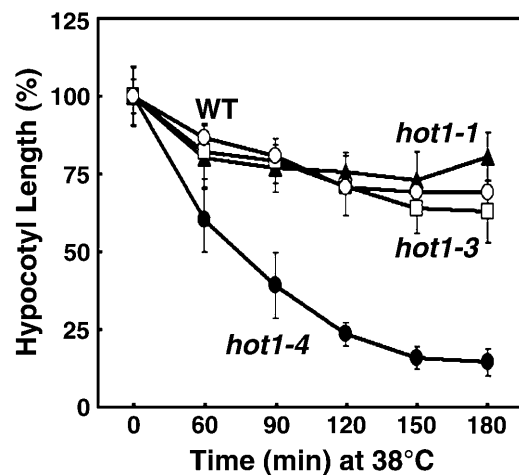

Figure 2. Unusual Sensitivity of *hot1-4* to 38°C.

Hypocotyl elongation of *hot1* mutant seedlings treated for different times at 38°C, as measured after an additional 2.5 d of growth in the dark at 22°C. Replicated measurements performed as in Figure 1C.

Table 1. Genetic Analysis of *hot1* Mutants

Cross	Generation	Total	WT	Semi-dominant	*hot*	χ^2	P
hot1-1	F1	23	23	0	0		
× WT	F2[a]	159	127	0	32	1.58	>0.1
hot1-3	F1	31	31	0	0		
× WT	F2	252	187	0	65	0.08	>0.5
hot1-4	F1	30	0	30	0		
× WT	F2	268	64	134	70	0.26	>0.5
hot1-5	F1	7	7	0	0		
× WT	F2	42	33	0	9	0.28	>0.5
hot1-6	F1	8	8	0	0		
× WT	F2	94	72	0	22	0.12	>0.5
hot1-7	F1	4	4	0	0		
× WT	F2	48	38	0	10	0.44	>0.5

The phenotypic scoring was based on the hypocotyl elongation phenotypes of the parental lines after 45°C for 2 h with pretreatment (38°C for 90 min). WT, wild type.

[a] The hypocotyl length ranges of the phenotypic classes of F2 progenies were 6.5 to 8 mm for the wild-type phenotype, 2.0 to 4.0 mm for the semidominant phenotype, and 0 mm for *hot1-4* mutant phenotype after heat stress.

shown in Figure 3, the phenotype of *hot1-4* can be recapitulated by expression of the *hot1-4* mutant protein in wild-type plants. Protein gel blot analysis showed that levels of HSP accumulation were unaffected in the transgenic plants. Thus, the *hot1-4* mutant protein functions in a dominant-negative fashion. In total, these results provide evidence that the coiled-coil domain is required for *At*Hsp101 function during an early step in the development of thermotolerance. In addition, the semidominant, gain-of-function phenotype of *hot1-4* suggests that this mutation results in a nonproductive interaction with a limiting substrate or cofactor.

Isolation of Suppressors of *hot1-4*

The dominant-negative, 38°C-sensitive phenotype of *hot1-4* provided an excellent opportunity to screen for suppressor mutations that could restore growth. Suppressor mutations should provide additional insight into structure-function relationships of *At*Hsp101 and potentially identify important *At*Hsp101 substrates or cofactors. Approximately 7500 homozygous *hot1-4* seeds were mutagenized with ethyl methanesulfonate, and nearly 110,000 dark-grown, 2.5-d-old seedlings from the M2 generation were screened for continued growth after a 2-h treatment at 38°C. In total, 43 lines were isolated that showed a significant reversion of *hot1-4* sensitivity to 38°C treatment and that exhibited the same phenotype in the subsequent M3 generation (data not shown).

To separate intragenic from extragenic suppressors, we sequenced the *At*Hsp101 gene from all 43 suppressors. Seven of the suppressors did not carry a second mutation in the *At*Hsp101 gene and are unlikely to be linked to the original *hot1-4* mutation. Presumably, they represent extragenic suppressors of *hot1-4*. These suppressors could define factors specific to plant stress tolerance and/or identify genes that encode *At*Hsp101 cofactors or critical substrates. Further ge-

netic and molecular analysis of these mutants is in progress. The other 34 suppressors had a second mutation in the *hot1-4* gene, resulting in 13 different amino acid substitutions: eight in the first nucleotide binding domain (NBD1) (R223K, A270T/A305T, A297T, G313D, E319K, A329V, G384S, and P388S), four within NBD2 (G611D, G649E, G653E, and A723V), and one in the C-terminal region (V813M) (Figure 4).

To confirm that the loss of sensitivity to a 38°C treatment in mutants carrying a second site mutation in the Hsp101 gene was because of this mutation and not to another extragenic mutation, a plant carrying each of the individual mutations was crossed to either the wild type or *hot1-4*. Segregation of the wild type, *hot1-4*, and suppressed phenotype was then scored in the F1 and F2 generations for each cross. As shown in Table 2 and Supplemental Tables 1 and 2 online, suppression of the 38°C phenotype was recessive and linked to the *hot1-4* mutation. These data confirm that the second site mutation in Hsp101 is the cause of the recovery of wild-type growth at 38°C for each of these suppressors.

The Suppressor Mutations Include Both Loss-of-Function and Restoration-of-Function Alleles

The phenotypes of intragenic suppressor mutants, representing each amino acid substitution, were retested at 38°C to quantify the degree of phenotypic reversion. All the intragenic suppressors
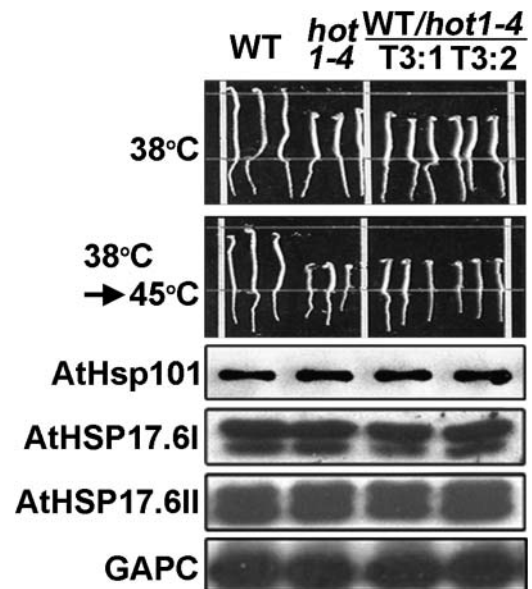

Figure 3. Expression of Mutant *HOT1-4* Protein in Wild-Type Plants Recapitulates the 38°C Sensitivity Phenotype of *hot1-4*.

The *hot1-4* genomic DNA under its own promoter was expressed in transgenic wild-type plants. Two independent transgenic lines (T3:1 and T3:2) are shown compared with the wild type and *hot1-4* (three seedlings each). The hypocotyl elongation assay was performed as in Figure 1C. Equal protein samples from heat-stressed seedlings (38°C for 90 min followed by 2 h at 22°C) were analyzed with the indicated antisera against AtHsp101, sHsps, or glyceraldehyde-3-phosphate dehydrogenase (GAPC).

showed a strong restoring phenotype of hypocotyl elongation after the same 38°C treatment that was used for their initial identification (Table 3). In addition, when tested for sensitivity to 38°C as 10-d-old seedlings, the suppressors also showed recovery of the wild-type phenotype (Figure 5A). Thus, all of the suppressors clearly eliminated the dominant-negative function of the *hot1-4* allele at two distinct growth stages.

The intragenic suppressor mutants were then tested to determine if the function of *At*Hsp101 in acquired thermotolerance had been restored by measurement of hypocotyl elongation after 2 h at 45°C of seedlings that had been preconditioned at 38°C (Table 3). The suppressors could clearly be separated into two classes based on their response to this assay. Suppressors in Class 1, which includes A270T/A305T, E319K, G384S, P388S, G611D, A723V, and V813M, were completely arrested in hypocotyl elongation after 2 h at 45°C with preconditioning. Genetic analysis confirmed that this phenotype also segregated with the *hot1-4* mutation, again confirming that it arises as a result of the second site mutation in *At*Hsp101 (see Supplemental Tables 1 and 2 online). This acquired thermotolerance test indicates that the Class 1 suppressors are most likely loss-of-function alleles because they eliminate the dominant-negative phenotype of *hot1-4* but do not restore function in acquired thermotolerance. By contrast, the second class of suppressors (Class 2) (R223K, A297T, G313D, A329V, G649E, and G653E) partially restored acquired thermotolerance of hypocotyl elongation, with the best suppression shown by A329V, which elongated to ~75% of wild-type levels (Table 3).

The same phenotypic behavior was observed when 10-d-old suppressor seedlings were tested for acquired thermotolerance. Class 1 suppressors failed to acquire thermotolerance; a 45°C treatment for 2 h (with preconditioning) completely blocked production of additional leaves, and existing leaves and cotyledons turned white (Figure 5B). Of the Class 2 suppressors, those which restored hypocotyl elongation after 45°C stress to <35% of the wild type (G313D, G649E, and G653E; Table 3) did not exhibit acquired thermotolerance at the 10-d-old seedling stage (Figure 5B). However, suppressors R223K, A297T, and A329V, which showed stronger reversion of the hypocotyl phenotype (>43% of the wild type), also showed acquired thermotolerance at the
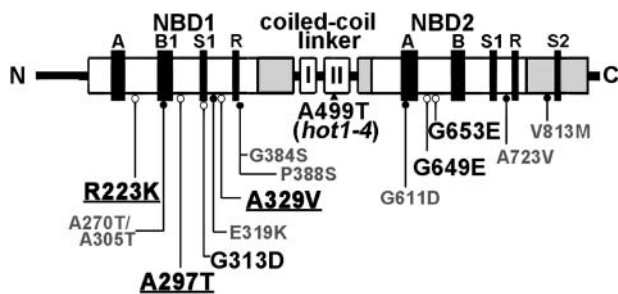

Figure 4. Location of *hot1-4* Suppressor Mutations in *At*Hsp101.

Class 1 loss-of-function suppressors are labeled in smaller gray font and indicated with closed circles. Restoration-of-function Class 2 suppressors are indicated in larger black font with open circles. The strongest Class 2 suppressors, as discussed in the text, are underlined (see also Supplemental Figure 1 online).

Table 2. Genetic Segregation Analysis of Intragenic Suppressors

Genotypes		Total	WT	*hot*	Sup.	χ^2	P
		No. of plants					
R223K	F1	5	5	0	0		
× WT	F2	36	36	0	0		
R223K	F1	5	0	0	5		
× hot1-4	F2	36	0	10	26	0.14	>0.9
A270/A305	F1	3	3	0	0		
× WT	F2	38	38	0	0		
A270/A305	F1	7	0	0	7		
× hot1-4	F2	40	0	9	31	0.13	>0.9
A297T	F1	7	7	0	0		
× WT	F2	36	36	0	0		
A297T	F1	6	0	0	6		
× hot1-4	F2	29	0	6	23	0.73	>0.5
G313D	F1	5	5	0	0		
× WT	F2	36	36	0	0		
G313D	F1	6	0	0	6		
× hot1-4	F2	32	0	7	25	0.16	>0.9
E319K	F1	7	7	0	0		
× WT	F2	32	32	0	0		
E319K	F1	2	0	0	2		
× hot1-4	F2	28	0	5	23	0.76	>0.5
A329V	F1	5	5	0	0		
× WT	F2	46	46	0	0		
A329V	F1	5	0	0	5		
× hot1-4	F2	39	0	8	31	0.14	>0.9
G384S	F1	6	6	0	0		
× WT	F2	36	36	0	0		
G384S	F1	5	0	0	5		
× hot1-4	F2	32	0	7	25	0.16	>0.9
P388S	F1	10	10	0	0		
× WT	F2	40	40	0	0		
P388S	F1	3	0	0	3		
× hot1-4	F2	40	0	9	31	0.13	>0.9
G611D	F1	4	4	0	0		
× WT	F2	46	46	0	0		
G611D	F1	5	0	0	5		
× hot1-4	F2	43	0	10	33	0.07	>0.9
G649E	F1	5	5	0	0		
× WT	F2	45	45	0	0		
G649E	F1	6	0	0	6		
× hot1-4	F2	44	0	8	36	0.36	>0.5
G653E	F1	5	5	0	0		
× WT	F2	37	37	0	0		
G653E	F1	5	0	0	5		
× hot1-4	F2	34	0	7	27	0.35	>0.5
A723V	F1	5	5	0	0		
× WT	F2	27	27	0	0		
A723V	F1	8	0	0	8		
× hot1-4	F2	35	0	8	27	0.08	>0.9
V813M	F1	5	5	0	0		
× WT	F2	46	46	0	0		
V813M	F1	5	0	0	5		
× hot1-4	F2	48	0	9	39	1	>0.5

Phenotype scoring was done by comparing the hypocotyl length distribution patterns of the F2 seedlings with those of the parental lines. The hypocotyl length ranges of the phenotypic classes of F2 progenies were 9.5 to 10.5 mm for the wild-type phenotype, 9.0 to 11.0 mm for the suppressor phenotype (Sup.), and 2.0 to 4.0 mm for *hot1-4* mutant phenotype after 38°C heat treatment for 2 h. WT, wild type.

Table 3. Hypocotyl Elongation Phenotype of Intragenic Suppressors

Genotype	No. of Isolates	Hypocotyl Length (%)[a]	
		38°C	38°C/45°C (%)
WT		91.8 ± 9.6	68.0 ± 9.2 (100)
hot1-4 (A499T)		26.2 ± 4.5	0 ± 0
hot1-3 (null)		90.8 ± 9.6	0 ± 0
Class 1 suppressors			
A270T/A305T	6	89.9 ± 6.4	0 ± 0
E319K	1	84.7 ± 6.6	0 ± 0
G384S	3	89.7 ± 8.3	0 ± 0
P388S	3	87.5 ± 6.8	0 ± 0
G611D	5	90.1 ± 6.1	0 ± 0
A723V	3	79.4 ± 9.8	0 ± 0
V813M	2	91.4 ± 8.3	0 ± 0
Class 2 suppressors			
R223K	2	83.6 ± 5.1	28.7 ± 5.8 (42)
A297T	2	82.1 ± 9.8	29.3 ± 6.3 (43)
G313D	3	75.3 ± 6.7	9.7 ± 4.1 (14)
A329V	1	94.1 ± 6.2	51.3 ± 6.7 (75)
G649E	1	86.7 ± 8.0	20.9 ± 6.8 (30)
G653E	2	85.2 ± 7.3	24.1 ± 7.3 (35)

[a] Dark-grown, 2.5-d-old seedlings were total hypocotyl elongation after the heat stress was measured as a percentage of the 22°C value. Mean and standard deviations were derived by measurement from three independent experiments performed with 60 or more seedlings per F3 seedling. The numbers in parentheses indicate percentage of elongation compared with that of wild-type plants.

10-d-old seedling stage (Figure 5B). Furthermore, the A329V suppressor recovered as much as the wild type, whereas the R223K and A297T suppressors had pale green leaves and were smaller than the A329V suppressor, also consistent with the relative growth of these suppressors in the hypocotyl assay. Thus, the strong suppressors, R223K, A297T, and A329V, clearly compensate for the primary functional defect caused by *hot1-4*, although with somewhat different effectiveness. These three mutations are all located in NBD1 (Figure 4), supporting a functional interaction of this domain with the coiled-coil domain containing the primary *hot1-4* mutation.

The V813M and G384S Suppressors Accumulate Truncated *At*Hsp101

To determine whether the restored growth phenotype of the suppressors was because of a dose effect of the mutant protein, protein gel blot analysis was performed on protein samples from heat-treated, 2.5-d-old dark-grown seedlings. Except for V813M and G384S, all the mutant proteins accumulated to similar levels when compared with the wild type and *hot1-4* (Figure 6). Although the Class 1 mutants V813M and G384S are obviously missense mutations, ∼90- and 41-kD bands, respectively, were detected with *At*Hsp101 antibody in these samples (Figure 6). Because the antibody is directed specifically to the N-terminal domain of *At*Hsp101 (Hong and Vierling, 2001), these bands most likely represent C-terminally truncated proteolytic fragments generated in vivo as a result of protease sensitivity near the mutation

site. Thus, these two suppressor mutations clearly produce a nonfunctional protein. This result supports the above conclusion that the defective phenotype in acquired thermotolerance of the Class 1 suppressors is caused by loss of *At*Hsp101 function.

Strong Suppressors Restore the Function of *At*Hsp101 in Protein Resolubilization

The current model for Hsp100/ClpB function in thermotolerance proposes that these proteins are required for reactivation of aggregated proteins after heat stress (Glover and Lindquist, 1998; Goloubinoff et al., 1999; Motohashi et al., 1999). Therefore, it can be assumed that during recovery from heat stress, heat-aggregated proteins requiring *At*Hsp101 function should remain in the insoluble cell fraction in *hot1* mutants, whereas these proteins should return to the soluble fraction in the wild type. It also follows that if resolubilization is an essential function of *At*Hsp101 for acquired thermotolerance, the strong *hot1-4* suppressor mutations should restore protein solubility after heat stress. As a first step to test this hypothesis, we sought conditions for comparing the insoluble protein fractions from wild-type and mutant plants during recovery from heat stress, along with a suitable protein to assay for solubility. We determined that in wild-type plants, the cytosolic Class I and II
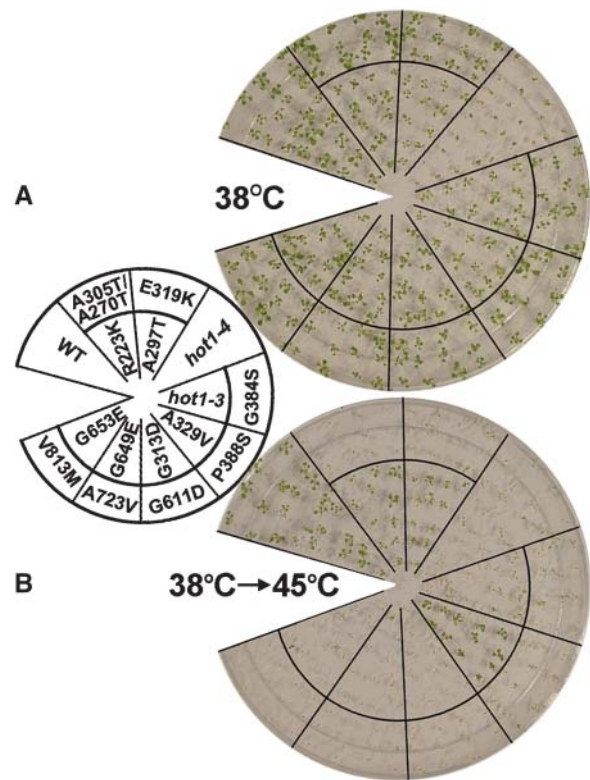
Figure 5. Thermotolerance Phenotype of 10-d-Old Suppressor Mutants.

After growth for 10 d in the light at 22°C, seedlings were treated either at 38°C for 3 h **(A)** or at 38°C for 90 min followed by 2 h at 22°C and then 2 h at 45°C **(B)**. Seedlings were photographed 7 d after treatment.
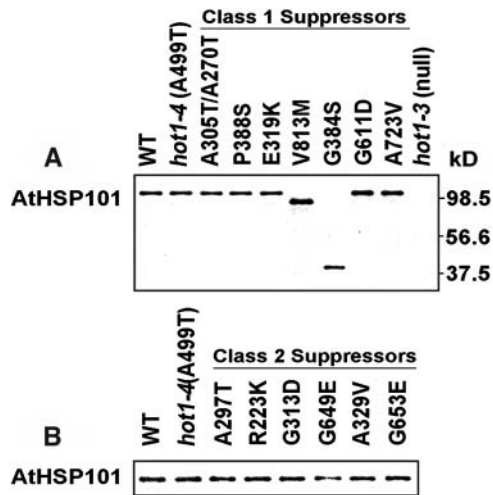
Figure 6. Accumulation of *At*Hsp101 Protein in the Suppressor Mutants.

Protein samples from heat-stressed seedlings (38°C for 90 min followed by 2 h at 22°C) were analyzed with *At*Hsp101 antiserum.
(A) Class 1 suppressor mutants.
(B) Class 2 suppressor mutants.

sHsps, partitioned during 45°C heat stress into the insoluble cell fraction and almost completely transitioned back to the soluble fraction after 3 h of recovery from the heat stress (data not shown). We therefore compared the solubility of either Class I *At*Hsp17.6C-I or Class II *At*Hsp17.6C-II in wild-type, *hot1-4*, and the strong suppressors, R223K, A297T, and A329V, after 3 h of recovery from a 38°C, 3-h heat stress or from a 45°C, 45-min heat stress with preconditioning. In contrast with the wild type, a majority of the sHsps remained in the pellet fraction during recovery from 45°C stress in the *hot1-4*, *hot1-1*, and *hot1-3* mutants (Figure 7). The mutants also showed detectably more pelleted sHsps after the 38°C stress. We then examined the solubility of these sHsps in the strong suppressors. sHsp solubility was directly correlated with the strength of the suppressor (Figure 7), with the most effective suppressor, A329V, being almost identical to the wild type and the weaker suppressors, R223K and A297T, showing slightly more sHsp retained in the insoluble fraction. The same samples were also used to examine the solubility of *At*Hsp101. In contrast with the sHsps, neither the wild type nor the *hot1-1* allele of *At*Hsp101 becomes insoluble during heat stress; similar behavior is reported for *E. coli* ClpB (Mogk et al., 2003a, 2003c). A fraction of *hot1-4* becomes insoluble during heat stress, but insolubility does not correspond to the phenotype, as at 38°C the *hot1-4* phenotype is already severe, whereas little protein is insoluble. Only the strongest suppressor reverses the *At*Hsp101 insolubility. Altogether, these results support the model that one essential thermotolerance function of *At*Hsp101 involves protein disaggregation and genetically link Hsp101 to the sHsp chaperone system.

DISCUSSION

We have been able to identify features critical to Hsp100/ClpB function in vivo using a sensitive, quantitative bioassay for thermotolerance. We identified four missense mutations in the Hsp100/ClpB protein, Arabidopsis *At*Hsp101, that compromise the function of *At*Hsp101 in thermotolerance, including an unusual allele, *hot1-4* (A499T), which shows a semidominant, gain-of-function sensitivity to a mild, usually nonlethal heat stress. This mutation, which lies in the coiled-coil domain of *At*Hsp101, supports an essential role for this structural domain in Hsp100/ClpB action in vivo. We also suggest that the gain-of-function phenotype of *hot1-4* is consistent with the interpretation that the mutation disrupts *At*Hsp101 activity such that a critical substrate or cofactor becomes limiting for the cell. Locations of the primary missense mutations *hot1-5*, *-6*, and *-7* and of suppressors of *hot1-4* define other features essential for in vivo function of *At*Hsp101 and point to protein domain interactions required for Hsp100/ClpB action. Finally, appearance of sHsps in the soluble cell fraction during recovery from heat stress does not occur in *hot1-4* but is restored by strong suppressor mutants, in line with the model that disruption of protein aggregates is an essential *At*Hsp101 function in thermotolerance and supporting the model that sHsps and Hsp100/ClpB proteins interact in a chaperone network in the cell.

The coiled-coil domain of Hsp100/ClpB is unique to this class of AAA+ proteins (Schirmer et al., 1996; Celerin et al., 1998). Although referred to alternatively as the linker, middle region, or spacer between AAA modules 1 and 2, it is actually an insertion into the C-terminal small domain of AAA module 1 (Figures 1 and 8; see Supplemental Figure 1 online). Deletion of the coiled-coil domain from *E. coli* ClpB has shown that it is not essential for hexamerization or ATPase activity but is required for thermotolerance in vivo and for disaggregation of firefly luciferase in vitro (Mogk et al., 2003b). The structure of *T. thermophilus* ClpB reveals that this domain is formed by four helices (L1 to L4) that
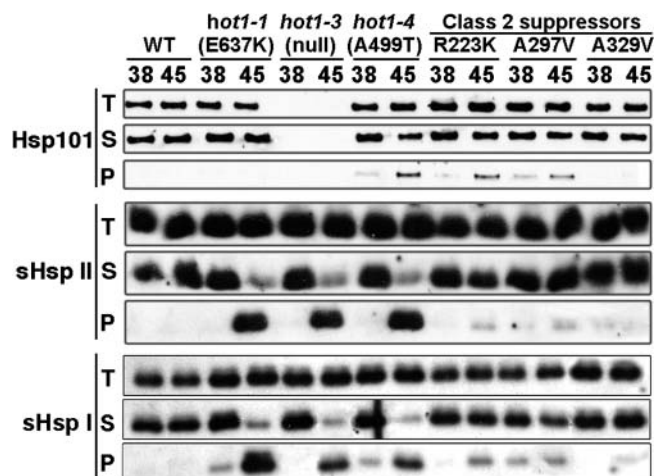

Figure 7. Reversal of sHsp Insolubility in the Suppressor Mutants.

Ten-day-old seedlings were treated either at 38°C for 3 h (38) or at 38°C for 90 min followed by 2 h at 22°C and then 60 min at 45°C (45), and total protein (T) was isolated after further recovery for 3 h at 22°C. The insoluble protein fraction (P) was separated from the soluble fraction (S) by centrifugation and analyzed with *At*Hsp101, *At*Hsp17.6C-I (sHsp I), and *At*Hsp17.6C-II (sHsp II) antiserum.
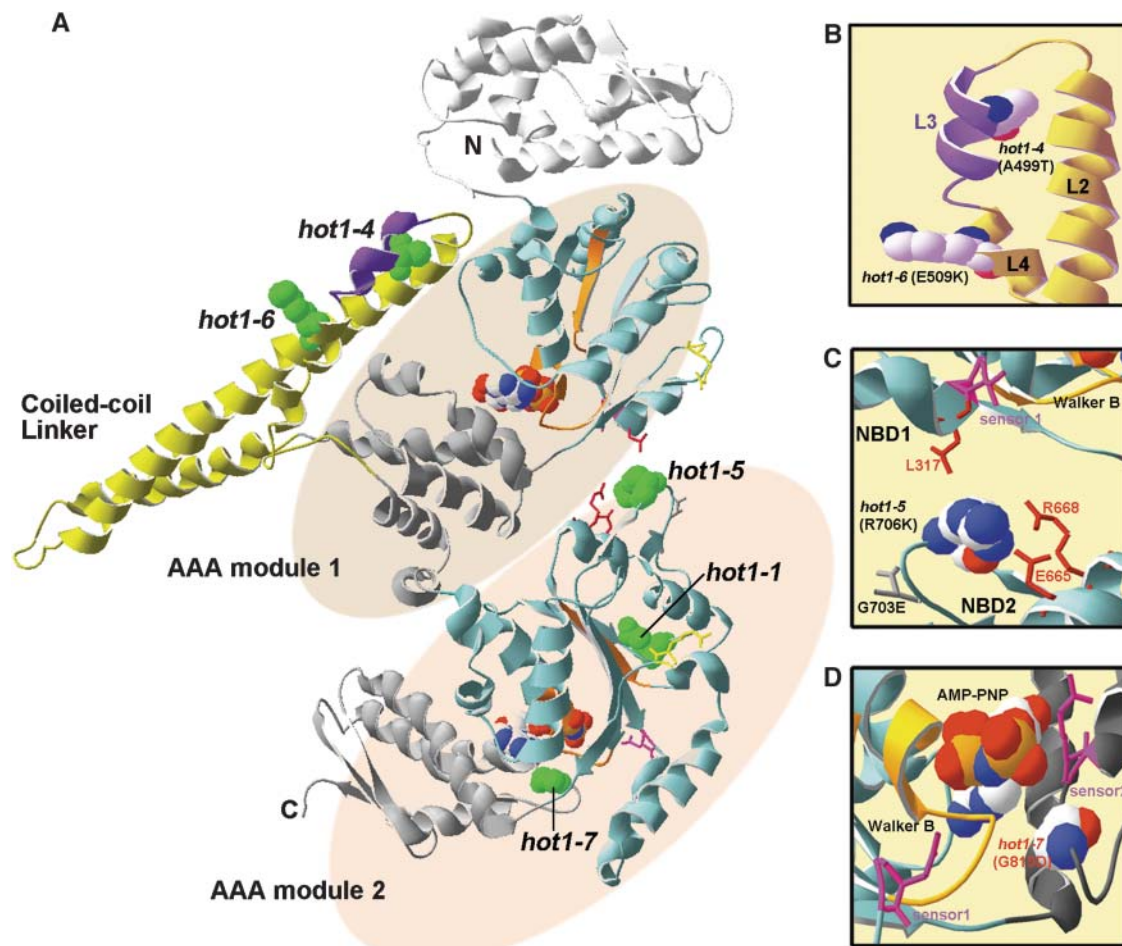
Figure 8. Location of the *hot1* Mutations on the *T. thermophilus* ClpB Structure.

(A) The available structure of the *T. thermophilus* ClpB monomer (PDB file 1QVR; chain in **[A]**) is shown with the coiled-coil domain and AAA modules indicated. Within each AAA module, the NBD domain is colored blue, with the Walker A and B motifs in orange, sensor 1 in pink, and the Arg finger in yellow (see also Supplemental Figure 1 online). The small, α-helical domain of each AAA module is shown in gray. Nucleotide (AMP-PNP) is space-filled in CPK coloring (hydrogen, white; oxygen, red; phosphorous, orange; sodium, blue). Motif 1 as defined by Schirmer et al. (1996) is colored purple in the coiled-coil domain. The residue corresponding to each of the *hot1* mutation sites is space-filled and colored green.
(B) Alternative view of the motif II region of the coiled-coil domain indicating the L2, L3, and L4 helices and the residue positions of the *hot1-4* and *hot1-6* mutations (in CPK coloring).
(C) View of interactions of the *hot1-5* position (in CPK coloring) with residues within NDB2 and NBD1 as discussed in the text.
(D) Position of *hot1-7* (CPK coloring) relative to the NBD2 ATP binding site. The sensor 2 Arg residue, which is part of the GAR motif, as well as sensor 1 are shown in pink. Residue numbering corresponds to *At*Hsp101. Figure was prepared with Swiss PDB viewer.

interact in two coiled-coil motifs comprised of α-helices L1 and L2, and L2 with L3/4 (Figures 8 and 9). Both coiled-coils have features typical of Leu zippers. The *hot1-4* mutation (A499T) lies in helix L3, within the conserved sequence segment 494YDLAR-**A**ADL502 (484YDLNRAAEL492, in *T. thermophilus*) (Figures 8A and 8B). This sequence motif was previously identified by Schirmer et al. (1996) as characteristic of Hsp100/ClpB chaperones. The weak mutation *hot1-6* (E509K) lies in the coiled-coil domain directly outside this conserved motif (Figure 8B). Neither the *hot1-4* nor *hot1-6* residues are predicted to contact residues in neighboring subunits, which along with the normal accumulation of the proteins and the similar solubility of *hot1-4* to the wild type, suggest that these mutations do not significantly disrupt the

hexamer structure. In vitro cross-linking experiments also produce the same large, presumably oligomer forms of *At*Hsp101 in the wild type and the *hot1-4* mutants (U. Lee, unpublished data). Eight other mutations in this domain have no effect on *At*Hsp101 function (Figure 1). Altogether, the *hot1-4* and *hot1-6* mutations provide direct genetic evidence for the importance of helix L3 and the coiled-coil domain in *At*Hsp101 function.

Interestingly, in a screen for mutants in *Saccharomyces cerevisiae* Hsp104, Schirmer et al. (2004) recently reported isolation of a mutation in the same motif, at the adjacent Ala residue (A503V; 497YDTATA**A**DL505 in *S. cerevisiae*). The Hsp104 A503V mutant was an active ATPase in vitro (Cashikar et al., 2002), exhibiting actually an approximately twofold
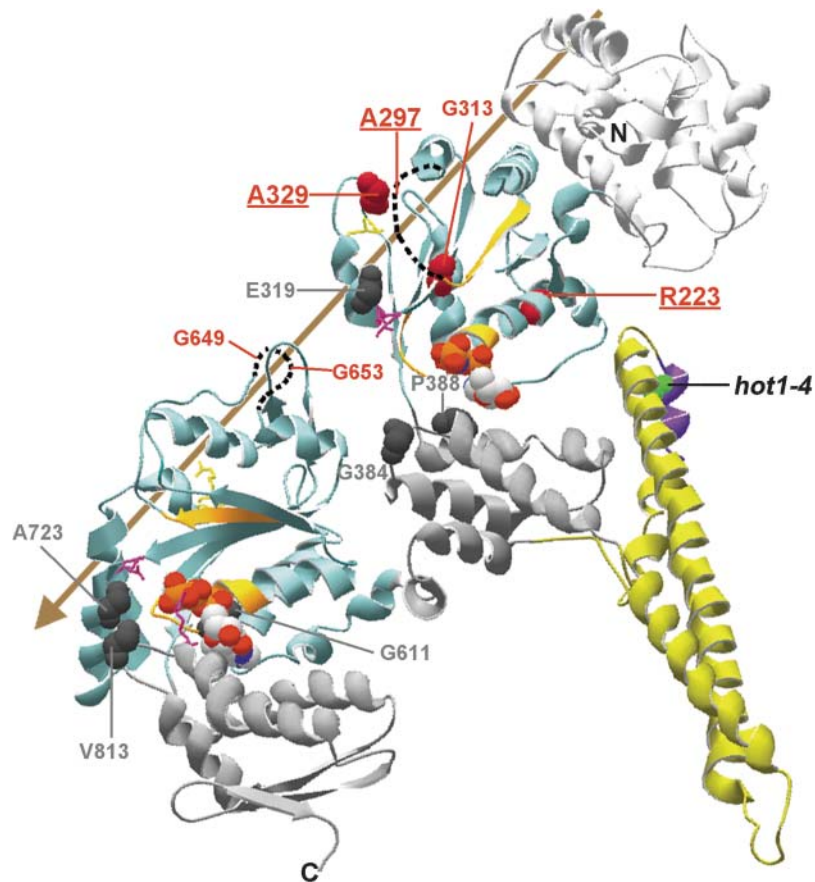
Figure 9. Location of the *hot1-4* Suppressor Mutations on the *T. thermophilus* ClpB Monomer Structure.

The major domains and motifs of HCP100/ClpB are colored as indicated and described in Figure 8. The sites of Class 1 suppressor mutations are space-filled dark gray, and sites of the Class 2 suppressor mutations are space-filled in red. The three strongest Class 2 suppressors are indicated in bold and are underlined. For A297, G653, and G649, which are in unresolved segments of the structure, dotted lines are used to indicate the relative positions of those segments. The arrow indicates the general position of the axial channel of the hexameric form of the protein. Residue numbering corresponds to *At*Hsp101. Figure was prepared with Swiss PDB viewer.

increase in basal ATPase activity (i.e., activity in the absence of substrate). Therefore, the A503V mutation cannot disrupt the hexameric structure, which is required for ATPase activity. This observation further supports our interpretation that the *hot1-4* mutant protein retains its hexameric structure. Furthermore, like *hot1-4*, the Hsp104 A503V mutation also exhibits a gain-of-function phenotype, leading to cell lethality when induced at the normally permissive temperature of 37°C (Schirmer et al., 2004). The similarity of phenotype of these *At*Hsp101 and yeast Hsp104 mutations, along with the ability of *At*Hsp101 to support thermotolerance in yeast (Schirmer et al., 1994), argue that these proteins are true functional homologs.

The other mutations studied also provide new information about interactions within the Hsp100/ClpB proteins. The strong *hot1-5* allele alters a residue (R706K) that is conserved in *E. coli* (R705) and *T. thermophilus* (R695), and which is in a loop between β-strands 4 and 5 in NBD2, positioned away from the active site (Figures 8A and 8C; see Supplemental Figure 1 online). Normal accumulation and solubility of the *hot1-5* protein argues

that the protein is not significantly compromised structurally. Based on the *T. thermophilus* structure, this Arg residue makes intramolecular contact with three other residues conserved in Arabidopsis and *E. coli*, including a Leu residue directly adjacent to sensor 1 in NBD1, and has side chain interactions with conserved Glu and Arg residues in NBD2 helix D7 (Figure 8C). Notably, a Lys residue is present at the *hot1-5* position in yeast Hsp104, and correspondingly the predicted interacting residues are different. The unique importance of *hot1-5* interactions, as opposed to variation in the loop itself, is also indirectly supported by the absence of a phenotype for another mutation in the loop connecting β-strands 4 and 5, G703E (Figures 1 and 8C). Hattendorf and Lindquist (2002a) have proposed that ATP hydrolysis at NBD1 depends on the nucleotide bound at NBD2 in yeast Hsp104. We speculate that *hot1-5* is in a position involved in communicating nucleotide status between NBD2 and NBD1 and note that the distance between the *hot1-5* Arg residue and the conserved Leu in NBD1 varies in the three ClpB molecules in the crystal structure, consistent with a dynamic interaction.

It is difficult to define the possible effects of some of the other *At*Hsp101 mutations. The *hot1-7* mutation represents a dramatic change (G815D) in a conserved motif (GAR) that includes the sensor 2 Arg residue already recognized as important for function (Figure 8D). The residues in the sensor 2 motif are in contact with nucleotides, and the Arg residue in yeast Hsp104 is proposed to contribute binding energy, but not discrimination between ATP and ADP (Hattendorf and Lindquist, 2002a). Mutations in sensor 2, or of all three residues of the GAR motif, do not severely disrupt hexamerization or ATPase activity, but prevent ability to dissociate protein aggregates (Hattendorf and Lindquist, 2002b; Mogk et al., 2003b). In vivo, the moderate phenotype of *hot1-7* is consistent with a moderate defect in thermotolerance observed for an R to M mutation of sensor 2 in yeast (Hattendorf and Lindquist, 2002b). The absence of phenotype for 35 other missense mutations analyzed is not surprising, given that many lie in nonconserved residues or represent conservative changes (Figure 1; see Supplemental Figure 1 online). Perhaps the most surprising of the aphenotypic mutations is P671L, which represents a Pro residue conserved not only in NBD2 of all ClpB proteins, but also found in ClpA.

The unusual sensitivity of the *hot1-4* allele to 38°C, which is otherwise permissive for wild-type and *At*Hsp101 null plants, led us to focus further on analysis of this mutation. We performed a screen that identified both loss-of-function (Class 1) and restoration-of-function (Class 2) intragenic suppressor mutations in *At*Hsp101. Genetic dissection of Hsp100/ClpB function by intragenic suppressor analysis has not been performed in any organism, and we are also unaware of any previous studies using intragenic suppressors for dissection of protein mechanism in higher plants. We argue that the seven Class 1 mutations that eliminated the 38°C heat sensitivity of *hot1-4*, but did not restore the acquired thermotolerance function, represent loss-of-function *At*Hsp101 mutants. Consistent with this interpretation, one of these suppressors is in the Walker A motif of NBD 2 (G611D), and three others are predicted to affect the nucleotide binding pocket (E319K, P388S, and A723V) (Figure 9), which should result in defective hexamerization or ATP hydrolysis as demonstrated in vitro for other Hsp100/ClpB proteins (Kim et al., 2000a; Watanabe et al., 2002; Mogk et al., 2003b). These findings suggest that in *hot1-4*, *At*Hsp101 retains normal ATP hydrolysis activity and hexamerization and indicates that these properties are essential for the dominant-negative effect of this allele. Two other Class 1 suppressors, G384S and V813M, are both located between the second and third helices in the C-terminal subdomain of NBD1 and NBD2, respectively (Figure 9; see Supplemental Figure 1 online). Both of these mutations lead to accumulation of a truncated *At*Hsp101 protein with sizes that predict that in vivo cleavage occurs in close proximity to the mutation. Proteolytic removal of the C terminus in V813M supports the requirement of this domain for function, which as stated before, may involve not only hexamerization, but also effector binding (Smith et al., 1999; Strub et al., 2003). Although our screen was not performed to saturation, it is notable that no loss-of-function suppressors were obtained in the N-terminal domain, which has also not been associated with an essential function in other organisms (Clarke and Eriksson, 2000; Beinker et al., 2002; Mogk et al., 2003b).

Whereas the coiled-coil domain is clearly essential for Hsp100/ClpB function, its role in the catalytic cycle is not known. The position of *hot1-4* (A499T) (and A503V in *S. cerevisiae* Hsp104) suggests that this mutation could disrupt interactions of L3 with L2 within a subunit. Lee et al. (2003) have proposed that motion of the coiled-coil domain is required for Hsp100/ClpB chaperone activity. They introduced Cys residues into *T. thermophilus* ClpB to effect cross-linking of the L2/L3-4 coiled-coil (in which *hot1-4* is located) to NBD1 and observed in vitro that ATP hydrolysis continued, but the protein disaggregation activity of ClpB was lost. The three *hot1-4* suppressors that restore >43% of acquired thermotolerance of hypocotyl growth and also restore acquired thermotolerance of 10-d-old seedlings are all located in NBD1 (R223K, A297T, and A329V). The strongest of these, A329V, has >70% wild-type activity in the hypocotyl assay and restores solubility of sHsps during recovery from heat stress. Interestingly, A329V lies in close proximity to the NBD1 Arg finger, which is believed to interact with the nucleotide in an adjacent subunit (Figure 9). Another weak suppressor, G313D, is also in NBD1 (Figure 9). Taken together, the location of the Class 2 restoration-of-function suppressors of *hot1-4* is consistent with the interpretation that dynamic interaction of the coiled-coil domain with NBD1 is indeed essential in vivo.

How Hsp100/ClpB proteins act to disaggregate proteins has been proposed to involve a crowbar action of the coiled-coil domain and/or threading of substrate through the axial channel of the hexamer, analogous to the mechanism of other hexameric ATPases (Lee et al., 2003; Lum et al., 2004; Schlieker et al., 2004). Location of the Class 2 suppressors provides potential support for the latter mechanism. The strong suppressor A297V is located in a presumably flexible, disordered segment that was not resolved in the *Tt*ClpB structure, but which is in the axial channel region. A329V, another strong suppressor, is positioned to potentially alter interactions with another unresolved axial channel loop (*Tt*ClpB residues 234 to 246; corresponding to 243 to 255 in *At*Hsp101), which has recently been proposed to contain residues that bind the *E. coli* ClpB substrate TrfA (Schlieker et al., 2004). The only restoration-of-function suppressors outside NBD1 are the two weak suppressors, G649E and G653E in NBD 2, which also lie in an unresolved axial channel loop (*Tt*ClpB residues 638 to 650; corresponding to *At*Hsp101 648 to 660) (Figure 9; see Supplemental Figure 1 online). This loop contains the conserved motif GYVG found in other AAA+ proteins (with the first G corresponding to G653 in *At*Hsp101). Mutation of the Tyr residue in this loop impairs function of *E. coli* ClpX (Siddiqui et al., 2004) and HslU (Wang et al., 2001b). Structural studies also indicate that this loop gates the axial channel of HslU during the ATPase cycle (Wang et al., 2001a). Recent experiments with yeast Hsp104 also support an essential role for this loop in the protein disaggregation reaction (Lum et al., 2004). In total, we suggest that these intragenic suppressors point to a model in which motions of the coiled-coil domain modulate the ATPase activity of NBD1 and the position of axial channel loops in *At*Hsp101, which may be involved in threading of substrate through the axial channel.

Analysis of the solubility of plant cytosolic sHsps in the *hot1-4* mutant and the strong suppressors also provides data to link these two chaperone systems in a eukaryote. Genetic

interactions of sHsps and ClpB have been reported in both *E. coli* and Synechocystis (Giese and Vierling, 2002; Mogk et al., 2003a) and are supported by in vitro analysis of *E. coli* ClpB activity in combination with *E. coli*, Synechocystis, and plant cytosolic class I sHsps (Mogk et al., 2003c). The current model for sHsp function proposes that sHsps bind and maintain denaturing proteins in a form that is accessible to ATP-dependent refolding chaperones. Furthermore, their presence in large protein aggregates makes these aggregates better substrates for the action of Hsp100/ClpB (Mogk et al., 2003c). However, although Hsp100/ClpB disaggregates sHsp containing complexes, there is no evidence for direct physical interaction of Hsp100/ClpB with sHsps (Mogk et al., 2003a, 2003c), and we did not observe association of *At*Hsp101 with sHsps in the insoluble protein fraction of the cell. In total, the data here indicate that the plant cytosolic Class II, as well as cytosolic Class I sHsps, which diverged on the order of 400 million years ago (Waters and Vierling, 1999), may both facilitate protein disaggregation by Hsp100/ClpB in plants.

At which step the *hot1-4* mutation affects catalysis will require additional biochemical studies. It does not appear that the dominant-negative phenotype of *hot1-4* is attributable to un-regulated ATPase activity of the mutant *At*Hsp101 protein, leading to depletion of cellular ATP. Measurement of ATP levels in the wild type, *hot1-3*, and *hot1-4* showed no significant differences in ATP levels between the genotypes during heat stress or recovery (U. Lee and E. Vierling, unpublished data). One possibility is that the mutation traps *At*Hsp101 in a nonfunctional protein complex such that another critical cofactor or substrate becomes limiting for the cell. Such a complex must still be soluble, as our analysis does not indicate that a significant fraction of *At*Hsp101 becomes insoluble at 38°C where the *hot1-4* phenotype is still severe. The Class 1 suppressors that eliminate the dominant-negative phenotype are predicted to disrupt the basic hexameric structure and ATPase activity, consistent with the requirement of these activities to form a trapped complex. By altering structural interactions of the coiled-coil domain with NBD1, the strong Class 2 suppressors may eliminate this trapped intermediate. Weaker Class 2 suppressors may affect the protein's conformation, but not enough to fully disrupt the formation of the nonfunctional complex between *hot1-4* and its bound protein(s). Another possibility is that the suppressors directly or indirectly alter a binding site for substrate or functional partner protein, such that a nonfunctional complex no longer forms, but sufficient substrate or cofactor interactions are retained for partial function in vivo. Attempts to coimmunoprecipitate *hot1-4* in complex with other proteins has not been successful. Biochemical characterization of *hot1-4* along with identity of extragenic suppressors should provide further insight into the nature of the *hot1-4* defect and the mechanism of Hsp100/ClpB action.

METHODS

Plant Material and Growth Conditions

Arabidopsis thaliana (Columbia accession) plants were grown on plates in the dark or under long-day conditions (16 h light/8 h dark) in a controlled-temperature growth chamber (22°C/18°C). Thermotolerance tests of 2.5-d-old dark-grown or 10-d-old light-grown seedlings were performed basically according to Hong and Vierling (2000).

Isolation and Genetic Analysis of *hot1* Mutant Alleles

The *hot1-4* mutation was isolated as a thermotolerance-defective mutant in a screen based on hypocotyl elongation of 2.5-d-old dark-grown seedlings (Hong and Vierling, 2000). Allelism tests showed that *hot1-4* was tightly linked to the previously described *At*Hsp101 loss-of-function missense allele, *hot1-1* (Hong and Vierling, 2000). For most of the experiments described in this work, the third backcrossed line of *hot1-4* to Columbia wild type was used. Tilling analysis (in the Columbia ecotype, carrying the *erecta* mutation) was performed on three segments of the *At*Hsp101gene, encompassing approximately amino acid residues 1 to 230, 355 to 595, and 660 to 911 (Arabidopsis Tilling Resource, http://tilling.fhcrc.org:9366) (see Supplemental Figure 1 online). The *hot1-5*, *hot1-6*, and *hot1-7* mutants were recovered from 37 missense mutations in *At*Hsp101 obtained from this analysis. Three stop codon mutations (Q409, Q422, and Q704) were also obtained, confirmed to be defective in thermotolerance, and not studied further. Each line was assayed for thermotolerance defects in the homozygous or heterozygous state, and homozygous lines were isolated for lines showing a phenotype (*hot1-5*, *hot1-6*, and *hot1-7*). One homozygous M4 plant from each mutant line was then backcrossed to Columbia *erecta* wild-type plants, and one homozygous F3 line for each mutation was used for quantitative analysis.

Isolation and Genetic Analysis of *hot1-4* Suppressor Mutations

Approximately 7500 homozygous seeds of *hot1-4* were mutagenized with ethyl methanesulfonate, and ~110,000 M2 seed were screened for suppressor mutants as follows. M2 seeds were surface-sterilized and plated on minimal medium containing 0.5% sucrose. Plates were incubated at 4°C for 3 d and then placed in a vertical position at 22°C for 2.5 d in the dark. The dark-grown seedlings were treated at 38°C for 2 h, and then the plates were returned to the dark at 22°C for 1.5 d. Seedlings that showed increased hypocotyl elongation compared with *hot1-4* were rescued by growth under light for approximately 1 week before being transplanted to soil. The selected seedlings were retested for tolerance to 38°C in the next generation (M3) as described above.

To distinguish if the suppressor mutations were intragenic or extra-genic, the entire *At*Hsp101 gene from the candidate suppressors was amplified by PCR using specific primers. The amplified DNA products were sequenced on both strands. Thirty-four lines were found to contain the original *hot1-4* mutation together with an additional missense mutation within the *At*Hsp101 coding region. The same suppressor mutation was found in more than two independent lines (R223K, A270/A305T, A297T, G313D, G384S, P388S, G611D, G653E, A723D, and V813M), whereas E319K, A329V, and G649E mutations, which were isolated only once, were confirmed by sequencing from two individual lines (Table 3).

To test genetic linkage between *hot1-4* and the suppressors, the homozygous M3 or M4 intragenic suppressors with second site mutations in *At*HSP101 were backcrossed to *hot1-4*. The F1 plants all showed the suppressing phenotype after 38°C heat treatment for 2 h. The subsequent F2 progenies completely segregated in a 3:1 ratio of the suppressor mutant to *hot1-4*, indicating that the suppressor mutations are tightly linked to the *hot1-4* mutation (Table 2; see Supplemental Tables 1 and 2 online).

To obtain F3 homozygous intragenic suppressor lines for phenotypic analysis, the homozygous M3 or M4 intragenic suppressors were out-crossed to wild-type plants. All F1 plants showed the wild-type pheno-type, and F2 progenies segregated either the *hot1-4* mutant phenotype (Class 1 suppressor) or the suppressor phenotype (Class 2 suppressor) at

a ratio of 1:3 to the wild-type phenotype when tested by heating at 45°C for 2 h with pretreatment (38°C for 90 min). Scoring of the hypocotyl length phenotype in F2 progenies was based on the hypocotyl length distribution of the parental lines.

Plant Transformation

A 4.7-kb *XhoI/XbaI* *At*Hsp101 genomic region containing the *hot1-4* mutation, including 1.5 kb of the promoter region, was cloned into pBin19 and transformed into Columbia wild-type plants by the floral dipping method (Clough and Bent, 1998). A total of 20 lines (T1 generation) were selected on minimal plates with kanamycin (30 μg/mL). The number of T-DNA insertion loci was determined in the T2 generation based on the segregation ratio of both kanamycin resistance and the *hot1-4* phenotype. Two independent T3 homozygous lines were used for phenotypic studies.

Fractionation of Heat-Denatured Proteins

Ten-day-old seedlings were pretreated at 38°C followed by 2 h at 22°C, and then heat shocked at 45°C for 60 min (denaturation phase), followed by 3 h of recovery at 22°C (recovery phase). Total protein was extracted before the denaturation phase and after the recovery phase in non-denaturing buffer (25 mM Hepes, pH 7.5, 0.5% Triton X-100, 200 mM NaCl, 0.5 mM EDTA, 5 mM ε-amino-*N*-caproic acid, and 1.0 mM benzamidine). Protein concentration was determined using a Coomassie Brilliant Blue dye binding assay (Ghosh et al., 1988) with BSA as a standard. Total protein solutions were immediately centrifuged at 19,000g for 15 min at 4°C. Supernatants were mixed with the same volume of SDS sample buffer (60 mM Tris-HCl, pH 7.5, 60 mM DTT, 2.0% [w/v] SDS, 15% [w/v] sucrose, 5 mM ε-amino-*N*-caproic acid, and 1.0 mM benzamidine). The pellet was suspended in nondenaturing buffer, washed six times, and then resuspended to the original volume in SDS sample buffer. Proteins were separated by SDS-PAGE on 15% acrylamide gels and processed for protein gel blot analysis (Hong and Vierling, 2001).

SDS-PAGE and Protein Gel Blot Analysis

The 2.5-d-old dark-grown seedlings were treated at 38°C for 90 min, and then total protein was extracted in SDS sample buffer and separated by SDS-PAGE on 7.5% or 15% acrylamide gels and processed for protein gel blot analysis (Hong and Vierling, 2001). Protein blots were probed with rabbit antiserum against *At*Hsp101 or against *At*Hsp17.6C-I or -II (Hong and Vierling, 2001). As a loading control, blots were probed for cytosolic glyceraldehyde-3-phosphate dehydrogenase using a GAPC antibody (gift of Ming-Che Shih, University of Iowa) as described (Chan et al., 2002). Blots were incubated with goat anti-rabbit horseradish peroxidase and bands visualized by enhanced chemiluminescence (Amersham International, Piscataway, NJ).

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NOTE ADDED IN PROOF

Additional published data support threading of the substrate through the axial channel.

Weibezahn, J., Tessarz, P., Schlieker, C., Zahn, R., Maglica, Z., Lee, S., Zentgraf, H., Weber-Ban, E.U., Dougan, D.A., Tsai, F.T.F., Mogk, A., and Bukau, B. (2004). Thermotolerance requires refolding of aggregated proteins by substrate translocation through the central pore of ClpB. Cell **119,** 653–665.