Interplay between Heat Shock Proteins HSP101 and HSA32 Prolongs Heat Acclimation Memory Posttranscriptionally in Arabidopsis[1][W][OA]

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Heat acclimation improves the tolerance of organisms to severe heat stress. Our previous work showed that in Arabidopsis (*Arabidopsis thaliana*), the "memory" of heat acclimation treatment decayed faster in the absence of the heat-stress-associated 32-kD protein HSA32, a heat-induced protein predominantly found in plants. The *HSA32* null mutant attains normal short-term acquired thermotolerance but is defective in long-term acquired thermotolerance. To further explore this phenomenon, we isolated Arabidopsis *defective in long-term acquired thermotolerance* (*dlt*) mutants using a forward genetic screen. Two recessive missense alleles, *dlt1-1* and *dlt1-2*, encode the molecular chaperone heat shock protein101 (HSP101). Results of immunoblot analyses suggest that HSP101 enhances the translation of HSA32 during recovery after heat treatment, and in turn, HSA32 retards the decay of HSP101. The *dlt1-1* mutation has little effect on HSP101 chaperone activity and thermotolerance function but compromises the regulation of HSA32. In contrast, *dlt1-2* impairs the chaperone activity and thermotolerance function of HSP101 but not the regulation of HSA32. These results suggest that HSP101 has a dual function, which could be decoupled by the mutations. Pulse-chase analysis showed that HSP101 degraded faster in the absence of HSA32. The autophagic proteolysis inhibitor E-64d, but not the proteasome inhibitor MG132, inhibited the degradation of HSP101. Ectopic expression of HSA32 confirmed its effect on the decay of HSP101 at the posttranscriptional level and showed that HSA32 was not sufficient to confer long-term acquired thermotolerance when the HSP101 level was low. Taken together, we propose that a positive feedback loop between HSP101 and HSA32 at the protein level is a novel mechanism for prolonging the memory of heat acclimation.

To survive and propagate, plants need to respond to various environmental cues by timely implementation of defense mechanisms or triggering of developmental procedures. Appropriate responses to recurrent, sometimes irregular, environmental changes seem to rely on the ability to keep "a memory" of prior exposure to certain conditions for a certain length of time (Bruce et al., 2007; Trewavas, 2009). Prior exposure to moderate heat stress enhances the tolerance of plants to a subsequent challenge with a more severe heat stress, a universal phenomenon called heat acclimation or acquired thermotolerance. However, just how plants keep the memory of heat acclimation is not clear. Our previous studies suggest that a heat-induced 32-kD protein, HSA32, is involved in prolonging the memory of heat acclimation in Arabidopsis (*Arabidopsis thaliana*; Charng et al., 2006).

HSA32 is not a canonical heat shock protein (HSP). Homologs of Arabidopsis HSA32 are only found in land plants and certain microorganisms. In contrast to the multigene families of canonical HSPs, a single-copy gene encodes HSA32 (Liu et al., 2006). Plant HSA32s share about 35% sequence similarity with (2*R*)-phospho-3-sulfolactate synthase, which catalyzes the first step in coenzyme M biosynthesis in *Methanococcus jannaschii*. However, plants do not have homologs of other genes involved in coenzyme M biosynthesis. The origin of *HSA32* in plants is not clear, but horizontal gene transfer has been proposed (Graham et al., 2002). The ubiquitous presence of the *HSA32* gene in plant species suggests that HSA32 may function as a unique protecting agent required for plants.

Reverse genetic studies have shed light on the biological function of HSA32. In the HSA32 null mutant of Arabidopsis, acquired thermotolerance is normally attained after a short recovery of 2 h, following heat acclimation treatment at 37°C for 1 h, but is significantly compromised after a long recovery for 48 h (Charng et al., 2006). Based on this phenotype, acquired thermotolerance attained after a long recovery period was named long-term acquired thermotolerance (LAT), as opposed to the

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Table I. *Phenotype segregation of selfed F2 seedlings from the wild type and dlt1 mutants*

Mutant	Cross to	F2 Phenotype			χ^2	*P*	Inheritance
		Wild Type	Mutant	Total			
dlt1-1	Col-0	285	81	366	1.61	0.2	Recessive
dlt1−2	Col-0	561	188	749	0.004	0.95	Recessive

ordinary short-term acquired thermotolerance (SAT; Yeh et al., 2012). The heat-inducible heat shock transcription factor HSFA2 and a peptidyl-prolyl cis-trans-isomerase (ROF1) have also been shown to be involved in LAT but not SAT (Charng et al., 2007; Meiri and Breiman, 2009). However, the molecular basis for LAT is still unclear.

HSP101 is a molecular chaperone belonging to the ClpB/HSP100 family of AAA+ proteins (Lee et al., 2007; Doyle and Wickner, 2009). HSP104, the yeast (*Saccharomyces cerevisiae*) counterpart of HSP101, was shown to resolubilize heat-denatured proteins from insoluble aggregates (Parsell et al., 1994; Glover and Lindquist, 1998) and confer thermotolerance (Sanchez and Lindquist, 1990). A yeast mutant lacking HSP104 could not acquire thermotolerance and could be complemented by plant HSP101 (Schirmer et al., 1994; Agarwal et al., 2003). In Arabidopsis, HSP101 is required for both SAT and LAT (Hu et al., 2012). In addition to its role in thermotolerance, HSP101 was reported to have translation-enhancing activity (Wells et al., 1998; Ling et al., 2000).

In this study, we used a forward genetic screen to further identify components of LAT. Arabidopsis seedlings defective in LAT were isolated from ethane methyl sulfonate (EMS) mutant pools. Among the isolates, two mutant lines were found to have different missense mutations in *HSP101*. Genetic and biochemical analyses show that HSP101 promoted the accumulation of HSA32 following heat acclimation treatment, and HSA32 in turn retarded the degradation of HSP101. Furthermore, we showed that the two mutations in HSP101 decoupled the regulation of HSA32 from its function in conferring thermotolerance. Our results suggest that the interplay between HSA32 and HSP101 confers more durable acquired thermotolerance, an important feature in plant adaptation to extreme temperature fluctuation.

RESULTS

Isolation of EMS Mutants with a Defective LAT Phenotype

EMS-mutagenized Arabidopsis M2 seedlings were subjected to a modified LAT assay: 3-d-old seedlings were treated at 37°C for 1 h, recovered at 24°C for 2 d, and then challenged at 44°C for 39 min. The conditions were not lethal to the *HSA32* knockout (KO) mutant, *hsa32-1*, yet were strong enough to bleach *hsa32-1* cotyledons but not those of the wild type. Thus, M2 seedlings showing bleached cotyledons were isolated as putatively defective in LAT. After first-round screening of approximately 38,600 M2 seedlings, followed by confirmation of the phenotype in the M3 generation of the putative mutants, seven lines were isolated and named *defective in long-term acquired thermotolerance* (*dlt*). Two mutants, *dlt1* and *dlt2*, were later found to be allelic and thus were renamed as *dlt1-1* and *dlt1-2*, respectively. The *dlt1* mutants were studied in detail as described below. *dlt3* to *dlt7* are mutations on loci different from that of the *dlt1* mutants and were characterized in separate studies.

dlt1-1 and *dlt1-2* Are Recessive Missense Mutations in *HSP101* But Have Different Thermotolerance Phenotypes

The *dlt1* mutants do not show obvious differences in comparison with the wild type at various growth stages under nonstress conditions. LAT assay analysis of the selfed F2 seedlings from a cross between the wild type and the *dlt1* mutants revealed an approximate 3:1

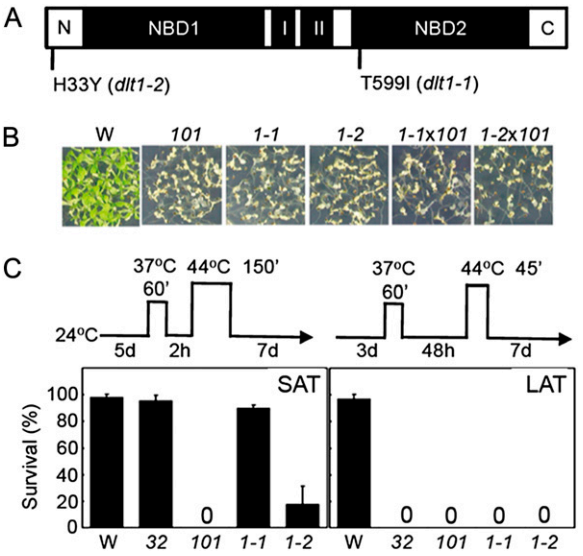

Figure 1. *dlt1-1* and *dlt1-2* missense mutations in *HSP101* result in differential defects in thermotolerance. A, Schematic showing the locations of mutated amino acid residues in *dlt1-1* and *dlt1-2*. N, NTD; NBD, nucleotide-binding domain; I and II, coiled-coil middle domain; C, C-terminal domain. B, Complementation test of the F2 offspring from crosses of *dlt1-1* (*1-1*) or *dlt1-2* (*1-2*) with *hsp101* (*101*). The images show the phenotypes of seedlings of the wild type (W); the indicated lines were subjected to LAT assay and recovery for 10 d. C, SAT and LAT levels of the wild type (W), *hsa32-1* (*32*), *hsp101* (*101*), and the *dlt1* mutant seedlings expressed as viability. Results are presented as mean values of three biological replicates ± SD ($n \geq 50$ each).

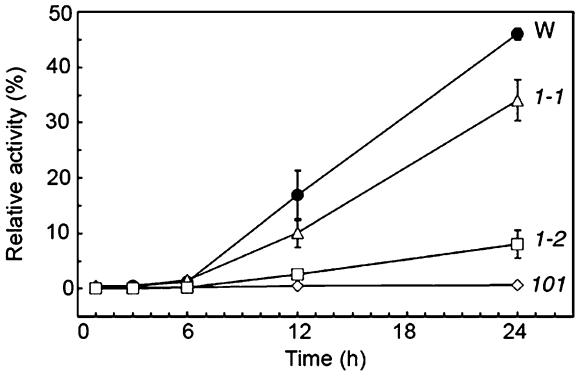

Figure 2. Recovery of Luc activity in transgenic plants after heat treatment. Seven-day-old seedlings of the Luc transgenic lines in wild-type (W), *hsp101* (*101*), *dlt1-1* (*1-1*), and *dlt1-2* (*1-2*) genetic backgrounds were treated at 37°C for 1 h, recovered at 22°C for 2 h, and then challenged at 44°C for 150 min. The Luc activities were measured at 0, 6, 12, and 24 h of recovery following heat treatment. Relative activities in recovery were calculated by using Luc-specific activities taken immediately before the 44°C heat treatment as 100% for each line. The data are means ± SD of three biological replicates.

segregation ratio of wild-type to mutant phenotype (Table I), indicating that *dlt1-1* and *dlt1-2* are caused by single recessive mutations in the nuclear genome. We directly sequenced genomic DNA of the *dlt* mutants at the coding regions of *HSA32*, *HSP101*, *HSFA2*, and *ROF1*, which are involved in LAT. The *dlt1* mutants contained no alteration in the sequences checked with the exception of *HSP101*. In *dlt1-1*, a single C-to-T mutation in *HSP101* caused a replacement of Thr by Ile at position 599, which is located near the N terminus of Nucleotide-Binding Domain2 (NBD2; Fig. 1A). Thr-599 is a conserved residue in cytosolic ClpB/HSP100s in plants (Supplemental Fig. S1). In *dlt1-2*, a C-to-T mutation resulted in a His-to-Tyr substitution at position 33 at the N-terminal domain (NTD). His-33 is conserved in all the ClpB/HSP100 class proteins known to date (Supplemental Fig. S1). On the other hand, the *dlt3* to *dlt7* mutants did not contain alteration in the sequences checked.

To confirm that the point mutations in the *HSP101* locus caused the mutant phenotype, *dlt1-1* and *dlt1-2* were crossed with the *HSP101* transfer DNA (T-DNA) KO line (SALK_066374; hereafter labeled as *hsp101*). All the F2 offspring showed the same defective phenotype in LAT as their parents (Fig. 1B), indicating that the phenotypes observed in *dlt1-1* and *dlt1-2* were due to the identified mutations at the *HSP101* locus.

Since HSP101 is also involved in SAT, we examined whether the *dlt1* mutations caused a defect in this type of thermotolerance in comparison with the wild type, *hsp101*, and *hsa32-1*. Figure 1C shows that *dlt1-2* is defective in both SAT and LAT, as is *hsp101*. In contrast, *dlt1-1* is severely defective in LAT but not in SAT, which is similar to *hsa32-1*. These results suggest that the different mutant alleles of *HSP101* in *dlt1-1* and *dlt1-2* affect SAT and LAT differently.

The Chaperone Function of HSP101 Was Substantially Impaired in *dlt1-2* But Not in *dlt1-1*

The chaperone activity of HSP101 is highly correlated with thermotolerance (Hong et al., 2003). To test HSP101 chaperone activity in these two mutants, we used an in vivo assay of HSP101 activity in Arabidopsis using the thermolabile firefly luciferase (Luc) as a reporter (Hong et al., 2003). Transgenic lines expressing Luc were generated in the wild-type, *hsp101*, *dlt1-1*, and *dlt1-2* backgrounds. The seedlings of homozygous T3 lines were subjected to 37°C for 1 h, recovered for 2 h at 24°C, then challenged at 44°C for 150 min. The HSP101 protein level did not change much after 2 h of recovery in the transgenic plants either in the *dlt1* mutants or the wild-type background (Supplemental Fig. S2). The activity of HSP101 was inferred by measuring Luc activity in the crude extracts of seedlings harvested during the recovery period after the challenge, when Luc activity gradually recovered. In the wild-type background, the Luc activity recovered from undetectable immediately after heat treatment to about 45% of that prior to challenge within 24 h (Fig. 2). In contrast, Luc activity did not recover at all in *hsp101*. In *dlt1-1* and *dlt1-2*, Luc activity recovered to 34% and 7%, respectively.

Since Arabidopsis HSP101 could complement the thermotolerance defect of the yeast mutant lacking HSP104 (Schirmer et al., 1994), the recombinant mutant enzymes were expressed in the yeast mutant for functional analysis. The *HSP101* complementary DNA (cDNA) constructs derived from *dlt1-1*, *dlt1-2*, and the wild type under the control of the yeast *HSP104* promoter were introduced into a yeast mutant with disrupted *HSP104*. Immunoblot analysis showed that the expression of these recombinant proteins was about the same in the transgenic lines (Fig. 3). A thermotolerance

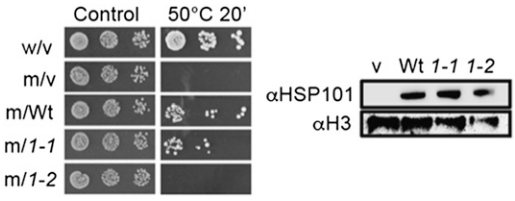

Figure 3. Complementation analysis of wild-type or mutant HSP101 expression in the yeast *hsp104* deletion mutant. Yeast transgenic lines containing different *HSP101* cDNA constructs were subjected to acclimation treatment at 37°C for 1 h and then challenged or not challenged at 50°C for 20 min. The cells were then serially 10-fold diluted and spotted onto agar medium from left to right with increased dilution to assess viability. Labeling of yeast lines is as follows: w/v, wild-type yeast with empty vector; m/v, mutant yeast with empty vector; m/Wt, mutant yeast with vector containing wild-type *HSP101* coding sequence; m/*1-1*, mutant yeast with vector containing *dlt1-1* coding sequence; m/*1-2*, mutant yeast with vector containing *dlt1-2* coding sequence. The panel on the right is an immunoblot of the crude extract of heated (37°C for 1 h) mutant yeast cells containing empty vector (v) or vector containing wild-type *HSP101* (Wt), *dlt1-1* (*1-1*), or *dlt1-2* (*1-2*) coding sequence. The blots were probed with antibodies against Arabidopsis HSP101 and yeast histone H3.

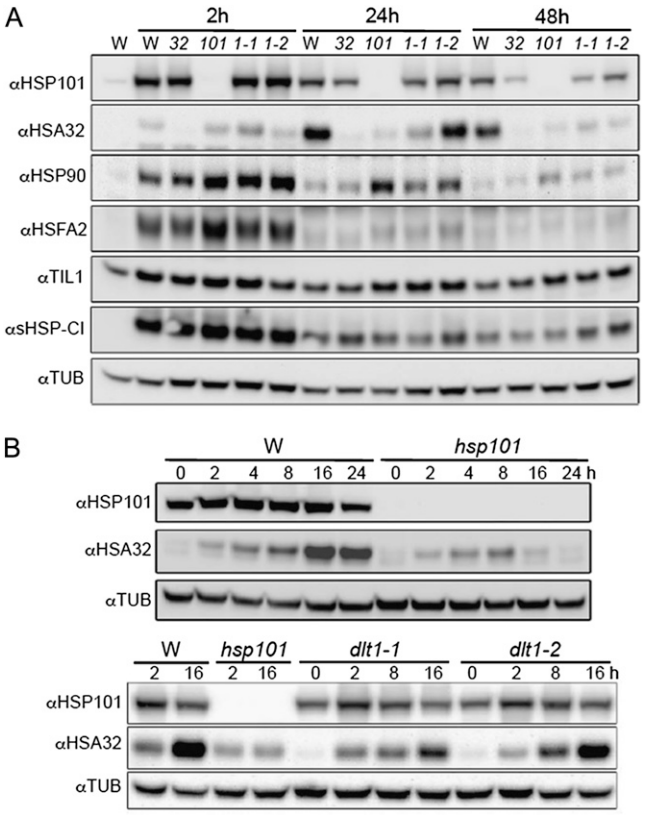

Figure 4. Immunoblot analysis of HSPs in *dlt1*, *hsp101*, and *hsa32-1* mutants reveals interplay between HSP101 and HSA32 during poststress recovery. A, HSPs in the crude extracts of wild-type (W), *hsa32-1* (*32*), *hsp101* (*101*), *dlt1-1* (*1-1*), and *dlt1-2* (*1-2*) seedlings after 2, 24, and 48 h of recovery following heat treatment. B, Time-course analysis of HSA32 levels in wild-type and mutant seedlings, from which the crude extracts were prepared at the indicated recovery times after heat treatment. Tubulin (TUB) was used as a loading control in all panels.

assay showed that *dlt1-1* cDNA could complement the yeast mutant nearly as well as the wild-type cDNA, while *dlt1-2* cDNA could not (Fig. 3). Taken together, these results suggest that the *dlt1-1* allele causes a minor defect in HSP101 chaperone function, whereas *dlt1-2* encodes severely impaired HSP101.

The Stability of HSP101 and Expression of HSA32 Were Simultaneously Reduced in the *dlt1-1* mutant, Revealing Interplay between These Two Proteins

Since the *dlt1-1* mutation did not result in a substantial loss of HSP101 activity, one possible cause of the LAT defect in this mutant could be a decrease in abundance of HSP101 after 48 h of recovery. To test this possibility, we examined HSP101 as well as other HSP levels in the mutant seedlings after a short and long recovery following heat treatment. Immunoblot analyses showed that the level of HSP101 in *dlt1-1* and *dlt1-2* mutants was similar to that of the wild type after recovery for 2 or 24 h following the 37°C, 1-h heat treatment (Fig. 4A). However, HSP101 declined significantly faster in *dlt1-1* than in the wild type when seedlings were allowed to recover for 48 h, whereas the abundance of HSP101 in *dlt1-2* fell between those of the wild type and *dlt1-1* (Fig. 4A). These results suggest that the mutated HSP101s are less stable than the wild-type protein.

Intriguingly, the level of HSA32 was substantially lower in *dlt1-1* and *dlt1-2* than that in the wild type after 48 h of recovery (Fig. 4A). HSA32 level was even lower in the *hsp101* mutant (Fig. 4A). These results suggest that HSP101 positively regulates the accumulation of heat-induced HSA32, which is compromised in the *dlt1* mutants. On the other hand, the decay of HSP101 in *hsa32-1* was faster than that in the wild type (Fig. 4A). HSA32 thus seems to maintain the stability of HSP101 during recovery. Similar interplay to that between HSP101 and HSA32 was not seen in the other HSPs tested (Fig. 4A).

We further compared the HSA32 expression pattern in the wild type, *hsp101*, and the *dlt1* mutants at additional time points. We found that the initial accumulation of low levels of HSA32 during 2 to 8 h of recovery after heat treatment was relatively independent of HSP101 (Fig. 4B). However, HSA32 continued to increase at 16 h of recovery in the wild type but dramatically decreased in *hsp101* (Fig. 4B). Another T-DNA KO mutant of *HSP101*, *hot1-3*, showed a similar result (Supplemental Fig. S3). The diminished HSA32 protein level in *hsp101* was not due to the decreased abundance of *HSA32* transcripts (Fig. 5), suggesting translational or posttranslational regulation of HSA32 by HSP101. In *dlt1-2*, HSA32 reached higher levels than in *dlt1-1*, and the level was similar to that seen in the wild type (Fig. 4B), suggesting that the mutation in *dlt1-1*, but

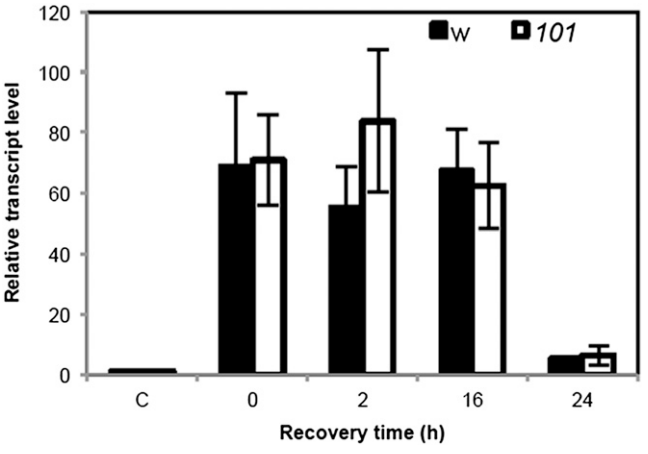

Figure 5. The transcript level of *HSA32* was not affected in the absence of HSP101. Real-time RT-PCR was performed to analyze *HSA32* transcripts in wild-type (W) and *hsp101* (*101*) seedlings in a time course of recovery from 0 to 24 h after heat treatment at 37°C for 1 h. Relative transcript levels of *HSA32* were obtained by normalization to the nonheated wild-type control (C). Results are presented as mean values of three replicates ± SD.

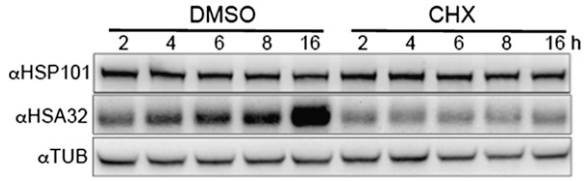

Figure 6. The effect of cycloheximide on the accumulation of HSA32. After heat treatment, cycloheximide (CHX) was applied to the wild-type seedlings after recovery at 22°C for 2 h. The seedlings were allowed to recover for the indicated times before harvest for immunoblotting. The same volume of DMSO solution was applied to the seedlings as the control. In these experiments, 4-d-old seedlings were used for the heat treatment (37°C for 1 h).

not *dlt1-2*, compromises the regulation of HSA32 production.

Poststress Accumulation of HSA32 Is Blocked by Cycloheximide

A previous report indicated that HSP101 could enhance translation by an unknown mechanism that requires the initiation factor eIF4G (Wells et al., 1998). To examine whether translation is involved in HSP101-dependent HSA32 accumulation, the translation inhibitor cycloheximide was applied to heat-treated wild-type seedlings after 2 h of recovery. Figure 6 shows that cycloheximide but not dimethyl sulfoxide (DMSO) control treatment prevented the accumulation of HSA32, suggesting that translation is required for HSA32 to accumulate. To determine whether the translation of HSA32 requires eIF4G in Arabidopsis, the level of HSA32 in the T-DNA KO mutant of *eIF4G*, as well as in the double KO mutant of *eIF(iso)4G1* and *eIF(iso)4G2*, homologs of *eIF4G*, was measured after heat treatment. However, none of these mutants showed different expression patterns of HSA32 from that of the wild type (Supplemental Fig. S4).

Degradation of HSP101 Is Accelerated in the Absence of HSA32 But Reduced in the Presence of the Autophagic Proteolysis Inhibitor E-64d

In Figure 4A, the results suggest that HSA32 retards the degradation of HSP101. To test this hypothesis, we compared the degradation of HSP101 in wild-type, *hsa32-1*, and *dlt1* mutant plants by pulse-chase analysis. The seedlings were fed with a ^{35}S isotope-labeled Met and Cys mixture during heat treatment and chased with cold amino acids during recovery. Our data show that pulse-labeled HSP101 accumulated to the same level at 2 h of recovery in the wild type and the mutants but diminished faster in the *hsa32-1* and *dlt1* mutants at 48 h of recovery (Fig. 7).

In an attempt to reveal how HSP101 is degraded in plant cells, we employed inhibitors of two protein degradation pathways, the proteasome inhibitor MG132 and the autophagic proteolysis inhibitor E-64d. Applying MG132 to wild-type or *hsa32-1* seedlings before or after heat treatment did not prevent the degradation of HSP101, as shown by the immunoblots (Supplemental Fig. S5, A and B), suggesting that HSP101 was not degraded via the proteasome-mediated pathway. The effect of MG132 was confirmed by the inhibition of hypocotyl elongation in seedlings grown in the dark (Supplemental Fig. S5C), consistent with a previous report (Wang et al., 2009). On the other hand, application of E-64d retarded the degradation of HSP101 in the wild-type and *hsa32-1* seedlings (Fig. 8). HSA32 level

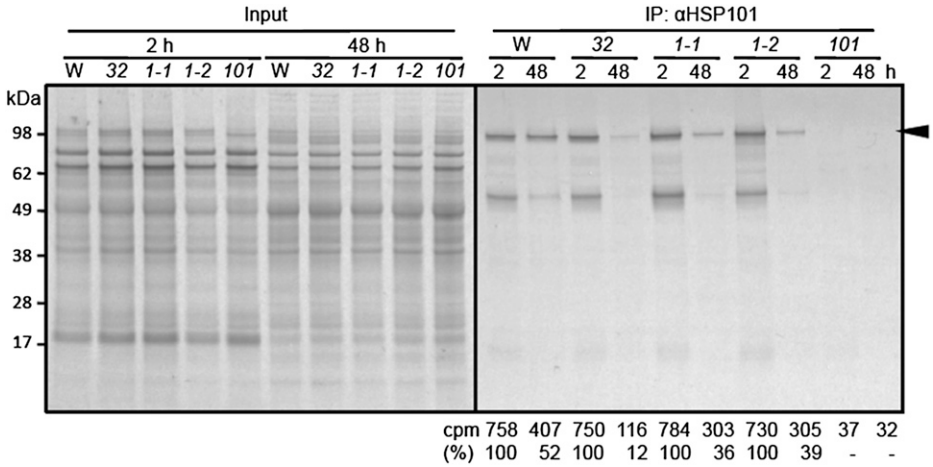

Figure 7. Pulse-chase analysis of HSP101 degradation in *hsa32-1* and *dlt1* mutants. Seedlings were heat treated and pulse-chase labeled with [^{35}S]Met/Cys as described in "Materials and Methods." The isotope-labeled HSP101 (arrowhead) in wild-type (W), *hsa32-1* (*32*), *dlt1-1* (*1-1*), and *dlt1-2* (*1-2*) seedlings after 2 and 48 h of recovery was immunoprecipitated from their crude extracts with specific antibodies. The total proteins (Input) and the immunoprecipitated HSP101 protein (IP:αHSP101) were resolved by SDS-PAGE and visualized by autoradiography. The HSP101 bands were sliced out afterward for radioactive counts in a scintillation counter. The absolute and relative radioactivities (cpm) are given at the bottom of the gel.

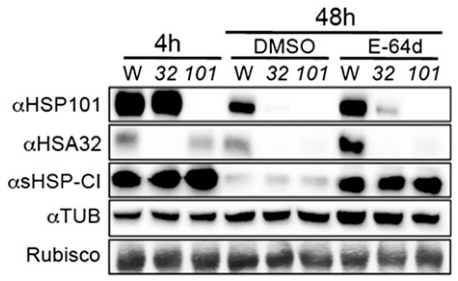

Figure 8. Proteolysis inhibitor E-64d retards the degradation of HSP101 and HSA32. Seedlings were treated with E-64d or DMSO as a control after 4 h of recovery following heat treatment at 37°C for 1 h and harvested after 48 h of recovery at 22°C. The effects of E-64d on the levels of HSP101, HSA32, and sHSP-CI in the wild type (W), *hsa32-1* (*32*), and *hsp101* (*101*) were assessed by immunoblots. Immunoblots of tubulin (TUB) and Coomassie blue stain of Rubisco are shown as loading controls.

was also higher in the heat-stressed wild-type and *hsp101* seedlings treated with E-64d, suggesting that HSA32 is also degraded via the autophagy-mediated pathway. However, E-64d did not inhibit the degradation of HSP101 and HSA32 in the *hsa32-1* and *hsp101* mutants, respectively, as effectively as it inhibited the degradation of class I small HSPs.

Late Induction of HSA32 Is Insufficient to Confer LAT When HSP101 Has Decayed to a Low Level

The positive interplay between HSA32 and HSP101 makes it difficult to determine whether HSA32 alone is sufficient to confer LAT, as the levels of both proteins are cohesively high in the wild type or simultaneously low in the mutants after a long recovery (Fig. 4A). To avoid this dilemma, we generated transgenic line Ei32, which harbors an estradiol-inducible *HSA32* cDNA construct in the *hsa32-1* background, so that HSA32 expression could be induced to a high level by estradiol when HSP101 level becomes low. In the absence of estradiol, the transgenic line showed a similar thermotolerance phenotype to that of *hsa32-1*, despite having a leaky but low expression of HSA32 (Supplemental Fig. S6). We observed that estradiol induced the production of HSA32 and retarded the degradation of HSP101 in Ei32 (Fig. 9A). As a control, estradiol did not significantly affect the levels of HSA32 or HSP101 in the wild type. Consistent with the protein levels, estradiol treatment rescued the LAT defect in Ei32 but not in *hsa32-1* (Fig. 9B). On the other hand, estradiol increased the transcript level of *HSA32* in Ei32 but not that of *HSP101* (Supplemental Fig. S7). These results indicate that the estradiol-inducible HSA32 was functional in the transgenic plants. To see whether HSA32 is sufficient for LAT, we tested the effect of a late induction of HSA32 after 46 h of recovery following heat treatment, when HSP101 level is lower in Ei32 than in the wild type without estrogen induction (data not shown). The high level of estrogen-induced HSA32 at this time could neither restore HSP101 to the wild-type level nor rescue the LAT defect (Fig. 9, C and D), suggesting that HSA32 alone is insufficient to confer LAT when HSP101 level is low.

We then generated an *hsp101 hsa32-1* double KO mutant to see whether the loss of both gene functions caused an additive effect. The double KO mutant was slightly more defective than *hsp101* in recovery growth, but no significant difference in hypocotyl elongation was seen under LAT assay conditions (Fig. 10). However, the defects in both recovery growth and hypocotyl elongation were much more severe in the double mutant than in *hsa32-1*. These data suggest that HSA32 exerts its effect on LAT mainly, if not solely, through reducing the decay rate of HSP101.

DISCUSSION

Acclimation to various environmental changes is crucial for plant survival and reproduction. However, how plants remember a stress acclimation experience is not

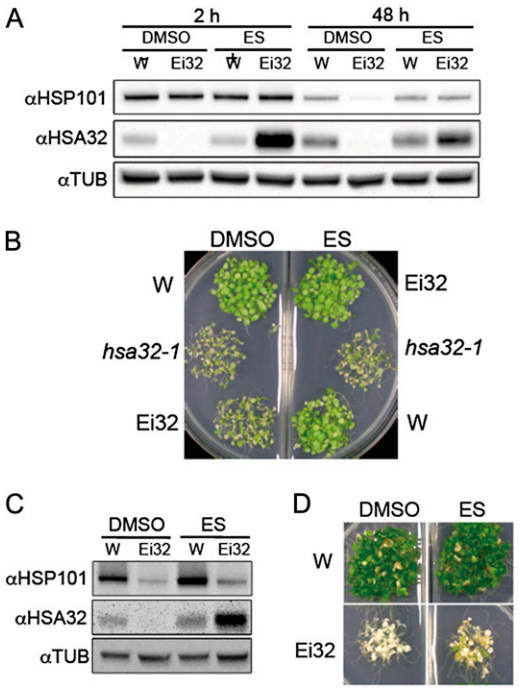

Figure 9. The temporal effects of HSA32 expression on HSP101 degradation and LAT. A, Immunoblots of HSP101 and HSA32 proteins in wild-type (W) and pEi32 transformant (Ei32) seedlings after 2 and 48 h of recovery following heat treatment at 37°C for 1 h. The seedlings were grown on medium with 1 μM estradiol (ES) or 0.1% DMSO solvent control. Tubulin (TUB) is shown as a loading control. B, The LAT phenotypes of wild-type, *hsa32-1*, and pEi32 seedlings in the presence and absence of 1 μM estradiol in the medium. C, Immunoblots of HSP101 and HSA32 in wild-type and pEi32 seedlings during 48 h of recovery after heat treatment. Ten micromolar estradiol solution or DMSO solution was applied to the heat-stressed seedlings after 46 h of recovery. D, The LAT phenotypes of wild-type and pEi32 seedlings with or without late induction with 10 μM estradiol as in C.

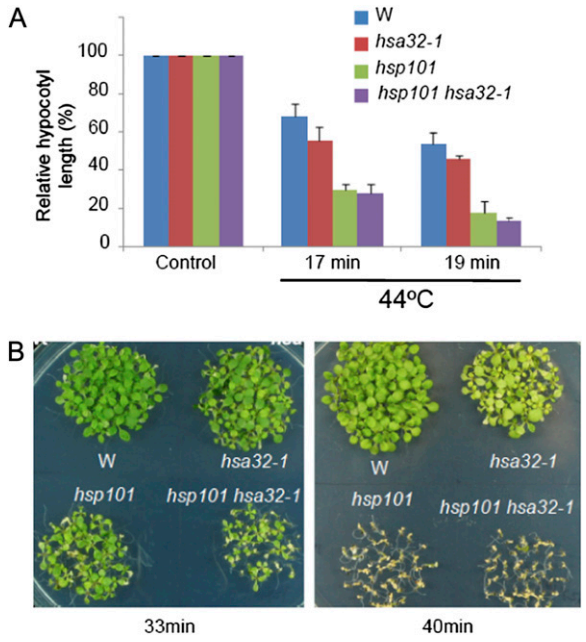

Figure 10. Epistatic analysis of the relationship between *HSP101* and *HSA32* by hypocotyl elongation and recovery growth assays. A, Dark-grown 2-d-old seedlings were heat acclimated at 37°C for 1 h, followed by recovery at 22°C for 48 h. Seedlings either remained at 22°C or were subjected to heat treatment at 44°C for 17 or 19 min. The elongation of hypocotyls during 2.5 d of recovery after 44°C treatment was measured and normalized to that without the severe heat treatment. Results are presented as mean values of three replicates ± SD ($n = 25$). B, Recovery growth of wild-type (Wt), *hsp101*, *hsa32-1*, and *hsp101 hsa32-1* plants after LAT assay treatment. The seedlings were subjected to LAT assay as described in "Materials and Methods" except that the plants were treated at 44°C for 33 or 40 min. The growth of heat-treated plants was assessed after 10 d of recovery at the normal condition.

yet understood. Recently, transcriptional memory to drought acclimation mediated by chromatin modification has been shown in Arabidopsis (Ding et al., 2012). Here, we demonstrated another example in which acclimation memory can be modulated by the interplay of stress proteins at the posttranscriptional level.

Using a forward genetic approach, we identified Arabidopsis *HSP101* missense mutations that cause a severe defect in LAT. So far, our screening did not find any mutant associated with *HSA32*, *HSFA2*, and *ROF1*, which were also shown to positively regulate LAT (Charng et al., 2006, 2007; Meiri and Breiman, 2009). It is likely that the mutation in HSP101 causes severe defects that are much easier to discover than the mutations in other genes. Isolation of the *dlt1* mutants provided new materials for the investigation of the structure-function relationship of HSP101. First, *dlt1-2* with a mutation in a highly conserved His residue (H33Y) of the NTD domain represents a mutation in the ClpB/HSP100 family of proteins that has not been reported previously (Supplemental Fig. S1). The NTD was shown to be dispensable for chaperone activity in *Escherichia coli* and *Thermus thermophilus* (Beinker et al., 2002; Mogk et al., 2003) and for thermotolerance in cyanobacteria and yeast (Clarke and Eriksson, 2000; Hung and Masison, 2006). However, work on *E. coli* ClpB showed that truncation of the NTD causes severe defects in molecular chaperone activity in vitro (Barnett et al., 2000; Li and Sha, 2003). Our results indicate that the NTD of Arabidopsis HSP101 is involved in thermotolerance and chaperone activity (Figs. 1–3).

The other HSP101 missense mutation, *dlt1-1* (T599I), which is located near the N-terminal end of NBD2, had only a minor effect on SAT (Fig. 1C) and chaperone activity (Fig. 2) but resulted in a substantial reduction in the poststress level of HSA32 (Fig. 4) and LAT (Fig. 1C). This is essentially the opposite phenotype to *dlt1-2*, which has severely reduced SAT and chaperone activity but shows a much less severe effect on the production of HSA32 at an early phase of recovery. These results suggest that the regulation of HSA32 accumulation by HSP101 does not correlate with its chaperone activity or function in thermotolerance and, therefore, that HSP101 has a dual function. In the *dlt1* mutants, regulation of HSA32 is decoupled from the thermotolerance function of HSP101. Of note, yeast HSP104 also has been reported for having dual functions in thermotolerance and the propagation of [*PSI*$^+$] prion, which could be decoupled by mutations (Takahashi et al., 2007).

The diminished accumulation of HSA32 in *dlt1-1* suggests that replacing Thr-599 by Ile disrupts the regulatory function of HSP101. Of note, the organelle-localized and prokaryotic ClpB/HSP100 homologs have an Ile residue at the position corresponding to Thr-599 in plant HSP101 (Supplemental Fig. S1). Thus, it is not unexpected that the T599I mutation in *dlt1-1*

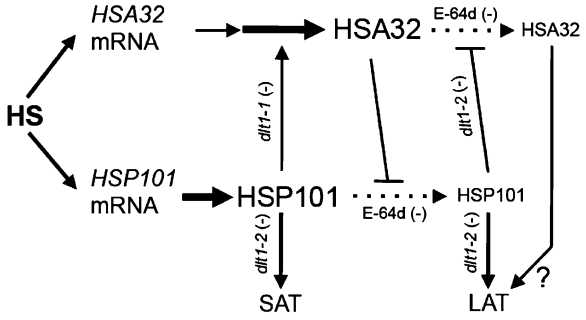

Figure 11. Model depicting the interplay between HSP101 and HSA32 and the effect of *dlt1-1* and *dlt-2* mutations. Heat shock (HS) induces the synthesis of *HSP101* and *HSA32* mRNA for the translation of corresponding proteins. HSP101 enhances the translation of HSA32 (thick arrow), and the *dlt1-1* mutation causes a reduction in this function. HSA32 retards the degradation (dotted arrow) of HSP101. The *dlt1-2* mutation causes a severe reduction in HSP101 chaperone function, which negatively affects SAT and LAT. HSP101 probably delays HSA32 degradation, which is compromised in *dlt1-2*. The reduced font sizes of HSP101 and HSA32 symbolize the reduced protein levels. The minus signs in parentheses indicate reduction in function or inhibition of a process caused by mutations or chemicals. The question mark indicates the uncertainty of a direct action.

led to only a minor loss in chaperone activity. It should be noted that the Thr residue is highly conserved in cytosolic ClpB/HSP100 in land plants and that HSA32 is also conserved in land plants (Liu et al., 2006). It is possible that the interplay between these two proteins was established during the terrestrial colonization of plants.

The effect of cycloheximide on HSP101-dependent HSA32 accumulation (Fig. 6) prompts us to speculate that HSP101 controls the translation of HSA32. Recently, HSP101 was identified in Cap-binding complexes of germinating maize (*Zea mays*; Lázaro-Mixteco et al., 2012), suggesting a role for HSP101 in protein synthesis. A role for HSP101 in translation regulation has been proposed by Gallie and coworkers (Wells et al., 1998; Gallie, 2002). However, direct genetic evidence to support this notion is still lacking. According to their working model, HSP101 could bind to the 5′ untranslated region (UTR) of *HSA32* mRNA and recruit the eukaryotic initiation factors eIF4E and eIF4G, as is the case for the tobacco mosaic virus RNA. However, the 5′ UTR of the Arabidopsis *HSA32* mRNA does not contain a sequence similar to the poly(CAA) element in Ω that has been identified as the HSP101 binding site (Tanguay and Gallie, 1996). Moreover, we found that neither eIF4G nor eIF(iso)4G proteins seem to be involved in the translation of HSA32 (Supplemental Fig. S4). However, a redundant role of these two isoforms in regulating the translation of HSA32 could not be excluded, as we did not check the triple KO mutant of *eIF4G*, *eIF(iso)4G1*, and *eIF(iso)4G2*, which could be lethal in Arabidopsis (Nicaise et al., 2007). In addition to translation, HSP101 may retard the degradation of HSA32. This is supported by the observation that HSA32 declines faster in *dlt1-2* than in the wild type after reaching similarly high levels at 16 h of recovery following heat treatment (Fig. 4). More experiments are needed to elucidate this complex relation in detail.

In this study, we showed that HSA32 possesses a unique function in affecting the decay of HSP101. The effect of HSA32 on HSP101 degradation is likely to be specific. Our data show that the absence of HSA32 led to no significant alteration in other tested HSPs (Fig. 4A). Occasionally, we observed faster degradation of sHSP-CI in the absence of HSA32 in some experiments, but the results were not as reproducible as those for HSP101. The mechanism by which HSA32 retards HSP101 degradation, and if it requires direct interaction of the two proteins, are not clear. We did not observe a protein corresponding in size to HSA32 in an immunoprecipitation of HSP101 (Fig. 7). This negative result suggests that HSA32 and HSP101 do not interact, but it is also very possible that their interaction was disrupted under our experimental conditions. Maize HSP101 was shown to coimmunoprecipitate with cytosolic HSC70 (Zhang and Guy, 2005). However, no distinct protein band corresponding to HSC70 was detected either. Nevertheless, a protein band of 54 kD was coimmunoprecipitated with HSP101 (Fig. 7). This band probably is a product of partially degraded HSP101, as it was not observed in *hsp101*. Alternatively, it may be a protein specifically interacting with HSP101. The identity of this protein band will be determined later.

Here, we showed that the decay of the memory of heat acclimation, as manifested by the decay of acquired thermotolerance, is negatively regulated by interplay between HSP101 and HSA32. This interplay consists of a positive feedback loop: HSP101 promotes the accumulation of HSA32 by affecting its synthesis and degradation, which subsequently retards the degradation of HSP101 (Fig. 11). By controlling HSP101 degradation, HSA32 prolongs the effect of heat acclimation, allowing plants to cope with rapid temperature changes in the terrestrial environment. This hypothesis is in line with recent studies of HSP101 effects in 10 Arabidopsis ecotypes, which suggest a primary role of HSP101 in emergency heat tolerance (Tonsor et al., 2008). In rice (*Oryza sativa*), heat-induced HSP101 decayed differentially in *indica* and *japonica* types (Agarwal et al., 2003), suggesting a role of regulating HSP101 decay in the adaptation to different climates. Recently, using rice *Tos17* retrotransposon insertion lines, we observed that disruption of *HSA32* also led to faster decay of HSP101 and to defective LAT, as was the case in Arabidopsis (M.-y. Lin and Y.-y. Charng, unpublished data). Further studies on the structure-function relationships of HSA32 and HSP101 in Arabidopsis and other plant species should provide more insight into this thermotolerance mechanism.

MATERIALS AND METHODS

Plants Materials and Mutant Screen

The Arabidopsis (*Arabidopsis thaliana*) T-DNA KO mutants *hsp101*, *hot1-3*, and *hsa32-1* were as described previously (Lee et al., 2005; Charng et al., 2006). The double KO mutant *hsp101 hsa32-1* was generated by crossing the single mutant lines and isolated from the offspring in the F2 generation. The T-DNA KO mutant of *eIF4G* and double KO mutants of *eIF(iso)4G1* and *eIF(iso)4G2* were described in detail previously (Nicaise et al., 2007) and confirmed by PCR analysis with gene-specific primers. EMS-mutagenized M2 seeds (Columbia [Col-0] ecotype) were purchased from Lehle Seeds. To screen for LAT-defective mutants, the M2 seedlings were subjected to heat treatment as described in "Results." The isolated mutants were transplanted to soil for M3 seed propagation. Plants were grown under the conditions described previously (Charng et al., 2006).

DNA and Protein Sequence Analysis

The genomic DNA sequences of *HSP101*, *HSA32*, *HSFA2*, and *ROF1* in the *dlt1* mutants were examined by PCR amplification and direct sequencing at both strands. Nucleotide sequence alignment was performed by using the AlignX program (Vector NTI 9.1.0; Invitrogen) to identify mutations. Amino acid sequences of ClpB/HSP100 homologs were from the public database of the National Center for Biotechnology Information and aligned with the HSP101 sequences of *dlt1-1* and *dlt1-2* using the ClustalW program in MEGA5 (Tamura et al., 2011). The classification of ClpB/HSP100 homologs mentioned in this work was described previously (Lee et al., 2007).

Genetic Analyses

The M3 plants of *dlt1-1* and *dlt1-2* were crossed to the wild-type Col-0 plants and *hsp101* separately. F2 seedlings from the cross between the *dlt1* mutants and the wild type were subjected to LAT assay to determine the

dominance of the mutant alleles by segregation rate. F2 seedlings from the cross between the *dlt1* mutants and *hsp101* were subjected to the LAT assay for the functional complementation study.

Plasmid Construction for Expressing HSP101 in Yeast

The full-length cDNAs of the wild type and *dlt1-1* and *dlt1-2* mutant alleles of Arabidopsis *HSP101* were amplified by reverse transcription (RT)-PCR using primers 5′-GCGGCCGCTTAATCCTCGATC-3′ and 5′-GCATGCATGAATCCAGAGAAATTC-3′. The promoter of yeast (*Saccharomyces cerevisiae*) *HSP104* was amplified by PCR using primers 5′-GGGTCAAAGCTTCGATTCAAAGGCGTTATTCAGCAT-3′ and 5′-GACTCAGCATGCATATTCTGTATATTTTATGGTACG-3′. PCR products were introduced into pGEM-T Easy vector (Promega) for sequencing confirmation. The cDNA and promoter were ligated in the intermediate plasmid and then subcloned to the YEp195 expression vector by replacing the *Hin*dIII/*Not*I fragment from YEp-CUP1-N1-GFP-T (King and Diaz-Avalos, 2004) to yield plasmids Yep-HSP104p-HSP101-T, Yep-HSP104p-DLT1-1-T, and Yep-HSP104p-DLT1-2-T for complementation analysis.

Thermotolerance Assays

For Arabidopsis, the SAT and LAT assays using viability or hypocotyl elongation as the output trait were performed as described previously (Charng et al., 2006). For yeast, the cells were first grown in the synthetic complete medium lacking uracil to maintain plasmid at 30°C to an optical density at 600 nm of 0.6 to 0.8. The cultures were then transferred to 37°C for 1 h, then with or without heat treatment at 50°C for 20 min, and then cooled on ice for 5 min. Cells were mixed by vortex, serially diluted, and spotted on the synthetic complete medium lacking uracil for viability assessment.

RNA Analysis

Quantitative analysis of *HSA32* and *HSP101* transcripts was performed using real-time RT-PCR as described previously (Liu et al., 2011).

Immunoblotting

The levels of HSPs in the plant samples were extracted and determined by immunoblotting with specific antibodies as described previously (Chi et al., 2009; Liu et al., 2011).

Generation of Transgenic Plants with the Luc Reporter Gene and Luc Activity Assay

Arabidopsis plants of Col-0, *hsp101*, *dlt1-1*, and *dlt1-2* were transformed with a modified Luc reporter gene in a plasmid, pB2GW7-LUCmc, by the floral dip method (Clough and Bent, 1998). The plasmid carries a modified firefly *Luc* cDNA derived from pJD301 (Luehrsen et al., 1992), which is fused downstream of the cauliflower mosaic virus 35S promoter in the pB2GW7 binary vector (Karimi et al., 2002). The modified *Luc* cDNA lacks the coding sequence for the three C-terminal amino acid residues required for peroxisomal targeting (Gould et al., 1989). Selection of transgenic plants was performed as described previously (Charng et al., 2007), and T3 generation seeds of a homozygous line with a single T-DNA insertion event were used for further studies. Luc activities in plant extracts derived from seedlings after heat treatment were determined by using a Luc assay kit according to the protocol of the manufacturer (Promega). Protein concentrations of samples were determined by using the DC protein assay kit (Bio-Rad), and the specific activities of Luc were estimated. The relative Luc activity (%) represents the ratio of specific activities of Luc determined immediately before and after a 44°C heat shock at each time point in each line.

Chemical Treatments

For poststress chemical treatment, about 50 heat-treated seedlings on solid medium were immersed in 50 μM cycloheximide, 50 μM MG132, or 100 μM E-64d in 0.1% DMSO solution for 15 min after evenly applying 250 μL of the solution onto the seedlings. Excess chemical solution was removed from the seedlings by pipetting. For control samples, 0.1% DMSO solution was applied in the same way. The seedlings were harvested for immunoblot analysis at the indicated time after treatment (as shown in Figs. 6 and 8). For prestress MG132 treatment, the compound was added to the one-half-strength Murashige and Skoog agar medium at a final concentration of 100 μM. Etiolated seedlings grown on the MG132-containing plate were treated at 37°C for 1 h and recovered for the indicated time at 22°C (as shown in Fig. S5) before harvest for protein extraction. The effect of MG132 was confirmed by the phenotype of retarded hypocotyl elongation, as described previously (Wang et al., 2009).

Pulse-Chase Analysis

Three-day-old seedlings grown on solid medium were fed with 1 μCi μL^{-1} [^{35}S] Met and [^{35}S]Cys (EasyTag Express Protein Labeling Mix; Perkin-Elmer) solution, then treated at 37°C for 1 h. Immediately after heat treatment, seedlings were removed from medium and washed three times with 5 mM nonradioactive Met and Cys solution for 2 min and transferred to a new agar plate for recovery at 22°C for 2 and 48 h. Labeled plant tissues were homogenized with buffer A (50 mM Tris-Cl, pH 7.5, 1 mM EDTA, 100 mM NaCl, 0.1% Triton X-100, 5% glycerol, and protease inhibitor cocktail [Sigma]). The homogenate was centrifuged at 13,000*g* for 30 min at 4°C. The supernatant was then centrifuged twice at 13,000*g* for 30 min. The protein concentration of the crude extract was determined by using the DC protein assay kit (Bio-Rad), and 1 mg of the protein extract was incubated with 50 μL of magnetic beads (1:1 mixture of PureProteome Protein A/G Magnetic Beads; Millipore) with immobilized anti-HSP101 antibody for 2.5 h at room temperature. The suspension was rinsed three times with 500 μL of buffer A. After the last wash, the beads was mixed with 45 μL of SDS sample buffer (60 mM Tris-Cl, 2% SDS, 2.5% glycerol, and 0.1 mM EDTA) and heated at 70°C for 10 min. An equal volume of the resulting supernatant was loaded onto a 4% to 12% SDS-PAGE minigel (Invitrogen). Following electrophoresis, the gels were transferred to Whatman 3MM chromatography paper, covered with plastic wrap, and vacuum dried using a slab gel drier (Bio-Rad). Isotope-labeled proteins were visualized by exposure to x-ray film.

Generation of Ei32 Transgenic Lines

The *hsa32-1* plant was transformed with *Agrobacterium tumefaciens* carrying pEi32 by the floral dip method. For preparation of the pEi32 construct, the full-length coding sequence of *HSA32* including the 3′ UTR was amplified by RT-PCR using primers 5′-ATGGCGGCTTACTACAGATGGAAGA-3′ and 5′-GATCACCGAAAAATCGCATATCACA-3′. The *HSA32* cDNA fragment was introduced into the Gateway entry vector pCR8 (Invitrogen) for sequencing confirmation and then subcloned to the estrogen-inducible expression vector pMDC7 (Zuo et al., 2000) to produce pEi32. T3 homozygous plants with single insertion events were used for studies. To induce the production of recombinant HSA32 in transgenic plants, β-estradiol of the indicated concentration was either added to the growth medium or at the indicated time (as shown in Fig. 9).

Detailed information about the genes mentioned in this article can be found in The Arabidopsis Information Resource database or the GenBank/EMBL database under the following accession numbers: *HSA32* (AT4G21320), *HSP101* (AT1G74310), *eIF4G* (AT3G60240), *eIF*(*iso*)*4G1* (AT5G57870), *eIF*(*iso*)*4G2* (AT2G24050).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Sequence alignment of ClpB/HSP100 family members from various species near the mutated sites in *dlt1-1* and *dlt1-2*.

Supplemental Figure S2. HSP101 protein levels in the luciferase transgenic lines.

Supplemental Figure S3. Accumulation of HSA32 during poststress recovery in two T-DNA KO lines, *hsp101* and *hot1-3*.

Supplemental Figure S4. The effect of disrupting the translational initiation factors on the poststress synthesis of HSA32.

Supplemental Figure S5. The effect of proteasome inhibitor MG132 on the degradation of HSP101.

Supplemental Figure S6. Characterization of the estradiol-inducible HSA32 (Ei32) transgenic line.

Supplemental Figure S7. The transcript levels of *HSP101* and *HSA32* in the wild type and the Ei32 seedlings.

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