A novel plant E3 ligase stabilizes *Escherichia coli* heat shock factor $\sigma^{32}$

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The heat shock response is crucial for organisms against heat-damaged proteins and maintaining homeostasis at a high temperature. Heterologous expression of eukaryotic molecular chaperones protects *Escherichia coli* from heat stress. Here we report that expression of the plant E3 ligase BnTR1 significantly increases the thermotolerance of *E. coli*. Different from eukaryotic chaperones, BnTR1 expression induces the accumulation of heat shock factor $\sigma^{32}$ and heat shock proteins. The active site of BnTR1 in *E. coli* is the zinc fingers of the RING domain, which interacts with DnaK resulting in stabilizing $\sigma^{32}$. Our findings indicate the expression of BnTR1 confers thermoprotective effects on *E. coli* cells, and it may provide useful clues to engineer thermophilic bacterial strains.

The heat shock response (HSR) is a universal signalling pathway in all organisms that maintains protein-folding homeostasis through the regulation of heat shock proteins (HSPs)$^{1,2}$. Although the HSR varies among species, a striking common feature is the rapid induction of evolutionarily conserved HSPs, including the chaperones and proteases that perform protein refolding and degradation, thereby protecting cells from stress-induced protein misfolding or aggregation$^{3,4}$. In *Escherichia coli*, the HSR is a complex circuit controlled by the alternative sigma factor ($\sigma^{32}$), encoded by *rpoH*, which guides RNA polymerase to HSP gene promoters in heat stress$^{5-7}$. In the canonical *E. coli* HSR, HSP synthesis rapidly increases owing to the transient accumulation of $\sigma^{32}$ (induction phase) and then gradually decreases during the adaptation phase to achieve a new steady state$^{8,9}$. During the induction phase, $\sigma^{32}$ synthesis is primarily regulated at the translational level, as heat opens an inhibitory region of *rpoH* mRNA$^{10-12}$, and $\sigma^{32}$ activity and stability increase$^{13}$. During the adaptation phase, the cytoplasmic chaperone teams DnaK/DnaJ/GrpE (KJE) and GroEL/GroES negatively regulate $\sigma^{32}$ activity by sequestering $\sigma^{32}$ from RNA polymerase$^{14-18}$. In addition, $\sigma^{32}$ stability is primarily controlled by the inner membrane protease FtsH$^{17,18}$. Recent studies have demonstrated that the interaction between the signal recognition particle (SRP) and $\sigma^{32}$ is indispensable for $\sigma^{32}$ localization at the cell membrane$^{19,20}$. It is widely accepted that a negative feedback loop exists such that HSR chaperones and proteases titrate free $\sigma^{32}$ by binding or degrading unfolded proteins, meanwhile the up-regulated $\sigma^{32}$ increases the transcription of HSPs which subsequently decrease the $\sigma^{32}$ activity and stability, thus facilitating *E. coli* cell viability and proliferation under heat stress$^{19}$.

In addition to endogenous HSPs, the heterologous expression of eukaryotic molecular chaperones increases *E. coli* cell viability at high temperatures$^{21-24}$. There is extensive support for the enhanced thermotolerance of transformed *E. coli* cells expressing plant small HSPs (sHSPs; 12–43 kDa), such as Oshsp16.9$^{21}$, CshSP17.5$^{22}$, and RcHSP17.8$^{23}$. Recent studies have shown that expression of CehSP17, a *Caenorhabditis elegans* sHSP, enables *E. coli* cell survival at lethal temperatures$^{24,25}$. In addition, the introduction of plant late embryogenesis abundant proteins$^{26}$ and human disulfide-isomerase$^{27}$ confers protection against heat stress to *E. coli* cells. Although the thermoprotective properties of various exogenous proteins have been extensively reported, the acquired thermotolerance is largely attributed to their conserved chaperone functions, raising the question of whether other types of eukaryotic proteins have similar protective effects.

Here, we report that heterologous expression of a RING (Really Interesting New Gene) domain E3 ligase from *Brassica napus*, named BnTR1, conferred pronounced thermoprotection on *E. coli* cells. BnTR1 dramatically increased the expression of numerous *E. coli* HSPs under both normal and heat stress conditions. Further
experiments revealed that BnTR1 expression induced the accumulation of heat shock factor $\sigma^{32}$. However, unlike molecular chaperones such as SHsPs, the RING domain of BnTR1 was the active site for its function in E. coli. We found that two zinc fingers in the RING domain were able to interact with DnaK and $\sigma^{32}$, respectively, resulting in $\sigma^{32}$ stabilization. Together, our findings reveal that heterologous expression of BnTR1 provides thermoprotective effects on E. coli cells, and it may yield useful insights into the development of engineered thermophilic bacteria.

Results
Heterologous expression of BnTR1 enhances Escherichia coli thermotolerance and up-regulates HSPs. Our previous study demonstrated that BnTR1 plays a key role in conferring thermal resistance among multiple plant species28. Surprisingly, we observed a similar trend when we expressed BnTR1 in E. coli. There was little change in growth rates between pET and pET-BnTR1 cells at the normal temperature (Fig. 1a), while transformed cells expressing BnTR1 showed superior growth over cells expressing the empty vector alone upon temperature up-shift. After 10 hours of heat stress, pET-BnTR1 cell growth was significantly greater than the total pET cell growth (Fig. 1a). Noticeably, after 1 hour of exposure at 48.8 °C, 67% of pET-BnTR1 cells survived, while only 22% of cells with the empty vector survived (Fig. 1b). Hence, heterologous expression of BnTR1 provided E. coli cells with tolerance against heat stress without affecting growth under normal culture conditions.

To further assess the impact of BnTR1 expression, we performed microarray analyses to explore the transcriptional changes of E. coli cells when cultured at 37 °C or 42 °C. Principal component analysis (PCA) was first applied to determine the distance between the transcriptomes (Fig. 1c). The first principal component (PC1) holding the largest variance (64%) distinctly clustered pET-BnTR1 cells and pET cells into two groups. We also noted that the second principal component (PC2) contributed 11% variance and slightly separated the samples by culture temperatures. These data demonstrated that changes to the transcriptome were primarily due to BnTR1 expression.

Next, to achieve a robust list of differentially expressed genes (DEGs), we used five independent statistical methods with stringent thresholds (Supplementary Fig. S1a). In consequence, we found that BnTR1 altered the expression levels of 112 and 122 genes at 37 °C and 42 °C, respectively (Supplementary Tables S1 and S2). Next, to achieve a robust list of differentially expressed genes (DEGs), we used five independent statistical methods with stringent thresholds (Supplementary Fig. S1a). In consequence, we found that BnTR1 altered the expression levels of 112 and 122 genes at 37 °C and 42 °C, respectively (Supplementary Tables S1 and S2). Intriguingly, nearly half (44 up-regulated and 17 down-regulated) of all DEGs were detected under both normal and heat stress conditions (Supplementary Fig. S1b), suggesting that BnTR1 expression induced conserved transcriptional changes at different temperatures. In particular, many bacterial HSPs were significantly up-regulated upon BnTR1 expression (Fig. 1d,e). Specifically, expression of the DnaK/DnaJ and GroEL/GroES chaperone teams, which function to re-fold and stabilize denatured proteins29, increased 16- and 24-fold in pET-BnTR1 cells compared with cells expressing the empty vector (Supplementary Table S3). Furthermore, the levels of proteases, such as HslU/HslV, which function in protein degradation, were approximately 14-fold higher in pET-BnTR1 cells (Fig. 1d). To explore the physiological functions of DEGs, we performed gene ontology (GO) analysis. Consistent with the increase in HSPs, the most significantly changed GO terms were closely related to “response to heat” and “protein folding” processes (Fig. 1f). To determine whether the transcriptome changes were due to the stress of over-expression proteins, we set a control group with E. coli cells expressing PUB18, which is a U-box E3 ligase from Arabidopsis thaliana30. We did not observe significant changes of the HSP gene dnaK in PUB18 expressing E. coli cells (Supplementary Fig. S2). Taken together, these data suggest that the heterologous expression of BnTR1 in E. coli specifically up-regulated bacterial HSPs.

BnTR1 expression induces $\sigma^{32}$ accumulation. Because the $\sigma^{32}$ is the central player in regulating HSP transcription8, we next investigated the changes of the $\sigma^{32}$ level in E. coli cells expressing BnTR1. Interestingly, more than half of the common up-regulated DEGs, together with HSP genes, were directly regulated by $\sigma^{32}$ (Fig. 2a and Supplementary Table S3). Three $\sigma^{32}$ regulators, dnaK, groEL, and iphA, were selected and validated their up-regulation using quantitative RT-PCR (qRT-PCR). Notably, rpoH (the gene encoding $\sigma^{32}$) remained at its basal transcriptional level, which was confirmed by both microarray and qRT-PCR (Fig. 2a,b). These data indicate that BnTR1 may not participate in the transcriptional regulation of $\sigma^{32}$.

Measuring the protein levels further supported the assumption of post-transcriptional regulation of $\sigma^{32}$. The BnTR1 expression rapidly triggered $\sigma^{32}$ accumulation within 30 min, and it persisted for at least 90 min (Fig. 2c). In accordance with the increased transcription, DnaK synthesis was concomitantly increased and reached its peak level after 1 hour (Fig. 2c), indicating that cellular $\sigma^{32}$ was in an active state. Because abnormal protein production in E. coli can also increase HSPs11, 31, we examined whether the increase in $\sigma^{32}$ was due to the aggregation of unfolded BnTR1. To minimize the basal BnTR1 protein level and to avoid its toxic effect on E. coli cells, BnTR1 with a tightly regulated pBAD promoter was generated and induced by L-arabinose under a low temperature in E. coli W3110 and C600 strains. Remarkably, $\sigma^{32}$ levels still increased dramatically even when BnTR1 was slightly induced at 30 °C (Fig. 2d). We also tested dosage effects of BnTR1 on the $\sigma^{32}$ accumulation by using different concentrations of inducer. When supplemented with L-arabinose, $\sigma^{32}$ levels increased in E. coli expressing BnTR1 compared with the control groups harbouring empty vectors. Moreover, when BnTR1 was induced, both the $\sigma^{32}$ level and the amount of BnTR1 were higher in E. coli cells treated with 1% L-arabinose than with 0.01% and 0.001% L-arabinose, respectively (Fig. 2e). In addition, cell lysate tests demonstrated that the majority of BnTR1 was concentrated in the supernatant and was barely detectable in the inclusion bodies (Fig. 2f).

The RING domain is the active site of BnTR1. As an E3 ligase, BnTR1 possesses a typical RING domain chelating two zinc atoms to form two zinc fingers28, which led us to explore whether the RING domain is essential for BnTR1 function in bacteria. The BnTR1 mutants, BnTR1ΔZn1 (BnTR1C66S/C69S), BnTR1ΔZn2 (BnTR1C82S/C84S), and BnTR1ΔZn1/2 (BnTR1C66S/C69S/C82S/C84S), had no influence on cell growth at the normal temperature (Fig. 3a). As expected, wild-type BnTR1 kept the in vitro E3 ubiquitin ligase activity, and E. coli cells benefited from the expression of wild-type BnTR1 at 42 °C (Fig. 3a and Supplementary Fig. S3). Interestingly, though BnTR1
mutants lost the ligase activity, they had different effects on *E. coli* cells. The growth rate of BnTR1ΔZn1 cells was similar but slightly lower than that of BnTR1 cells (Fig. 3a). In sharp contrast, the growth of BnTR1ΔZn2 and BnTR1ΔZn1/2 cells significantly decreased (Fig. 3a). The cell growth results indicated that mutations in the first zinc finger (Zn1) did not substantially affect BnTR1 activity, but the second zinc finger (Zn2) was more crucial.

To identify the distinct functions of the two zinc fingers, the levels of HSPs and σ32 were measured. HSPs in BnTR1ΔZn1/2 and BnTR1ΔZn2 cells dropped to basal levels; however, mutation of Zn1 had little effect on HSPs as the HSP levels were similar in BnTR1ΔZn1 and wild-type BnTR1 cells. We observed a positive correlation between σ32 levels and HSP synthesis. The expression of wild-type BnTR1 and BnTR1ΔZn1 induced the accumulation of σ32 over 10 to 30 min, whereas no significant changes of σ32 levels were detected in BnTR1ΔZn1/2 and BnTR1ΔZn2 cells (Fig. 3b). It is important to note that the cells expressing mutant BnTR1 were cultured under...
non-stress conditions (30 °C). These results were in strong agreement with the growth rate experiments, suggesting that zinc fingers play an important role in the function of BnTR1; in particular, Zn2 was indispensable for the up-regulation of HSPs and σ32.

To further understand BnTR1 activity in *E. coli*, we next explored the distribution of its homologues. By mapping BnTR1 homologues to the reconstructed phylogenetic tree, we found that BnTR1 homologues emerged in ferns (*Selaginella moellendorffii*) but are missing in mosses (*Physcomitrella patens*), green algae, and red algae. Interestingly, unlike full-length BnTR1, the RING domain was widely spread among the *Plantae* and was even detected in yeast (*Saccharomyces cerevisiae*) (Fig. 3c and Supplementary Fig. S4a). Considering the importance of the RING domain, we next conducted a domain search in prokaryotic genomes. In *E. coli*, DnaJ chaperone contained a zinc-finger domain next to the J-domain 33. Moreover, other types of zinc fingers, such as the B-box and AN1, were integrated with the J-domain in specific bacterial and archaeal species34 (Fig. 3d), raising the possibility that BnTR1 and DnaJ are analogous in terms of their structural topology or enzymatic activity. The comparison of the structures of BnTR1 and DnaJ by molecular modelling indicated that the eight residues (cysteine/histidine) of BnTR1 coordinated the zinc ions in a cross-braced shape (Supplementary Fig. S4b). By contrast, the two zinc fingers in DnaJ constituted a right angle and formed a V-shaped molecule33 (Supplementary Fig. S4c).

The dissimilar topology led us to extend the comparison of zinc fingers in vivo. A complementary experiment using the *dnaJ* mutant *E. coli* strain MF634 revealed that cells expressing wild-type BnTR1 formed very few clones at 43 °C, indicating that BnTR1 alone was unable to compensate for the loss of DnaJ (Supplementary Fig. S5).

**Figure 2.** The σ32 level is increased during the expression of BnTR1. (a) Distribution of σ32 regulons among the common DEGs (right panel). The expression level of *rpoH* measured by microarray is denoted as an orange dot. (b) Validation of microarray data by qRT-PCR and two-tailed Student’s *t*-test was used for the comparison (*p*-value < 0.05 and **p*-value < 0.001). Six genes were selected, including three heat shock genes (*dnaK*, *groEL*, and *ipbA*), one down-regulated DEG (*prfH*), and two genes without significance (*hyfF* and *rpoH*). (c) Whole-cell extracts from pET and pET-BnTR1 cells cultured at 37 °C with 0.1 mM IPTG were analysed using Western blotting (probed with anti-DnaK, anti-σ32, and anti-His for BnTR1). (d) Wild-type *E. coli* W3110 and C600 cells harbouring the pBAD24 empty vector or pBAD-BnTR1 were grown at 30 °C with 0.1% L-arabinose. Whole-cell extracts taken at the indicated time were loaded and probed with anti-σ32 and anti-His for BnTR1. (e) Whole-cell extracts were from *E. coli* C600 cells induced with different concentration of L-arabinose for 30 min at 30 °C. Immunoblots were shown and probed with antibodies indicated. (f) Western blotting (probed with anti-σ32 and anti-His for BnTR1) of whole cell, supernatant, and pellet proteins that were extracted as described in (d). OmpC was detected in parallel by anti-OmpC and used as a loading control in (c,d,e). The data are presented as means ± s.d. of three independent experiments (b). Full-length blots are presented in Supplementary Figure 6.

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Because the J-domain and glycine/phenylalanine-rich (G/F) region are crucial for DnaJ chaperone functions,
a chimeric protein made by concatenating BnTR1 with the J-domain and G/F region of DnaJ was engineered
and named JdBnTR1 (Fig. 3e). Surprisingly, JdBnTR1 rescued the defective cells at the high temperature, but
mutations in both zinc fingers (JdBnTR1ΔZn1/2) were incapable of rescuing the growth defect at 43 °C (Fig. 3e).
Taken together, these results suggest that the up-regulation of HSPs and σ32 was not a result of over-expression
of foreign proteins or possible BnTR1 solubility problems, but rather the physiological function of BnTR1 in the
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**BnTR1 stabilizes σ32 in vivo through its RING domain.** Because σ32 activity is negatively controlled
by the KJE chaperone team, it is unknown whether BnTR1 could hinder KJE-dependent regulation of σ32.
The assay of KJE refolding of denatured luciferase substrate was employed to test the function of two zinc fingers
of BnTR1. BnTR1 and its mutant forms sharply decreased the reactivation activity of the KJE chaperone team in
10 minutes (Fig. 4a). However, along with increasing the reaction time, BnTR1 mutants displayed different effects.
After 20 min, BnTR1ΔZn2 and BnTR1ΔZn1/2 showed distinguishably higher levels of reactivated luciferase than
the wild-type BnTR1 and BnTR1ΔZn1. To confirm our observations, series of BnTR1 mutant concentrations were supplied into the reaction. We confirmed the inhibitory effects, which were even more significant when we used a low concentration (0.0125 μM) of BnTR1 mutant (Fig. 4b). Based on these results, BnTR1 could act as an intruder in the KJE refolding system in vitro and confirmed the distinct functions of two zinc fingers.

As the Zn2 of BnTR1 is more important for its inhibitory effect, we hypothesized that this region physically interacts with the DnaK chaperone. Therefore, 6His-BnTR1 (N terminus) and BnTR1 mutants were purified and used as prey in co-immunoprecipitation (Co-IP) experiments. Indeed, DnaK and BnTR1 formed a stable complex, which remained intact when Zn1 was mutated (BnTR1ΔZn1) (Fig. 4c). As expected, the DnaK-BnTR1ΔZn2 or DnaK-BnTR1ΔZn1/2 complex was barely detectable, suggesting that DnaK interacted with the Zn2 of BnTR1 in vivo. We next used a bacterial two-hybrid system to confirm their interaction. We identified a clear signal for the DnaK-BnTR1 and DnaK-BnTR1ΔZn1 interactions (Fig. 4d).

It is known that σ32 is a substrate of DnaK and the co-chaperone DnaJ. We observed different growth rates (Fig. 3a) and inhibition effects (Fig. 4a) between BnTR1ΔZn1 and BnTR1ΔZn1/2, suggesting that Zn1 may play a minor role in the stabilization of σ32. Surprisingly, BnTR1 and σ32 formed a rather tight complex, and mutation of Zn2 had no effect on their interaction (Fig. 4e). The interaction between BnTR1 and σ32 was dependent on Zn1, as mutation in this region completely disrupted the interaction, which was demonstrated by Co-IP and bacterial two-hybrid experiments (Fig. 4e,f).

**Discussion**

In this study, we report heterologous expression BnTR1, a plant E3 ligase containing a RING domain, could effectively protect *E. coli* cells from heat stress. BnTR1 expression in *E. coli* dramatically increased the level of heat shock factor σ32, even at low temperatures, resulting in the significant up-regulation of HSPs. It has been well established that HSP expression rapidly increases following temperature upshift to protect *E. coli* cells from heat-damaged proteins. In our study, several HSPs were induced by BnTR1 expression, including DnaK, GroEL, ClpB and HtpG, which are molecular chaperones with functions in deterring unfolded protein aggregation and assisting in their refolding. Other HSPs, such as HslU/HslV and ClpP, are proteases that function
to degrade and dissolve heat-denatured proteins. Thus, the increased heat resistance largely depends on the cumulative effects of multiple molecular chaperones and proteases, which are essential to refold and degrade heat-damaged proteins. Therefore, cells expressing BnTR1 seem less affected and damaged by heat stress. Consistent with this idea is a recent report that over-expression of the GroEL/GroES chaperones increase the maximum growth temperature of wild-type E. coli to 47.5 °C. Heat shock factor σ32 is known to be the key regulator of E. coli HSPs. Although BnTR1 triggered considerable activation of σ32, which is sufficient to induce HSPs, σ32 transcription remained relatively constant. Thus, the apparent linkage between BnTR1 and σ32 is at the post-transcriptional level.

It could be argued that the enhanced HSP levels are not due to a specific BnTR1 function, as previous studies have shown that over-expression of abnormal proteins increases HSP levels. The simplest explanation for the induction of HSPs is the accumulation of unfolded BnTR1. However, this simple model seems inconsistent with our current data. These reported abnormal proteins are misfolded or unfolded with aberrant high-order structure, and are found in inclusion fractions, while the majority of BnTR1 protein was soluble. In addition, BnTR1 in E. coli remained active rather than being unfolded. Thus, the thermotolerance of E. coli was not due to the stress of the over-expression of exogenous proteins.

The heterologous expression of eukaryotic molecular chaperones enables E. coli cells to resist heat stress at lethal temperatures. This protective effect is closely related to chaperone activity in maintaining proteins in a folded state or preventing unfolded proteins from aggregation. However, this is not the case for BnTR1, as purified BnTR1 exhibited no chaperone function in refolding denatured luciferases with KJE in vitro; instead, the efficiency of BnTR1 largely depended on its RING domain. Thus, we propose an alternative model. The KJE chaperone team interacts with σ32, instead of DnaK, thereby inhibiting the efficiency of BnTR1 largely depended on its RING domain. Thus, we propose an alternative model. The KJE chaperone team inhibits the KJE chaperone activity in vitro by directly binding to σ32 in vivo and in vitro. BnTR1 interacts with DnaK to form a stable complex mainly through the Zn2, which may inhibit the negative effect of KJE on σ32, resulting in the accumulation of active σ32. Moreover, although mutation in Zn1 had little effect on σ32 and HSP levels, our in vivo interaction studies suggest that Zn1 selectively interacts with σ32. It is not clear whether Zn1 and/or Zn2 could influence pathways associated with SRP and the protease FtsH, key regulators for σ32 membrane localization and degradation. Thus, we suggest that the increased amount of active σ32 is largely attributed to the Zn2 of BnTR1.

BnTR1 is identified as a key player in the heat stress response of B. napus, and constitutive BnTR1 expression significantly increases the heat tolerance of multiple plant species. Interestingly, we observed that heterologous expression of BnTR1 also conferred pronounced thermoprotective effects on E. coli cells. In plant the function of BnTR1 tightly relies on its E3 ligase activity, but BnTR1 may work in a different way in E. coli. All BnTR1 mutants used in this study lost their in vitro E3 ligase activity, whereas the effect of BnTR1ΔZn1 in E. coli was slightly affected. Although no significant homologues of BnTR1 have been detected in prokaryotes, we noted that the E. coli chaperone DnaJ possesses a domain containing two zinc fingers. Importantly, analogous to BnTR1, the two DnaJ zinc fingers play different roles such that one is important for DnaK-independent chaperone activity, while the other is essential for the interaction with DnaK. A rather surprising finding was that the expression of the JdBnTR1 chimeric protein (a fusion of the J-domain and BnTR1) rescued the dnaJ mutant strains at a high temperature. Thus, it suggested that BnTR1 and other proteins possessing similar zinc finger domains may be useful resources in the genetic engineering of thermophilic bacteria for industrial application.

Methods
Further details of bacterial viability assays, two-hybrid assays, recombinant protein expression and purification, in vitro assay of E3 ubiquitin ligase activity, Co-IP assays, and RNA extraction and qRT-PCR can be found in the Supplementary Methods.

Strains, plasmids and growth condition. The E. coli strains and plasmids were commercially obtained from the American Type Culture Collection (ATCC) and the Coli Genetic Stock Center (CGSC) (Supplementary Table S4). The strains and their transformants were grown aerobically in Luria–Bertani (LB) medium (tryptophane 10 g L−1, NaCl 10 g L−1 and yeast extract 5 g L−1, pH 7.4) and were supplemented with ampicillin (100 μg mL−1) and kanamycin (50 μg mL−1) when necessary. To validate the in vitro interaction of DnaK and σ32 with BnTR1,
the expression of recombinant proteins from *E. coli* Rosetta (DE3) was induced at the mid-logarithmic phase (OD$_{600nm}$ = 0.6) using 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 30 min.

**Vectors construction.** pET-28a and pBAD24 vectors were used to express polyhistidine (6His)-tagged proteins (Supplementary Table S5). The construct expressing 6His-BnTR1 (N-terminal) was generated as follows. *BnTR1* and *PUB18* (AT1G10560) was amplified by PCR from *B. napus* and *A. thaliana*, respectively, and ligated into pET-28a (digested with BamHI and HindIII). 6His-BnTR1 was then sub-cloned into the pBAD24 vector (digested with Ncol and HindIII) to obtain pBAD24-BnTR1. Site-specific mutations were introduced into BnTR1 using QuikChange® Lightning Site-Directed Mutagenesis Kits (Stratagene) with the resultant plasmid pET-28a-BnTR1 as the template. The constructs containing mutant BnTR1 (pET-28a-/pBAD24-BnTR1_C86S/C95S, pET-28a-/pBAD24-BnTR1_C82S/C84S , and pET-28a-/pBAD24-BnTR1_C86S/C95S/C82S/C84S ) were transformed into *E. coli* and induced to express the target proteins.

The 6His-dnaJ was amplified by using *E. coli* C600 as the template and ligated into pET-28a (digested by BamHI and Xhol). pBAD24-dnaJ was then constructed by subcloning of the Ncol/Xhol fragment from pET-28a-dnaJ. For the construction of pBAD24-JdBnTR1, the J domain of dnaJ (312 bp) was amplified from pET-28a-dnaJ and then ligated into pBAD24-BnTR1 (digested by Ndel and BamHI). The gene encoding for σ^32 (rpoH) was obtained by amplifying its entire coding region (855 bp) from *E. coli* C600 and then cloned into pET-28a (digested by BamHI and Xhol) to get the pET-28a-rpoH. To create pET-28a-luc, the luciferase gene was amplified and ligated into pET-28a (digest by BamHI and Xhol). Successful transformants were analyzed by colony PCR and constructs containing correct inserts were sequenced to ensure the accuracy.

**Luciferase refolding assays.** Luciferase refolding was evaluated as described previously with modifications. Specifically, luciferase (25 μM) was denatured and diluted 100-fold into a premix containing 10 mM MOPS (pH 7.2), 50 mM KCl, 5 mM MgCl$_2$, 0.015% (w/v) bovine serum albumin (BSA), 0.1 mM γ-creatinine kinase, 20 mM creatine phosphate, 5 mM ATP, 2 μM DnaK, 0.5 μM DnaJ, 0.125 μM GrpE, and a serial concentration of BnTR1. Luciferase activity was continuously monitored at 22°C using a luciferase assay system from Molecular Devices (L Max™).

**Immunoblotting.** Western blotting was employed to determine the translational level of σ^32 and heat shock proteins. Briefly, *E. coli* C600 and W3110 pBAD24 transformants (OD$_{600nm}$ = 0.6) were induced with 0.1% L-arabinose for 30 minutes. Cultures were harvested and divided into two equal aliquots. One aliquot was immediately precipitated with 5% trichloroacetic acid (TCA) for protein concentration quantification. The precipitate was collected by centrifugation and washed with 80% iced acetone. Pellets were dried under vacuum and re-suspended with double distilled water. The concentration of proteins was quantified using Pierce BCA protein assay kit (Thermo). The other aliquot was collected and re-suspended in SDS-PAGE sample buffer for western blotting analysis.

Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad). Membranes were blocked with 10% bovine serum albumin (BSA) diluted in TBST (TBS and 0.1% (w/v) Tween 20) for 1 hour at room temperature and then incubated overnight at 4°C with anti-σ^32 (1:1,000) (Neoclon), anti-DnaK (1:10,000) (Abcam), anti-GroEL (1:1,000) (Abcam), anti-His (1:1,000) (Abcam), and anti-OmpC (1:1,000) (Biorbyt) antibodies (diluted in TBST). Membranes were washed three times with TBST, incubated for 1 hour at room temperature with a 1:1,000 dilution of HRP-conjugated secondary antibodies in TBST, and washed three times with TBST. Immunoreactive bands were detected using Pierce ECL Western Blotting Substrate (Thermo Scientific) and ChemiDoc XRS Plus (Bio-Rad). Images were captured with Image Lab Software version 3.0 (Bio-Rad).

**Phylogenetic and protein domain analysis.** We constructed a phylogenetic tree of 51 *Plantae* species according to the method described by Ciccarelli et al. We selected highly conserved proteins without horizontal gene transfers effects, and the list included ribosomal proteins (S2, S3, S4, S5, S7, S8, S11, S12, L3, L5, L11, L9E/L22, L1O, and L15), aminoacyl-tRNA synthetases (Seryl-, Phenylalanine-, and Leucyl-tRNA synthetase), metal-dependent proteases with chaperone activity, and predicted GTPase probable translation factor. The MUSCLE program was used to conduct multiple sequence alignments with iteration number set to 100, and then aligned sequences were concatenated. The phylogenetic tree was constructed using RAXML parameters: -f a -T 6 -m PROTGAMMAJTTX -p -x -autoMRE. *Saccharomyces cerevisiae* was set as the outgroup.

Significant homologs of full-length BnTR1 was detected by BLASTP (E-value < 10^-10). The Kyoto Encyclopedia of Genes and Genomes (KEGG) database was used to construct the distribution of J-domain, RING, AN1, and B-box protein domains (E-value < 10^-4).

**Microarray procedures.** Cy3 fluorescently-labeled cRNA was prepared and hybridized to Agilent *E. coli* whole-genome gene expression microarrays (8*15 K) according to the single channel microarray-based protocol (Agilent Technologies Inc.). The biological repeats were randomly distributed onto two microarray slides. The array images were scanned by the G2565BA Microarray Scanner System, and raw data were then normalized (quantile method), merged and filtered by Feature Extraction Software (Agilent Technologies Inc.).

The control probes and probes without annotations were dropped out as they were considered to have non-transcriptome biological meanings. As the Agilent *E. coli* microarray contains three other types of probes (designed for *E. coli* O157:H7, CF0703 and EDL933), these probes were neglected before the downstream analysis. For the “sibling probe-set”, one gene corresponding to multiple probes, the average value to represent the gene expression intensity. A total of 4108 genes were finally selected, the intensity values were log2 transformed.

To identify robust DEGs, five parametric and non-parametric methods were independently applied. First, three traditional processing methods, the Student’s t-test, the Mann-Whitney U-test, and fold-change calculation
were applied. The DEGs in each comparison were identified with the false discovery rate (FDR) < 0.05 for the t-test and U-test, together with fold-change cut-off values of 2.0-fold decrease and increase. Two more sophisticated methods in R/Bioconductor packages “limma”48 and “rankProd”49 were used (Supplementary Table S6). The intersection of DEGs identified using the five methods were considered to be significantly up- or down-regulated in BnTR1 strains at 37 °C and 42 °C, respectively. The σ32 regulons were summarized from Nonaka et al.50.

The latest GO information (submission date: 7/11/2015) of E. coli was retrieved from Gene Ontology Consortium. The minimum number of genes in each GO term was set as 5. The Fisher’s exact test was used to determine the significant GO terms with threshold of p-value < 0.05.

For microarray data validation, the transcriptional levels of genes were detected (Supplementary Table S7). RNA was extracted as described above, cDNA was generated using an iScript cDNA synthesis kit (Bio-Rad) and qPCR was performed using SYBR Green Supermix (Bio-Rad). The results were analysed, and the mRNA levels were normalized against that of 16s rRNA using the ΔΔCT method51,52.

**Statistical analysis.** All statistical analysis was conducted by the R software.

**Data availability.** The microarray data has been deposited in the Gene Expression Omnibus with the accession number GSE85807.

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Author Contributions
Y.Y. generated ideas of this study and supervised the project. Y.N. analyzed the gene expression data and other bioinformatic tasks. X.X. carried out the majority of experiments with the help from C.L., T.W., and K.L., J.M., Z.L., and X.L. provided intellectual discussions. Y.Y. and X.X. prepared the figures. Y.Y. and Y.N. wrote the manuscript.

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