Plastid Translation Elongation Factor Tu Is Prone to Heat-Induced Aggregation Despite Its Critical Role in Plant Heat Tolerance[1][OPEN]

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Translation elongation factor Tu (EF-Tu) is a conserved GTP-binding protein essential for protein translation in prokaryotes and in eukaryotic mitochondria and plastids. EF-Tu also has a GTP/GDP-independent chaperone activity that may function in acclimation to heat. Here, we report that the Arabidopsis (*Arabidopsis thaliana*) plastid EF-Tu, Rabe1b, rapidly becomes insoluble at temperatures as low as 35°C in vitro and 41°C in vivo, with more than 90% aggregation after 9 h at 45°C in vivo. Based on its established function in protein translation, heat-induced aggregation likely inactivates Rabe1b. To determine the effect of heat-induced aggregation, we isolated an Arabidopsis *rabe1b* knockdown mutant and discovered it to be highly compromised in heat tolerance. Overexpression of constitutive GTP- or GDP-bound mutant forms of Rabe1b in Arabidopsis and virus-induced silencing of *Rabe1b* in tomato (*Solanum lycopersicum*) also reduced heat tolerance. Compromised heat tolerance in the Arabidopsis *rabe1b* mutant and in the lines overexpressing constitutive GTP- or GDP-bound mutant Rabe1b proteins was associated with reduced plastid translation under heat stress. The Arabidopsis *rabe1b* mutant also showed compromised heat-induced expression of *HsfA2* and its target genes. Constitutive overexpression of HsfA2 activated its target genes but only partially restored the heat tolerance of the *rabe1b* mutant. These results strongly suggest that the plastid protein EF-Tu is heat sensitive and acts as a critical limiting factor in plant heat stress responses, primarily functioning in plastid protein translation but also in protein folding and retrograde signaling of nuclear heat-responsive gene expression.

In plants, moderate heat disturbs cellular homeostasis, causing inhibition of growth and development; moreover, severe heat can cause irreversible damage and death. Chloroplasts are highly sensitive to heat, which affects chlorophyll biosynthesis, photochemical reactions, electron transport, thylakoid membrane fluidity, photophosphorylation, and CO_2 assimilation (Wahid et al., 2007). Heat impairment of chloroplast functions often is the result of the inactivation of heat-sensitive proteins (e.g. oxygen-evolving complex of PSII and Rubisco activase) and the down-regulation of important chloroplast components (e.g. enzymes involved in tetrapyrrole metabolism), which can lead to decreased photosynthesis, redox imbalance, other associated damage, or even cell death (Eamus et al., 1995; Klimov et al., 1997; Salvucci and Crafts-Brandner, 2004a, 2004b; Allakhverdiev et al., 2008).

Because heat is such a fundamental threat to all life, heat stress responses characterized by rapid expression of heat shock proteins (HSPs) are found universally (Craig et al., 1993; Georgopoulos and Welch, 1993; Jakob et al., 1993; Arrigo, 2005; Latijnhouwers et al., 2010; Xu et al., 2012; Reddy et al., 2014). In plants, heat stress rapidly induces the production of well-characterized HSPs, including Hsp101, Hsp70, and small HSPs, which act as molecular chaperones in protein quality control by promoting the folding and refolding of nonnative proteins (Baniwal et al., 2007; Kotak et al., 2007; von Koskull-Döring et al., 2007; Schramm et al., 2008). Protein chaperones also are involved in monitoring misfolded/damaged proteins and targeting their degradation by the ubiquitin proteasome system, autophagy, and other pathways (Arias and Cuervo, 2011; Amm et al., 2014). A great deal has been learned about the signaling network of heat shock-responsive gene expression and protein quality control

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in important cellular compartments such as the cytosol and endoplasmic reticulum (Brodsky and McCracken, 1999; Schäfer and Wolf, 2005; Kruse et al., 2006; Radke et al., 2008; Liu and Howell, 2010; Okiyoneda et al., 2011; Needham and Brodsky, 2013).

Heat shock transcription factors (HSFs) are the terminal regulators of heat stress signal transduction, mediating the expression of HSPs and other heat-induced genes (Kotak et al., 2007; von Koskull-Döring et al., 2007). In plants, HSFs are encoded by a multigene family with more than 20 members in Arabidopsis (*Arabidopsis thaliana*) and can be grouped into three evolutionary classes: A, B, and C (Nover et al., 2001). Studies in plants including Arabidopsis and tomato (*Solanum lycopersicum*) have shown that several HSFs, including HsfA1a, HsfA2, and HsfB1, play critical roles in the transcriptional regulatory network responsible for the expression of nuclear heat-responsive genes (Kotak et al., 2007). HsfA1a is constitutively expressed and regulates the heat-induced expression of HsfA2 and HsfB1 (Mishra et al., 2002). HsfA2, which is highly responsive to heat stress, is a major HSF in the heat-induced expression of nuclear heat-responsive genes (Kotak et al., 2007). HsfA1a can function as a coactivator of HsfA2 by forming an HsfA1a-HsfA2 protein complex (Kotak et al., 2007). Disruption of Arabidopsis *HsfA2* decreased plant thermotolerance, whereas its overexpression increased thermotolerance in transgenic plants (Li et al., 2005; Nishizawa et al., 2006; Charng et al., 2007; Ogawa et al., 2007).

It has long been recognized that heat stress responses are associated with altered protein biosynthesis, a fundamental biological process that provides the driving force for the growth and development of all living organisms (Matts and Hurst, 1992; Matts et al., 1992; Shalgi et al., 2013). Protein translation is accomplished by the combined actions of the ribosomes and ancillary proteins such as initiation, elongation, and termination factors. A central step in protein biosynthesis is the elongation cycle. In this process, the elongation factors EF-Tu (in bacteria) and eEFIA (in eukarya) play pivotal roles by carrying the aminoacyl-tRNAs (aa-tRNAs) to the ribosomes (Brisio et al., 2010). EF-Tu and EFIA belong to the large family of GTP-binding proteins, or G-proteins, that catalyze the binding of an aa-tRNA to the ribosome (Brisio et al., 2010). The ribosome has three sites for tRNA binding: the aminoacyl/acceptor or A site, the peptidyl or P site, and the exit or E site. In prokaryotes, GTP-bound EF-Tu forms complexes with aa-tRNA and transports the correct aa-tRNA to the A site of the ribosome, where the anticodon of the tRNA binds to the codon of the mRNA (Krab and Parmeggiani, 1998, 2002). Following the correct anticodon/codon binding, the ribosome changes configuration to activate the GTPase of EF-Tu, resulting in the hydrolysis of bound GTP to GDP. GTP hydrolysis results in a drastic change in the conformation of EF-Tu, causing its dissociation from the aa-tRNA and ribosome complex (Krab and Parmeggiani, 2002). This allows the aa-tRNA to fully enter the A site, where its amino acid is brought near the P site's polypeptide so that the ribosome can catalyze the covalent transfer of the polypeptide onto the amino acid (Krab and Parmeggiani, 2002). Upon leaving the ribosome in the cytoplasm, the deactivated GDP-bound EF-Tu is activated by the elongation factor Ts (EF-Ts), which belongs to the family of guanine nucleotide exchange factors, resulting in the restoration of its GTP-bound form, which can then associate with another aa-tRNA to start the next cycle (Krab and Parmeggiani, 2002).

Apart from the pivotal role in transporting aa-tRNA to the ribosome in protein biosynthesis, EF-Tu contributes to translational accuracy through several mechanisms (Krab and Parmeggiani, 2002). In addition, EF-Tu can act as a protein chaperone that promotes the refolding of a number of denatured proteins in vitro. Two earlier studies suggested that the chaperone activity of a bacterial EF-Tu was enhanced by GTP (Kudlicki et al., 1997; Caldas et al., 2000). However, those studies were not reproducible, and subsequent studies with bacterial, mitochondrial, and chloroplast EF-Tu proteins have established that the chaperone activity of EF-Tu is independent of GTP or GDP (Malki et al., 2002; Rao et al., 2004; Ristic et al., 2007; Suzuki et al., 2007). In plants, genes encoding EF-Tu are induced by abiotic stresses (Bhadula et al., 2001; Momcilovic and Ristic, 2007; Ristic et al., 2008), and the levels of plant plastid EF-Tu in plants have been correlated with the levels of heat tolerance (Ristic et al., 2004; Fu et al., 2008; Fu and Ristic, 2010). It is unclear whether the role of the plant EF-Tu protein in heat tolerance is mediated by its role as a protein translation factor in protein biosynthesis, as a protein chaperone in protein refolding, or unknown activities.

We have studied the protein degradation pathways, including the 26S proteasome, autophagy, and multivesicular bodies, in the degradation of nonnative proteins under biotic and abiotic stresses, including heat stress (Zhou et al., 2013, 2014b; Wang et al., 2014, 2015). We became interested in plastid EF-Tu from our research on the mechanisms by which autophagy targets the degradation of ubiquitinated protein aggregates derived from heat-denatured proteins (Zhou et al., 2013, 2014b). Through proteomic profiling of heat-induced protein aggregates accumulated under heat stress, we discovered that the Arabidopsis plastid EF-Tu, Rabe1b, was one of the most abundant aggregates under heat stress (Zhou et al., 2014b). Here, we report that Arabidopsis Rabe1b proteins rapidly became insoluble both in vitro and in vivo during heat stress. The heat-induced aggregation of Rabe1b would inactivate the elongation factor based on its established mode during the elongation cycle of protein translation. To determine the impact of reduced levels of soluble, active plastid EF-Tu, we isolated an Arabidopsis *rabe1b* knockdown mutant and discovered that it was highly compromised in heat tolerance. Overexpression of constitutive GTP- and GDP-bound mutant forms of Rabe1b in Arabidopsis and virus-induced silencing of *Rabe1b* in tomato also led to reduced heat tolerance. We

also found that the compromised heat tolerance of the Arabidopsis *rabe1b* mutant and overexpression lines for the constitutive GTP- and GDP-bound mutant Rabe1b was associated with reduced plastid translation under heat stress, and the Arabidopsis *rabe1b* knockdown mutant also was compromised in heat-induced expression of HsfA2 and its target genes. Overexpression of HsfA2 could partially restore the heat tolerance of the *rabe1b* mutant, with activated expression of HsfA2 target genes. Based on these results, we propose that heat-sensitive plastid EF-Tu is a critical limiting factor of plant heat stress responses primarily as a plastid translation factor but also through positive roles in protein folding and retrograde signaling of nuclear heat-responsive gene expression.

RESULTS

Heat-Induced Rapid Aggregation of AtRabe1b Both in Vitro and in Vivo

In our previously reported proteomic profiling of insoluble protein aggregates in heat-treated plants, we discovered that the Arabidopsis plastid EF-Tu, Rabe1b, is one of the most abundant aggregates under heat stress (Zhou et al., 2014b). Other abundant protein aggregates include Rubisco activase and catalases (Zhou et al., 2014b), which are known to be heat sensitive and aggregate prone (Feinstein et al., 1967; Chen et al., 1993a, 1993b; Salvucci et al., 2001). The heat hypersensitivity of Rubisco activase and catalases contributes to reduced photosynthetic efficiency and increased reactive oxygen species (ROS) accumulation under heat stress, respectively (Rizhsky et al., 2002; Kurek et al., 2007). The aggregate proneness of plastid EF-Tu raises important questions about its structural and functional properties under heat stress.

To confirm and characterize the possible aggregate-prone nature of the Arabidopsis plastid EF-Tu, we generated a myc-tagged *AtRabe1b* gene construct under the control of the cauliflower mosaic virus (*CaMV*) *35S* promoter and transformed it into Arabidopsis Columbia-0 (Col-0) wild-type plants. As Rabe1b is a G protein, we were also interested in determining whether its GTP and GDP binding and exchange affect temperature-induced aggregation. Therefore, we also made myc-tagged gene constructs for two mutant Rabe1b proteins: AtRabe1bH152L and AtRabe1bN203I. AtRabe1bH152L is a constitutive GTP-bound form of AtRabe1b (AtRabe1b-GTP) due to its GTPase deficiency as a result of the substitution of the catalytic residue His-152, which corresponds to His-84 in *Escherichia coli* EF-Tu (Fig. 1; Daviter et al., 2003). Indeed, recombinant AtRabe1bN203I produced in *E. coli* showed deficiency in its GTPase activity (Supplemental Fig. S1A). AtRabe1bN203I is a constitutive GDP-bound form of AtRabe1b (AtRabe1b-GDP) due to the substitution at Asn-203, which corresponds to the Asn-135 in *E. coli* EF-Tu (Fig. 1) that is required for binding to the guanine nucleotide exchange factor EF-Ts (Hwang et al., 1992). We confirmed using yeast two-hybrid assays that both wild-type AtRabe1b and the GTP-bound AtRabe1bH152L mutant protein, but not the GDP-bound AtRabe1bN203I mutant protein, interacted with the plastid EF-Ts guanine nucleotide exchange factor (Supplemental Fig. S1B). The AtRabe1b-GTP-myc and AtRabe1b-GDP-myc constructs under the control of the *CaMV 35S* promoter also were transformed into Arabidopsis. For each construct, we generated more than 30 independent transformants. Surprisingly, more than 70% of T1 transformants for each construct developed bleached leaves (Supplemental Fig. S2, A and B). The bleached areas often expanded to stem, inflorescence, and other tissues to various degrees (Supplemental Fig. S2A). Both reverse transcription quantitative PCR (RT-qPCR) and immunoblotting analysis (Supplemental Fig. S2C) indicated that the bleaching phenotype of the transformants was strictly correlated with silencing of the *Rabe1b* gene. From the remaining transgenic plants with no detectable bleaching phenotype, we identified two lines for each transgene with similar levels of *AtRabe1b* transgene transcripts based on RT-qPCR (Supplemental Fig. S3) and AtRabe1b-myc proteins based on immunoblotting using an anti-myc antibody. These lines were used to investigate the temperature-induced aggregation of the plastid translation elongation factor.

We first examined the temperature-induced aggregation of AtRabe1b in vitro. Total soluble proteins were extracted from transgenic Arabidopsis plants overexpressing myc-tagged AtRabe1b (AtRabe1b-OE), AtRabe1b-GTP (AtRabe1b-GTP-OE), and AtRabe1b-GDP (AtRabe1b-GDP-OE). The extracted proteins were incubated at different temperatures for 0.5, 1, and 2 h. Soluble and insoluble proteins in the protein extracts were then separated by low-speed centrifugation, and the relative amounts of AtRabe1b-myc in the soluble and insoluble fractions were analyzed by immunoblotting. When incubated at 23°C, AtRab1b-myc and its constitutive GTP- and GDP-bound mutant forms remained soluble, with little aggregation detected after the 2-h incubation (Fig. 2). At 35°C, on the other hand, incubation for as short as 0.5 h caused aggregation of more than 50% of the myc-tagged AtRabe1b and its GTP- and GDP-binding mutant forms (Fig. 2). Extended incubation at 35°C led to a further increase in the aggregated form of the proteins (Fig. 2). After a 2-h incubation at 35°C, more than 80% of myc-tagged AtRabe1b proteins became insoluble (Fig. 2). Interestingly, when incubated at 42°C, the rates of insolubilization of wild-type Rabe1b proteins were not substantially different from those at 35°C. However, insolubilization of the GTP- and GDP-bound forms of the plastid EF-Tu were enhanced substantially when the temperature increased from 35°C to 42°C (Fig. 2). Almost all the GTP- and GDP-bound forms of Rabe1b became aggregated after incubation for as short as 0.5 h at 42°C (Fig. 2). Inclusion of 20 μM GTP in the protein extracts had no detectable effect on the heat-

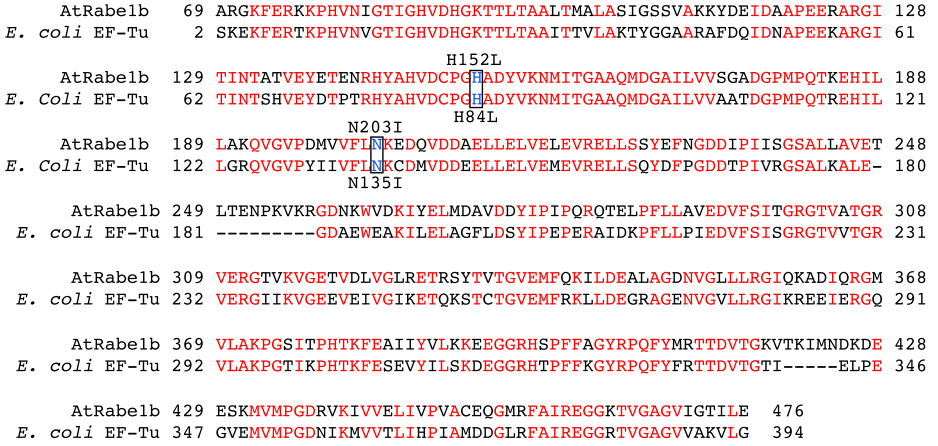

Figure 1. Sequence comparison between Arabidopsis chloroplast EF-Tu and *E. coli* E-Tu proteins. Identical amino acid residues are in red. Previously established GTPase-deficient, constitutive GTP-bound *E. coli* EF-TuH84L and constitutive GDP-bound EF-TuN135I mutant proteins are indicated. The corresponding amino acid residues His-84 and Asn-135 of *E. coli* EF-Tu are His-152 and Asn-135, respectively, in Arabidopsis plastid EF-Tu, and their constitutive GTP- and GDP-bound mutant forms also are indicated.

induced aggregation of either the wild-type or mutant form of Rabe1b at 42°C (Supplemental Fig. S4). By contrast, less than 5% of the total proteins in the extract became aggregated in the insoluble fraction after incubation at 42°C for 2 h. For comparison, Rubisco large subunit proteins, which were used as the loading control in immunoblotting, remained mostly soluble after the 2-h incubation at both 35°C and 42°C (Fig. 2). These results indicated that AtRabe1b proteins were highly prone to aggregation at elevated temperatures in vitro. In fact, a substantial percentage of Rabe1b proteins became aggregated when incubated for 2 h at a temperature as low as 25°C in vitro (Supplemental Fig. S5).

We also examined the temperature-induced aggregation of AtRabe1b in vivo. Stable transgenic AtRabe1b-OE, AtRabe1b-GTP-OE, and AtRabe1b-GDP-OE plants were treated at different temperatures for 3, 6, and 9 h, and total proteins were extracted and separated into soluble and insoluble fractions by low-speed centrifugation. As shown in Figure 3, treatment of these transgenic plants at a temperature up to 39°C for up to 9 h caused little accumulation of Rabe1b aggregates. When incubated at 41°C, no detectable levels of aggregates of Rabe1b and its GTP- and GDP-binding forms were observed during the first 3 h (Fig. 3). Extension of the incubation time at 41°C to 6 and 9 h, however, resulted in detectable accumulation of Rabe1b protein aggregates (Fig. 3). Increasing the temperature to 43°C and 45°C further increased the aggregation of the Rabe1b proteins (Fig. 3). Again, the constitutive GTP- and GDP-bound mutant forms of Rabe1b were substantially more prone to aggregation than the wild-type Rabe1b proteins (Fig. 3). For example, at 43°C, almost all constitutive GTP- and GDP-bound mutant forms of Rabe1b became aggregated after a 6-h incubation but less than 50% of the wild-type Rabe1b proteins were in the insoluble fraction (Fig. 3). At 45°C, incubation of only 3 h caused complete aggregation of the GTP- and GDP-bound forms of Rabe1b (Fig. 3). On the other hand, complete aggregation of wild-type Rabe1b proteins occurred after 9 h at 45°C (Fig. 3). It should be noted again that, even after a 9-h incubation at 45°C, when almost all Rabe1b proteins became insoluble, only a small percentage of the total proteins (less than 5%) became aggregated in the insoluble fraction. Also, little aggregation of Rubisco large

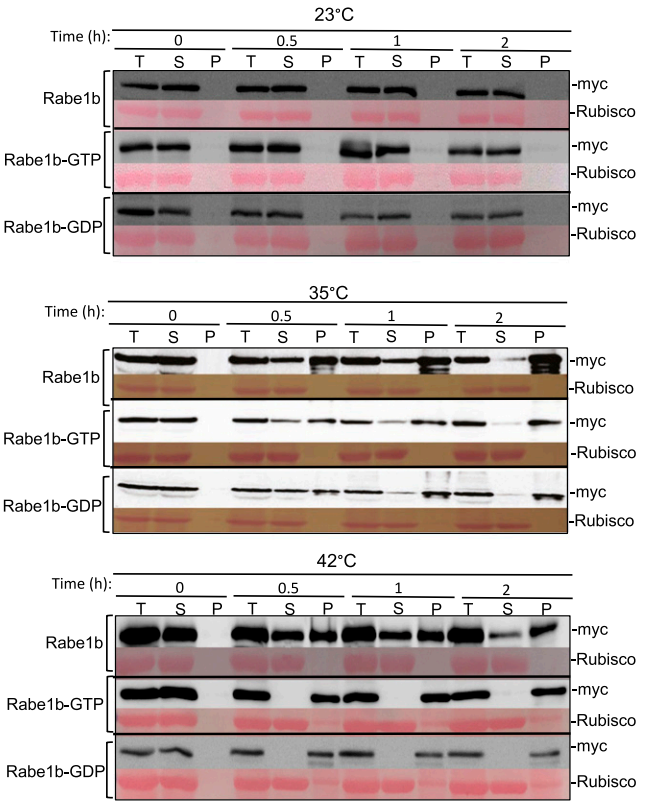

Figure 2. Heat-induced rapid aggregation of Arabidopsis plastid EF-Tu in vitro. Total proteins (T) were extracted from transgenic *AtRabe1b-OE*, *AtRabe1b-GTP-OE*, and *AtRabe1b-GDP-OE* Arabidopsis plants and were incubated at the indicated temperatures for the indicated hours. Soluble proteins in the supernatants (S) and insoluble proteins in the pellets (P) were separated by low-speed centrifugation, and the relative amounts of myc-tagged AtRabe1b, AtRabe1b-GTP, and AtRabe1b-GDP proteins in the protein fractions were analyzed by immunoblotting using an anti-myc monoclonal antibody. Rubisco large subunit proteins stained with Ponceau S are shown as the loading control.

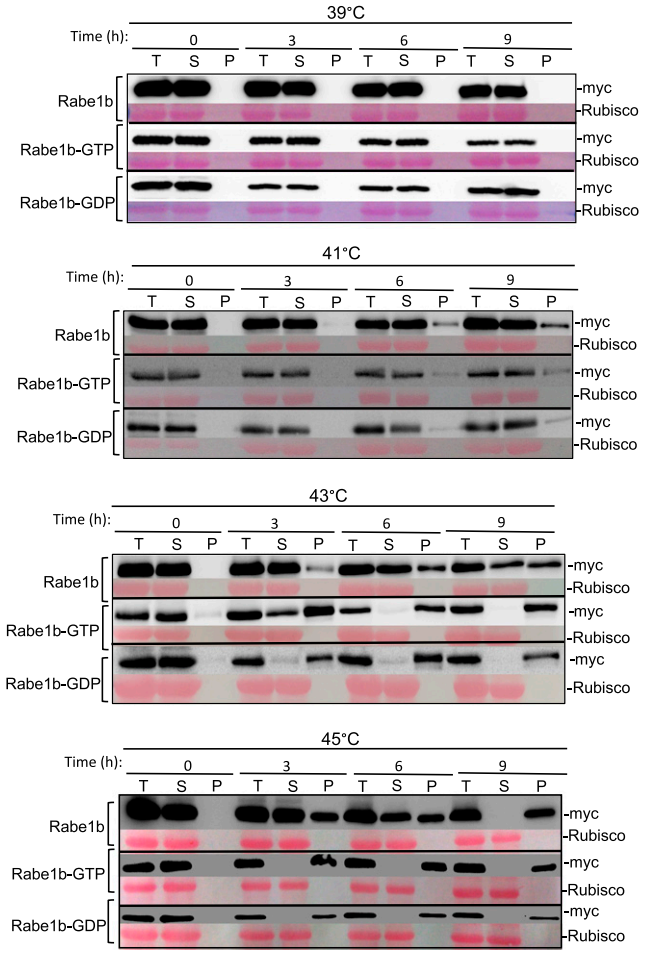

Figure 3. Heat-induced rapid aggregation of Arabidopsis plastid EF-Tu in vivo. Transgenic *AtRabe1b-OE, AtRabe1b-GTP-OE,* and *AtRabe1b-GDP-OE* Arabidopsis plants were treated at the indicated temperatures for the indicated hours. Total proteins (T) were extracted from treated transgenic plants and separated by low-speed centrifugation into soluble proteins in the supernatants (S) and insoluble proteins in the pellets (P). Myc-tagged AtRabe1b, AtRabe1b-GTP, and AtRabe1b-GDP proteins in the total, soluble, and insoluble fractions were analyzed by immunoblotting. Rubisco large subunit proteins stained with Ponceau S are shown as the loading control.

subunit proteins, which were used as the loading control in immunoblotting, was detected after a 9-h incubation of the transgenic plants at 45°C (Fig. 3). Thus, even though Rabe1b proteins were more stable in vivo than in vitro, they were still exceptionally heat sensitive and prone to aggregation in vivo at high temperatures.

Heat-Induced Aggregation of Rabe1b Was Irreversible in Vitro and Likely in Vivo as Well

To determine whether the heat-induced aggregation of Rabe1b proteins is reversible in vitro, we first extracted total proteins from transgenic *AtRabe1b-OE*, *AtRabe1b-GTP-OE*, and *AtRabe1b-GDP-OE* plants and incubated the proteins at 42°C for 2 h to induce Rabe1b aggregation. The heat-treated protein extracts were then incubated at a low temperature (23°C) for various amounts of time and analyzed for possible solubilization of Rabe1b aggregates. As shown in Figure 4A, incubation at 23°C for 2 h did not lead to a reduction in Rabe1b aggregates or an increase in the soluble forms. Further increase in the incubation time at the low temperature for up to 24 h did not increase the amount of soluble Rabe1 proteins either, even though protein degradation became apparent after the extended incubation in vitro (data not shown). Thus, heat-induced aggregation of Rabe1b proteins was irreversible in vitro.

We also analyzed the fate of heat-induced Rabe1b protein aggregates in vivo after recovery at a low room temperature. Transgenic *AtRabe1b-OE*, *AtRabe1b-GTP-OE*, and *AtRabe1b-GDP-OE* plants were first treated for 6 h at 45°C to induce Rabe1b aggregation and then were transferred to 23°C. After a 24-h recovery at 23°C, total proteins were extracted and the relative amounts of soluble and insoluble Rabe1b proteins were analyzed by low-speed centrifugation and immunoblotting. As shown in Figure 4B, recovery of heat-treated transgenic plants at 23°C for 24 h led to an increase in soluble Rabe1b and a corresponding reduction in insoluble Rabe1b proteins. However, it was unclear whether the increased accumulation of soluble Rabe1b during the recovery at the low temperature after heat treatment was due to the solubilization of heat-induced Rabe1b aggregates or to new Rabe1b synthesis. Likewise, reduction of heat-induced Rabe1b aggregates during the recovery at 23°C could result from their solubilization or from their degradation. To examine these possibilities, we treated the transgenic plants first with the protein synthesis inhibitor CHX before heat treatment at 45°C. After the 6-h heat treatment, the plants were again transferred to 23°C and Rabe1b proteins were analyzed after the 24-h recovery at low temperature. Interestingly, we observed that CHX treatment prevented not only an increase in soluble Rab1b proteins but also a reduction of heat-induced Rabe1b aggregates during the 24-h recovery at 23°C. The blocking of the reduction of heat-induced Rabe1b aggregates during the recovery by a protein synthesis inhibitor would argue against their ability to spontaneously refold into soluble forms at room temperature. The observed reduction of heat-induced Rabe1b aggregates during the recovery in the absence of CHX likely could result from their degradation by autophagy or other protein degradation pathways whose induction or activation may be dependent on protein synthesis. In addition, some heat-induced Rabe1b aggregates may undergo facilitated, not spontaneous, refolding into soluble forms with the aid of other proteins, such as protein chaperones, whose synthesis is sensitive to CHX.

Overexpression of Constitutive GTP- and GDP-Bound Mutant Rabe1b Proteins Compromised Heat Tolerance

As a translation factor, Rabe1b plays an essential role in plastid protein synthesis, and its rapid aggregation

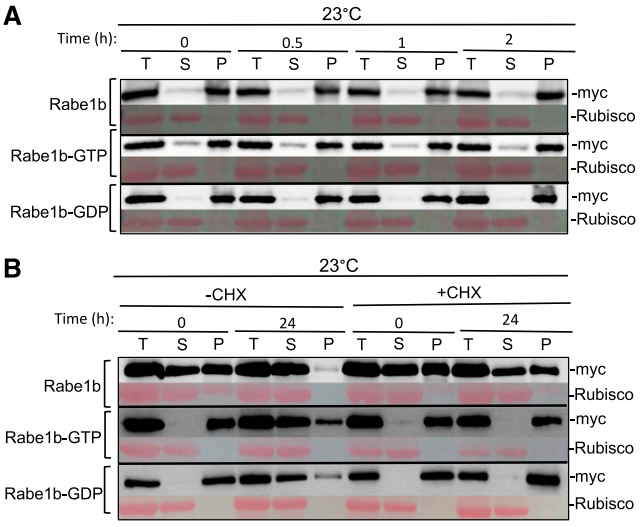

Figure 4. Analysis of heat-induced Rabe1b aggregates during recovery. A, Heat-induced Rabe1b aggregates during recovery in vitro. Total proteins were extracted from transgenic *AtRabe1b-OE*, *AtRabe1b-GTP-OE*, and *AtRabe1b-GDP-OE* Arabidopsis plants incubated at 42°C for 2 h to induce Rabe1b aggregation and then placed at 23°C for the indicated hours. Total proteins (T), soluble proteins in the supernatants (S), and insoluble proteins in the pellets (P) were separated, and the amount of myc-tagged AtRabe1b proteins in the fractions was analyzed by immunoblotting. Rubisco proteins stained with Ponceau S are shown as the loading control. B, Effects of a protein synthesis inhibitor on heat-induced Rabe1b aggregates during recovery in vivo. Transgenic *AtRabe1b-OE*, *AtRabe1b-GTP-OE*, and *AtRabe1b-GDP-OE* Arabidopsis plants were treated with the protein synthesis inhibitor cycloheximide (CHX; 100 μM) and then incubated at 45°C for 6 h. The plants were then transferred to 23°C for recovery. Total proteins were extracted from treated plants at 0 and 24 h of recovery. Soluble proteins in the supernatants and insoluble proteins in the pellets were separated, and myc-tagged AtRabe1b proteins were analyzed by immunoblotting. Rubisco proteins stained with Ponceau S are shown as the loading control.

under heat stress could have important effects on the growth and survival of plants under heat stress. To determine this possibility, we analyzed the effects of the overexpression of wild-type Rabe1b and its constitutive GTP- and GDP-bound mutant forms on plant heat tolerance. During the elongation phase of protein translation, EF-Tu alternates between GTP- and GDP-bound forms, which are required for its dynamic interactions with other factors, including aa-tRNAs, ribosomes, and other proteins (Krab and Parmeggiani, 2002). Unlike wild-type EF-Tu, constitutive GTP- and GDP-bound forms of EF-Tu would interact with only certain cellular factors but not with other cellular factors required for protein elongation in a dynamic manner and, therefore, could act as dominant negative proteins that interfere with plastid translation. To determine heat tolerance, we used the transgenic *AtRabe1b-OE*, *AtRabe1b-GTP-OE*, and *AtRabe1b-GDP-OE* lines with similar levels of the expressed AtRabe1b proteins (Fig. 5A), placed 3-week-old seedlings in a 45°C growth chamber for 9 h, and scored for survival rates after recovery for 5 d at room temperature. As shown in Figure 5, B and C, more than 30% of wild-type Col-0 seedlings survived after the heat stress. Overexpression of wild-type Rabe1b significantly increased heat tolerance, as their survival rates were about 10% higher than those of wild-type plants (Fig. 5, B and C). By contrast, the survival rates of transgenic *AtRabe1b-GTP-OE* and *AtRabe1b-GDP-OE* lines were less than 5% after heat stress (Fig. 5, B and C).

We also compared mature plants of the wild type and overexpression lines for heat tolerance. Five-week-old mature plants were treated at 45°C for 10 h and observed for growth and survival after 5 d of recovery at room temperature. As shown in Figure 6A, while Col-0 wild-type and transgenic *AtRabe1b-OE* plants survived after heat stress, most *AtRabe1b-GTP-OE* and *AtRab1lb-GDP-OE* plants displayed wilting symptoms during heat stress and died during the recovery at room temperature. At the biochemical level, heat stress causes damage to the light-harvesting complexes of PSII and inhibition of PSII (Allakhverdiev et al., 1996, 2008). Therefore, we compared the ratio of variable chlorophyll fluorescence to maximum fluorescence (F_v/F_m), indicative of the maximum quantum yield of PSII, in fully expanded leaves of the overexpression lines with those of wild-type plants immediately before and after 2, 4, and 6 h of heat treatment at 45°C. As shown in Figure 6, B and C, no significant difference in F_v/F_m was observed among the wild type and the Rabe1b-overexpressing lines prior to the heat treatment. After the 6-h heat treatment, both wild-type and AtRabe1-b-OE plants displayed a reduction of about 35% in F_v/F_m when compared with those before heat treatment (Fig. 6, B and C). On the other hand, AtRabe1b-GTP-OE and AtRabe1b-GDP-OE plants showed a greater than 70% reduction in F_v/F_m from heat treatment (Fig. 6, B and C). Thus, overexpression of constitutive GTP- and GDP-bound mutant Rabe1b compromised heat tolerance in transgenic Arabidopsis plants.

Knockdown of *Rabe1b* Increased Heat Sensitivity

To further analyze the role of Rabe1b in plant heat tolerance, we isolated T-DNA insertion mutants for the gene. Two T-DNA insertion lines, SAIL_415_E05 and SAIL_659_G09, were screened using PCR with primers flanking the insertion sites. SAIL_415_E05 contains a T-DNA insertion in the middle of the single-exon gene, but a PCR screen failed to identify homozygous plants, most likely due to their lethal phenotype. SAIL_659_G09 contains a T-DNA insertion in the 5′ region of the gene, approximately 120 nucleotides upstream of its translation start codon (Fig. 7A). RT-qPCR showed that homozygous SAIL_659_G09 contains about 25% of the wild-type levels of the *Rabe1b* transcripts (Fig. 7B). Therefore, SAIL_659_G09 is a knockdown mutant and was named *rabe1b-1* (Fig. 7A). The *rabe1b-1* mutant plants were reduced in size and were slightly pale green when grown at normal conditions

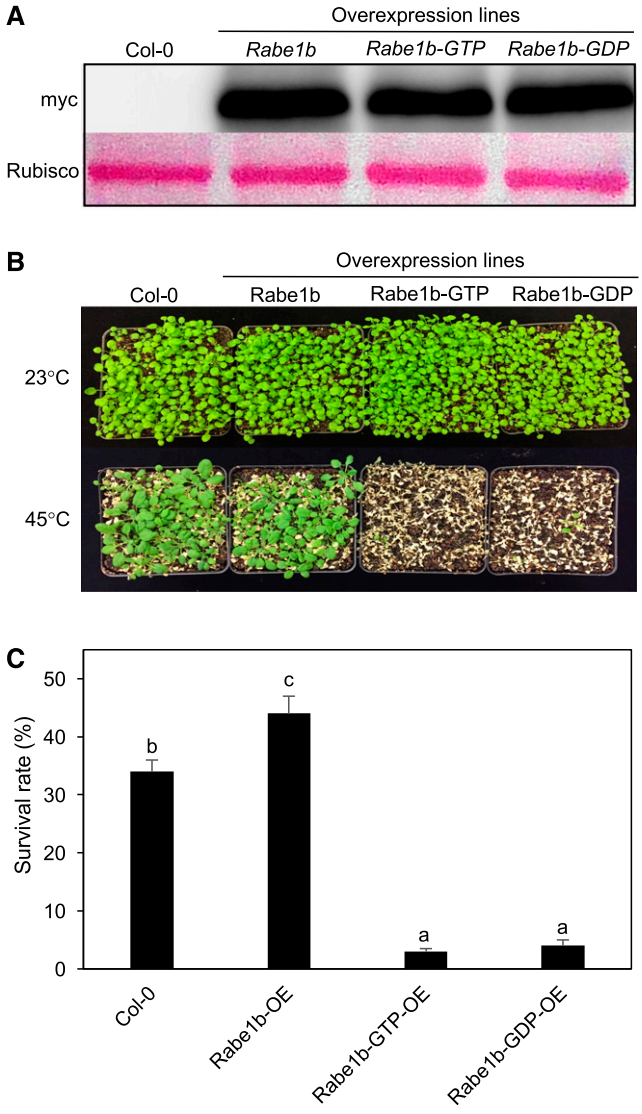

Figure 5. Effects of overexpression of Rabe1b, Rabe1b-GTP, and Rabe1b-GDP on the heat tolerance of Arabidopsis seedlings. A, Immunoblotting of myc-tagged AtRabe1b, AtRabe1b-GTP, and AtRabe1b-GDP proteins in transgenic plants. Total proteins were extracted from transgenic *AtRabe1b-OE*, *AtRabe1b-GTP-OE*, and *AtRabe1b-GDP-OE* Arabidopsis plants with similar levels of the transgene transcripts and analyzed by immunoblotting using an anti-myc antibody. Rubisco large subunit proteins stained with Ponceau S are shown as the loading control. B, Assays of young seedling heat tolerance. Two-week-old seedlings were placed in a 45°C growth chamber for 9 h. The heat-treated plants were then moved to a 23°C growth chamber for recovery. The photograph was taken 5 d after the heat treatment. C, Survival rates of heat-stressed young seedlings determined after treatment at 45°C for 9 h followed by 23°C for 5 d. Means and SE were calculated from survival rates determined from three experiments with approximately 100 seedlings per experiment for each genotype. According to Duncan's multiple range test ($P < 0.01$), mean survival rates do not differ significantly if they are indicated with the same letter.

(Fig. 7C). To determine that the growth phenotype of the *rabe1b-1* mutant was due to the reduced expression of *Rabe1b*, we transformed myc-tagged *AtRabe1b*, *AtRabe1b-GTP*, and *AtRabe1b-GDP* genes into the mutant. As observed in the wild type, a substantial percentage of transformants displayed bleached phenotypes due to cosuppression. Among those lines that expressed the transgenes based on both RT-qPCR and immunoblotting, we observed that myc-tagged AtRabe1b, but not its constitutive GTP- or GDP-bound mutant form, could complement the mutant (Fig. 7C). This result indicates that the constitutive GTP- and GDP-bound mutant forms of Rabe1b are inactive as translation factors.

To compare the wild type and the *rabe1b* mutant for heat tolerance, we again tested both seedlings and mature plants for survival rates after heat treatment. As shown in Figure 8, about 35% of wild-type seedlings survived after heat treatment at 45°C for 9 h. By contrast, none of the *rabe1b-1* mutant seedlings survived the same heat treatment (Fig. 8). Introduction of the myc-tagged *AtRabe1b*, but not the *AtRabe1b-GTP* or *AtRabe1b-GDP* transgene, could completely restore the heat tolerance of the *rabe1b* mutant seedlings (Fig. 8). Similar results also were obtained with mature plants based on an analysis of the survival and the F_v/F_m ratios after heat treatment (Fig. 9). Thus, the *rabe1b* knockdown mutant, despite the substantial residual levels of the *Rabe1b* transcripts, is highly compromised in heat tolerance.

We also transformed the *rabe1b* mutant with the wild-type *Rabe1b* gene under the control of its native promoter. Again, we observed a substantial percentage of transformants displaying bleached phenotypes due to cosuppression. The remaining lines that expressed the transgene based on both RT-qPCR and immunoblotting were indistinguishable from the wild type (Supplemental Fig. S6). Furthermore, both the heat tolerance (Supplemental Fig. S6) and heat induction of *HsfA2* and its target genes (data not shown) of the *rabe1b* mutant were fully restored to that of wild-type plants. Thus, the *rabe1b* mutant could be fully complemented by the wild-type *Rabe1b* gene under the control of its native promoter.

Silencing of *Rabe1b* Compromised Heat Tolerance in Tomato

To determine whether the critical role of plastid EF-Tu in plant heat tolerance is evolutionarily conserved, we extended our functional analysis to tomato using virus-induced gene silencing (VIGS). From the sequenced tomato genome, we identified two tomato *Rabe1b* genes, *SlRabe1b-1* (Solyc03g112150) and *SlRabe1b-2* (Solyc06g071790). Tomato *Rabe1b-1* and *Rabe1b-2* genes encode two proteins of 477 and 478 amino acid residues, respectively, that share approximately 88% sequence identify with each other and about 85% with Arabidopsis *Rabe1b*, with most of the variable amino acid residues in the N-terminal plastid-targeting signal peptides (Supplemental Fig. S7). For VIGS, we cloned a DNA fragment specific to the tomato plastid *Rabe1b* genes into the pTRV vector and infiltrated

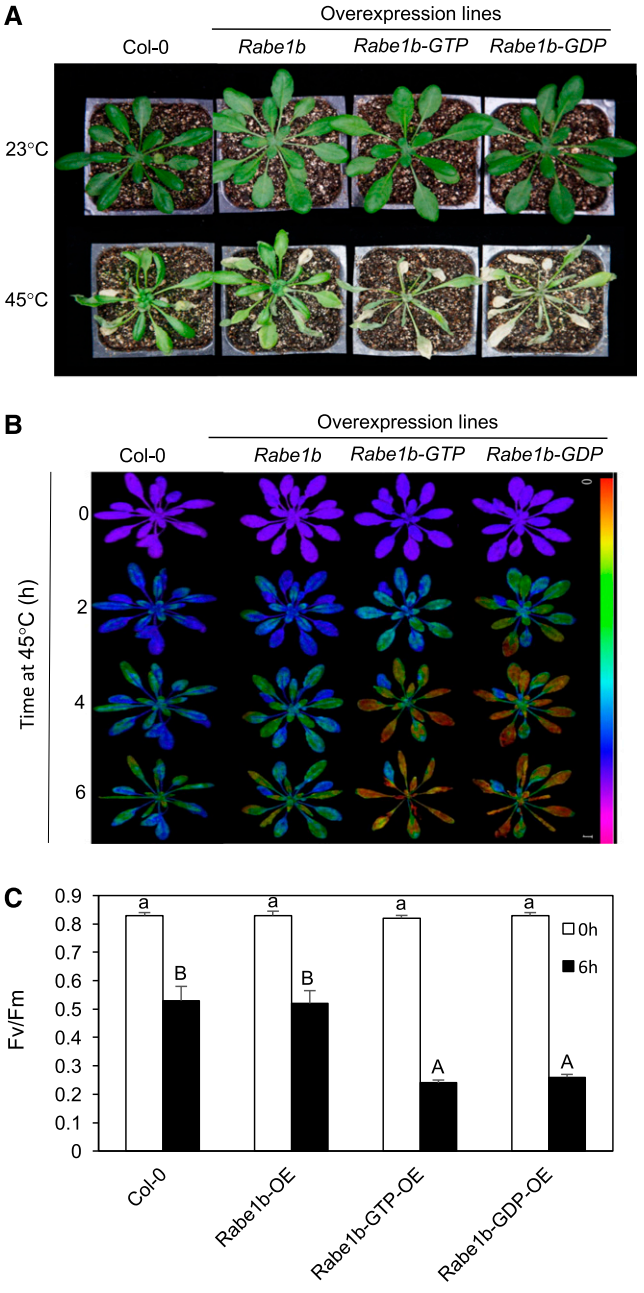

Figure 6. Effects of overexpression of Rabe1b, Rabe1b-GTP, and Rabe1b-GDP on the heat tolerance of Arabidopsis mature plants. A, Six-week-old mature plants were placed in a 45°C growth chamber for 10 h. The heat-treated plants were then moved to a 23°C growth chamber for recovery. The photograph was taken 5 d after the heat treatment. B, F_v/F_m images of fully expanded leaves determined immediately after the indicated hours (h) at 45°C heat treatment. The color code in the images ranged from 0 (black) to 1 (purple). C, F_v/F_m values of fully expanded leaves determined immediately after the indicated hours of heat treatment at 45°C. Means and SE were calculated from average F_v/F_m values determined from three experiments with 10 leaves per experiment for each genotype. According to Duncan's multiple range test ($P < 0.01$), means of F_v/F_m at 0 h do not differ significantly if they are indicated with the same lowercase letter; means of F_v/F_m at 6 h do not differ significantly if they are indicated with the same uppercase letter.

Agrobacterium tumefaciens cells harboring the VIGS vector into tomato cotyledons. Approximately 3 weeks after the infiltration, tomato plants infiltrated with the pTRV-Rabe1b vector started to develop pale green leaves with bleached patches (Fig. 10A). No such phenotypes were observed in the tomato plants infiltrated with the pTRV empty vector (Fig. 10A). RT-qPCR analysis showed that transcript levels of the tomato *Rabe1b* genes in the plants infiltrated with pTRV-Rabe1b were reduced by about 85% when compared with those in the control plants infiltrated with the pTRV empty vector (Fig. 10). To assess the effect of silencing of the *Rabe1b* genes on heat tolerance, we placed the tomato plants in a 45°C growth chamber for 8 h and then moved them to room temperature for a 3-d recovery. For heat-treated pTRV control plants, we observed few symptoms after recovery (Fig. 10B). On the other hand, almost all expanded leaves of pTRV-Rabe1b plants displayed wilting after recovery (Fig. 10B). The severe wilting symptoms in *Rabe1b*-silenced tomato plants after heat stress were consistent with the drastic increase in electrolyte leakage in the silenced plants relative to that in the pTRV control plants (Fig. 10C). These results indicated that the plastid Rabe1b proteins also play a critical role in tomato heat tolerance.

Knockdown of *Rabe1b* Inhibits Nuclear Heat-Responsive Gene Expression

In plants, heat stress rapidly induces the production of well-characterized HSPs, including Hsp101, Hsp70, and small HSPs, which act as molecular chaperones in protein quality control by promoting the folding and refolding of nonnative proteins (Whitley et al., 1999). The regulation of HSP gene expression is mediated by the conserved HSFs. In Arabidopsis, HsfA2 plays a key role in the triggering and amplification of plant heat stress responses (Kotak et al., 2007). Interestingly, the basal transcript levels of Arabidopsis *HsfA2* in the Arabidopsis *rabe1b-1* mutant were only about 30% of those in the wild type in the absence of heat stress. At 45°C, the transcript levels of *HsfA2* in the wild type increased rapidly, with an approximately 800-fold induction at the 1-h peak point under heat stress (Fig. 11). In the *rabe1b* mutant, the induction of *HsfA2* gene expression was inhibited, with less than a 200-fold induction at the 1-h peak point during heat treatment (Fig. 11). We also analyzed a subset of HsfA2 target genes for their expression before and after heat treatment. These target genes encode APX2, GolS1, and several HSPs (Hsp17.6, Hsp18.2, Hsp21, Hsp70, and Hsp101). We again observed that the basal levels of these HsfA2 target genes in the absence of heat stress were all reduced by 60% to 80% when compared with those in the wild type. All these HsfA2 target genes were strongly induced in the wild type, where the transcript levels peaked 1 to 2 h after heat stress (Fig. 11). As with *HsfA2*, the heat induction of these

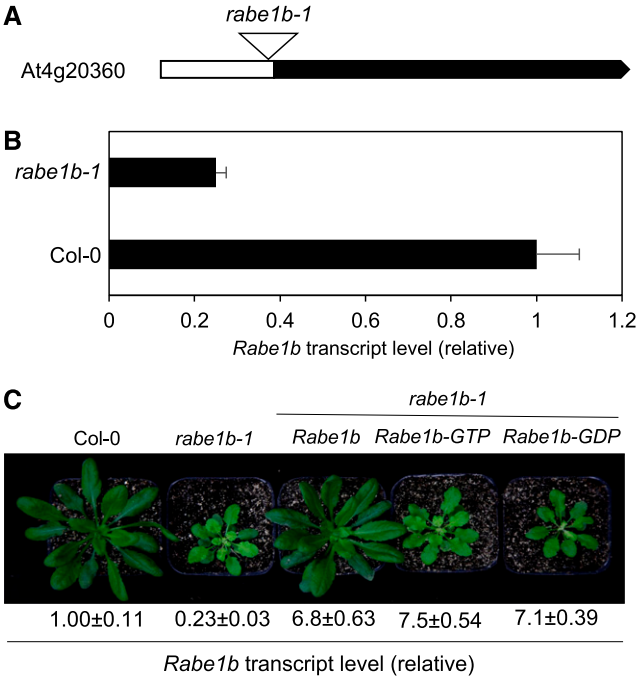

Figure 7. Identification and growth of Arabidopsis *rabe1b-1* mutants. A, Diagram of the *Rabe1b* gene and the T-DNA insertion site. B, Transcript levels of *Rabe1b* in the Col-0 wild type and the *rabe1b-1* mutant as determined using RT-qPCR. Means and SE were calculated from three replicates. C, Growth of Col-0, the *rabe1b-1* mutant, and the *rabe1b-1* mutant complemented with the wild-type *Rabe1b* or its constitutive GTP- and GDP-bound mutant forms. The photograph was taken 4 weeks after germination. Means and SE of the relative *Rabe1b* transcript levels in the plants determined by RT-qPCR from three replicates also are shown at the bottom.

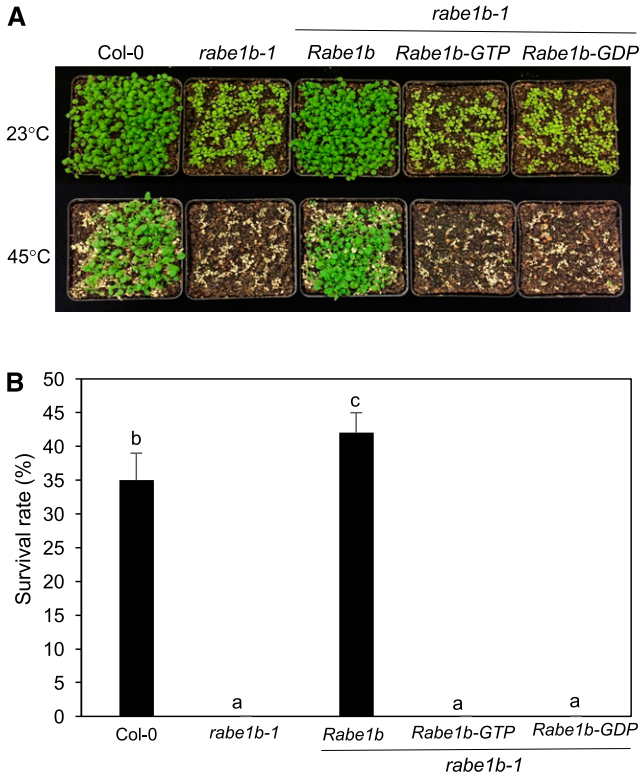

Figure 8. Functional analysis of Arabidopsis *Rabe1b* in the heat tolerance of Arabidopsis seedlings. A, Assays of the heat tolerance of young seedlings. Approximately 50 2-week-old seedlings of wild-type Col-0, the *rabe1b-1* mutant, and the *rabe1b* mutant complemented with the *Rabe1b*, *Rabe1b-GTP*, or *Rabe1b-GDP* transgene were placed in a 45°C growth chamber for 9 h. The heat-treated plants were then moved to a 23°C growth chamber for recovery. The photograph was taken 5 d after the heat treatment. B, Survival rates of heat-stressed young seedlings determined after treatment at 45°C for 9 h followed by 23°C for 5 d for recovery. Means and SE were calculated from survival rates determined from three experiments with approximately 100 seedlings per experiment for each genotype. According to Duncan's multiple range test ($P < 0.01$), means of survival rates do not differ significantly if they are indicated with the same letter.

HsfA2 target genes was all reduced substantially in the *rabel1-1* knockdown mutant relative to that in the wild type (Fig. 11). Thus, the reduced expression of Rabe1b in the *rabe1b* mutant caused substantial inhibition of both basal and induced expression of *HsfA2* and its target genes.

Constitutive Expression of HsfA2 Partially Restored the Heat Tolerance of *rabe1b-1*

HsfA2 regulates the expression of a variety of genes involved in stress responses with protective roles in scavenging ROS, production of osmolytes, and protein quality control (Shigeoka et al., 2002; Taji et al., 2002; Nishizawa et al., 2006). Reduced basal and induced expression of HsfA2 and its target genes in *rabe1b-1* could lead to reduced capacities of these protective mechanisms and, consequently, growth defects and compromised heat tolerance in the mutant. To determine this possibility, we generated transgenic *rabe1b* mutant plants harboring an *HsfA2* transgene under the control of the constitutive *CaMV 35S* promoter. Transgenic lines constitutively expressing the *HsfA2* gene were obtained based on RT-qPCR (Fig. 12A). Notably, constitutive expression of *HsfA2* in the *rabe1b-1* mutant did not rescue the phenotypes of reduced size and pale green leaves of the mutant (Fig. 12B).

To determine the impact of the constitutive expression of *HsfA2* on the heat tolerance of the *rabe1b-1* mutant, we compared F_v/F_m values before and after heat treatment at 45°C for 10 h among wild-type and *rabe1b* mutant plants with or without the *HstA2-myc* transgene. The F_v/F_m value for the *rabe1b* mutant was more than 10% lower than that for the wild type even when grown at normal conditions (Fig. 12, B and C). Constitutive expression of *HsfA2* in the *rabe1b* mutant did not significantly affect the F_v/F_m value of the mutant prior to heating. After the 4-h heat treatment at 45°C, the F_v/F_m value of the *rabe1b* mutant was reduced by more than 85%, compared with less than a 30% reduction in wild-type plants (Fig. 12, B and C). In the *rabe1b* plants

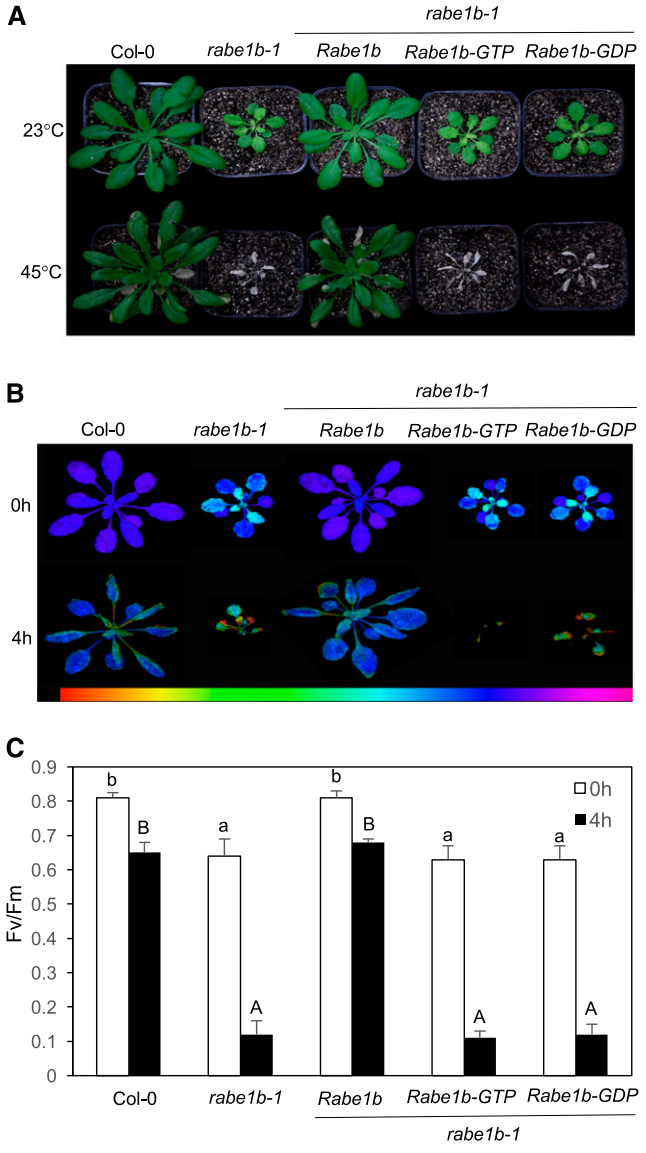

Figure 9. Functional analysis of Arabidopsis Rabe1b in the heat tolerance of mature plants. A, Six-week-old mature plants of wild-type Col-0, the *rabe1b-1* mutant, and the *rabe1b* mutant complemented with the *Rabe1b*, *Rabe1b-GTP*, or *Rabe1b-GDP* transgene were placed in a 45°C growth chamber for 10 h. The heat-treated plants were then moved to a 23°C growth chamber for recovery. The photograph was taken 5 d after the heat treatment. B, F_v/F_m images of fully expanded leaves determined immediately after 0 and 4 h at 45°C. The color code in the images ranged from 0 (black) to 1 (purple). C, F_v/F_m values of fully expanded leaves determined immediately after the indicated hours of heat treatment at 45°C. Means and SE were calculated from five replicates. According to Duncan's multiple range test ($P < 0.01$), means of F_v/F_m at 0 h do not differ significantly if they are indicated with the same lowercase letter; means of F_v/F_m at 4 h do not differ significantly if they are indicated with the same uppercase letter.

constitutively expressing *HsfA2*, the F_v/F_m value was reduced by approximately 50% after 4 h at 45°C. RT-qPCR analysis, on the other hand, showed that constitutive expression of *HsfA2* in the *rabe1b* mutant increased both the basal and heat-induced expression of *HsfA2* and *HsfA2* target genes to levels close to or even higher than those in wild-type plants (Fig. 13). Thus, constitutive expression of *HsfA2* had no significant effect on the growth of the *rabe1b* mutant and only partially restored the heat tolerance of the mutant despite fully restored induction of *HsfA2* and *HsfA2* target genes.

Activity of Rabe1b Proteins in Plastid Protein Translation and Protein Folding

To further study how Arabidopsis Rabe1b plays such a critical role in plant heat stress responses, we examined the effects of the *rabe1b-1* mutation and the overexpression of wild-type Rabe1b and its constitutive GTP- and GDP-bound mutant forms on plastid protein translation at normal conditions and during heat stress. Plastids were isolated from the Arabidopsis wild type, transgenic *Rabe1b-OE*, *Rabe1b-GTP-OE*, and *Rabe1b-GDP-OE* plants, and the *rabe1b-1* mutant, and plastid protein translation was assayed in isolated plastids using [^{35}S]Met. As shown in Figure 14, at normal conditions, overexpression of wild-type Rabe1b increased plastid protein synthesis by about 10%, whereas overexpression of the constitutive GTP- and GDP-bound mutant forms has no significant effect on plastid protein synthesis. After 6 h at 45°C, protein synthesis in plastids from wild-type plants was reduced by about 65% when compared with that at normal conditions (Fig. 14). In transgenic plants overexpressing wild-type Rabe1b, the heat treatment also reduced plastid translation by approximately 55% and, as a result, plastid protein synthesis in the transgenic plants was about 40% higher than that in wild-type plants after heat treatment (Fig. 14). In transgenic plants overexpressing the constitutive GTP- and GDP-bound mutant Rabe1b forms, heat stress reduced plastid protein translation by about 80% and, as a result, plastid protein synthesis in these transgenic plants was about 40% lower than that in wild-type plants after heat treatment (Fig. 14). In the *rabe1b-1* mutant, plastid protein synthesis was reduced to about 30% of the wild-type level at normal conditions (Fig. 14). After heat treatment at 45°C for 6 h, plastid protein synthesis in the *rabe1b-1* mutant was reduced further to about 20% of the wild-type level (Fig. 14). Thus, the levels of plastid protein synthesis under heat stress were highly associated with the levels of heat tolerance among the wild type, the *rabe1b* mutant, and the three types of transgenic overexpression lines (Figs. 5, 6, 8, and 9).

To examine how wild-type Rabe1b and the constitutive GTP- and GDP-bound mutant forms differed in their abilities to promote heat tolerance, we were interested in determining their effects on plastid protein folding and stability, which, however, is currently still not feasible in vivo. Therefore, we compared the chaperone activity of the three proteins in vitro. We generated Rabe1b proteins in *E. coli* and assayed their

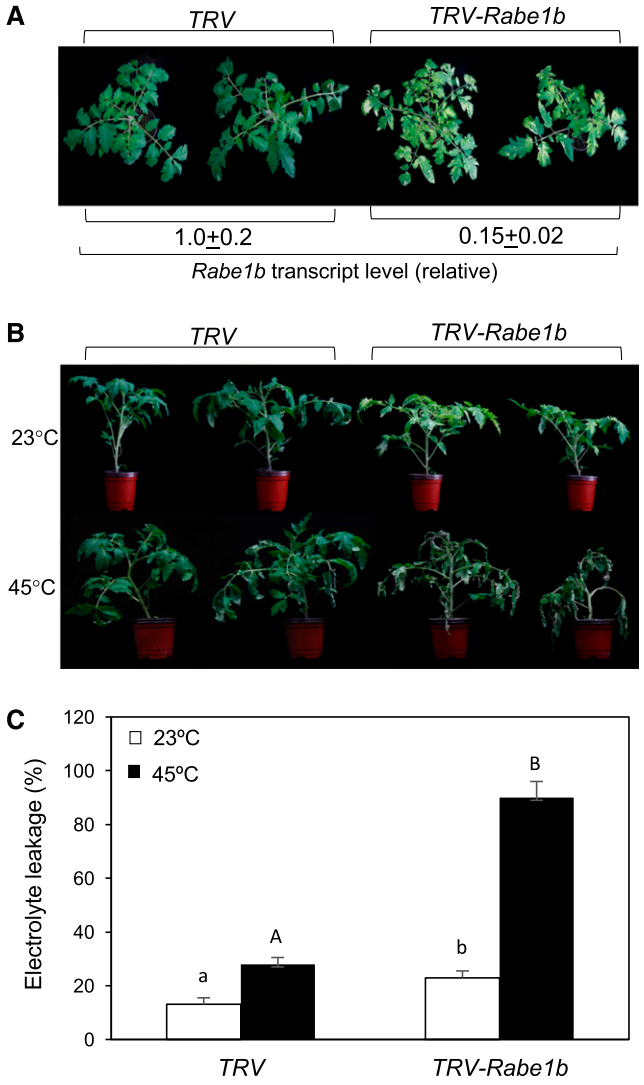

Figure 10. Functional analysis of tomato *Rabe1b* in tomato heat tolerance using TRV-mediated gene silencing. A, Tomato plants infiltrated with *A. tumefaciens* cells harboring the empty pTRV vector or the silencing pTRV-*Rabe1b* vector were placed in a 23°C growth room. Photographs of the representative plants were taken 4 weeks after *A. tumefaciens* infiltration. Tomato Rabe1b transcript levels in tomato plants infiltrated with *A. tumefaciens* cells harboring the empty pTRV vector or the pTRV-Rabe1b silencing vector were determined by RT-qPCR analysis using total RNA isolated from the terminal leaflets of the fifth leaves of *A. tumefaciens*-infiltrated tomato plants and are listed below the images. Means and SE were calculated from approximately 10 leaflets from five plants. B, *A. tumefaciens*-infiltrated tomato plants were placed in a 23°C or 45°C growth chamber for 8 h. Photographs of the whole plants were taken after the 5-d recovery. C, Electrolyte leakage (EL) of the terminal leaflets of the fifth leaves was determined immediately after 8 h at 23°C or 45°C heat treatment. Means and SE were calculated from average EL values determined from three experiments with10 leaves per experiment for each genotype. According to Duncan's multiple range test ($P < 0.01$), means of EL at 23°C do not differ significantly if they are indicated with the same lowercase letter; means of EL at 45°C do not differ significantly if they are indicated with the same uppercase letter.

activity in protecting the highly heat-sensitive malate dehydrogenase (MDH) at 43°C, which has been widely used to determine protein chaperone activity. MDH was incubated with Rabe1b or bovine serum albumin (BSA) at 43°C for 30 min and then analyzed immediately for residual MDH activity. As shown in Supplemental Figure S8, wild-type Rabe1 and its constitutive GTP- and GDP-bound mutant forms had similar levels of activity, protecting heat-sensitive MDH at the high temperature when compared with the BSA control. This result is consistent with the previous reports that EF-Tu proteins from bacteria, mitochondria, and plastids act as protein chaperones in a GTP/GDP-independent manner (Malki et al., 2002; Rao et al., 2004; Ristic et al., 2007; Suzuki et al., 2007). Thus, despite their differential ability in promoting plant heat tolerance, wild-type Rabe1b and its constitutive GTP- and GDP-bound mutant forms appeared to have similar levels of protein chaperone activity.

DISCUSSION

As a translation factor, EF-Tu plays an essential role in protein biosynthesis in prokaryotes and in eukaryotic organelles of mitochondria and plastids. EF-Tu also can act as a protein chaperone, and chloroplast EF-Tu from plants plays a role in plant heat tolerance based on the expression of their corresponding genes and changed heat tolerance from altered levels of the translation elongation factor (Bhadula et al., 2001; Rao et al., 2004; Ristic et al., 2004, 2007, 2008; Momcilovic and Ristic, 2007; Fu et al., 2008; Brisio et al., 2010; Fu and Ristic, 2010). Here, we extended these studies by providing important information about the dynamic states of chloroplast EF-Tu under heat stress and its important role and possible mode of action in plant responses to heat stress.

Heat-Induced Aggregation of Chloroplast EF-Tu

We reported previously from proteomic profiling that the Arabidopsis plastid EF-Tu, Rabe1b, accumulates as aggregates in heat-treated plants (Zhou et al., 2014b). Through comprehensive analysis of its total, soluble, and insoluble forms using immunoblotting, we confirmed that aggregation of Arabidopsis Rabe1b proteins occurred in vitro at a temperature as low as 25°C (Supplemental Fig. S5) and that more than 50% of the proteins became insoluble in vitro after 30 min at 35°C (Fig. 2). In vivo, the plastid EF-Tu also aggregated when the plants were treated with a temperature above 41°C (Fig. 3). Thus, plastid Rabe1b proteins were highly prone to aggregate at elevated temperatures. In vitro and in vivo protein aggregation is a topic of high importance to the understanding of protein structures and functions, production of recombinant proteins in biotechnology, and human health (Philo and Arakawa, 2009). Among the possible mechanisms of protein aggregation, the predominant one involves

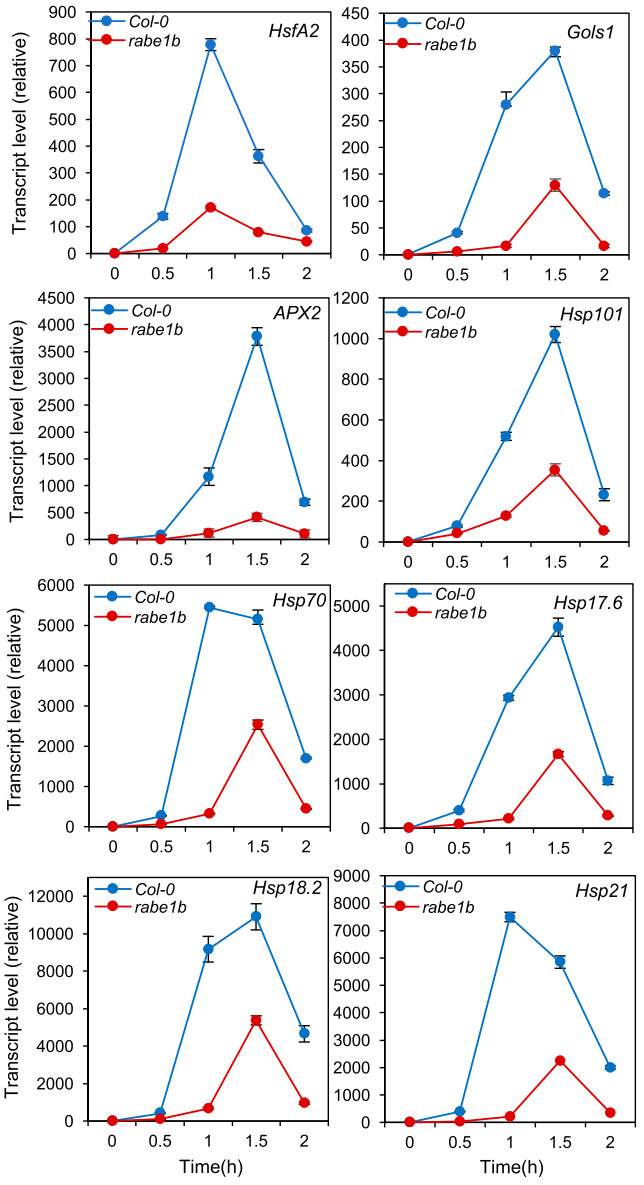

Figure 11. Induction of Arabidopsis *HsfA2* and its target genes by heat stress. Five-week-old Arabidopsis Col-0 wild-type and *rabe1b-1* mutant plants were placed in a 45°C growth chamber, and total RNA was isolated from leaf samples collected at the indicated times. Transcript levels were determined using RT-qPCR. Means and SE were calculated from three replicates.

conformational changes that expose hydrophobic portions of the proteins, which may interact with the exposed hydrophobic patches of other proteins (Philo and Arakawa, 2009). The EF-Tu from prokaryotes, mitochondria, and plastids is a triangular three-domain protein that undergoes substantial conformational changes during the elongation cycle of protein translation (Brisio et al., 2010). The GTP-bound EF-Tu forms a ternary complex with aa-tRNA, and upon codon-anticodon recognition and GTP hydrolysis to GDP, EF-Tu can undergo a conformational change necessary for the release of the aa-tRNA and dissociation from the ribosome in the EF-Tu-GDP form (Brisio et al., 2010). Biochemical and structural analyses of EF-Tu from mesophilic bacteria like *E. coli* have revealed that the translation factor, particularly its N-terminal GTP/GDP-binding domain, is prone to secondary structure shift (Brisio et al., 2010). On the other hand, EF-Tu proteins from hyperthermophilic bacteria are highly structured and more resistant to high temperatures (Katava et al., 2016).

During the translation elongation, EF-Tu undergoes dynamic association with aa-tRNAs, ribosomes, and other proteins, including guanidine exchange factor EF-Ts (Brisio et al., 2010). Therefore, aggregation of EF-Tu at high temperatures would inactivate its activity as a translation elongation factor. The high propensity of plastid EF-Tu to aggregate in vitro even under slightly elevated temperatures above 25°C would be highly detrimental if it was also the case in vivo, given its essential role in plastid protein synthesis. Apparently, there are mechanisms that promote stability and reduce the aggregation of plastid Rabe1b proteins in plant cells, as evident from the substantially higher temperatures required to induce Rabe1b protein aggregation in vivo than in vitro (Figs. 2 and 3). One possible mechanism could be the active engagement of soluble Rabe1b proteins in protein translation with dynamic interactions with GTP, GDP, aa-tRNA, ribosomes, and other proteins, which would thermodynamically increase the resistance to forming insoluble aggregates by reducing their interactions with unfolded or partially unfolded proteins under heat stress. This possible mechanism is consistent with the observation that constitutive GTP- and GDP-bound forms of Rabe1b proteins were more prone to aggregate, possibly due to their lack of dynamic interactions with different guanine nucleotides, aa-RNA, ribosomal, and other proteins. In addition, the stronger in vivo stability of plastid Rabe1b proteins could be aided by the protective chaperone proteins in chloroplasts. We have observed previously that the Rabe1b protein aggregates accumulate to higher levels in the mutants for the CARBOXY TERMINUS OF HSC70 INTERACTING PROTEIN (CHIP) ubiquitin E3 ligase and the autophagy-selective receptor NEIGHBOR OF BREAST CANCER1 (NBR1; Zhou et al., 2014b), suggesting that Rabe1b protein aggregates are targeted for degradation by the CHIP-mediated 26S proteasome system and NBR1-mediated selective autophagy. Likewise, aggregates of heat-sensitive Rubisco activase and catalase proteins also accumulated at higher levels in the *chip nbr1* mutant than in wild-type plants (Zhou et al., 2014b). Interestingly, CHIP and NBR1 deficiency in the *chip nbr1* mutant also increased total catalase and Rubisco protein levels but reduced the levels of their soluble forms (Zhou et al., 2014b). Thus, CHIP- and NBR-mediated degradation pathways not only remove insoluble protein aggregates but also protect soluble native proteins. By promoting the removal of aggregated forms, these pathways could possibly reduce the

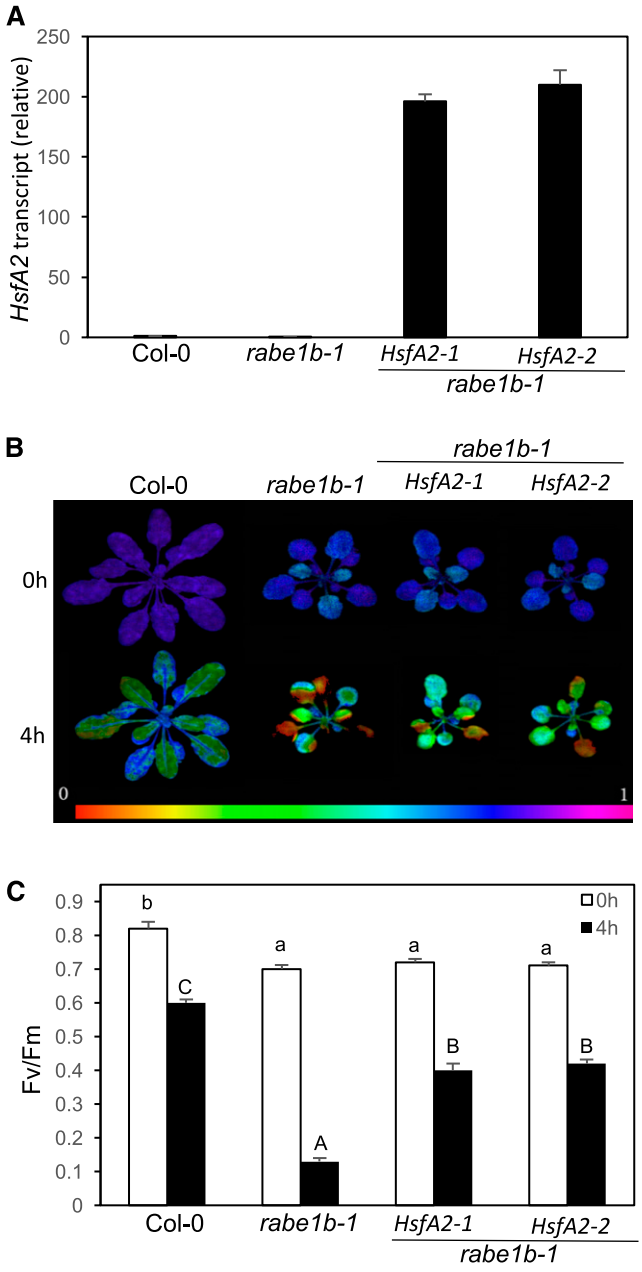

Figure 12. Effects of the constitutive expression of *HsfA2* on the growth and heat tolerance of *rabe1b-1*. A, Constitutive expression of the *HsfA2* transgene in transgenic *rabe1b* plants. Total RNA was isolated from Arabidopsis Col-0 wild type, the *rabe1b* mutant, and two independent lines of transgenic *rabe1b* mutant plants harboring the *HsfA2* transgene under the control of the *CaMV 35S* promoter. *HsfA2* transcript levels were determined using RT-qPCR. Means and SE were calculated from three replicates. B, F_v/F_m images of fully expanded leaves determined immediately after 0 and 4 h at 45°C. The color code in the images ranged from 0 (black) to 1 (purple). C, F_v/F_m values of fully expanded leaves determined immediately after the indicated hours of heat treatment at 45°C. According to Duncan's multiple range test ($P < 0.01$), means of F_v/F_m at 0 h do not differ significantly if they are indicated with the same lowercase letter; means of F_v/F_m at 4 h do not differ significantly if they are indicated with the same uppercase letter.

interactions of protein aggregates with soluble proteins, thereby decreasing the inactivation and aggregation of soluble proteins. CHIP- and NBR1-mediated pathways could play a similar role in protecting plastid Rabe1b proteins from aggregation by removing highly interactive plastid protein aggregates.

The Role of Plastid EF-Tu in Plant Growth, Photoprotection, and Heat Tolerance

In this study, we have comprehensively analyzed the biological functions of plastid EF-Tu in plant growth and heat tolerance using gene silencing in both Arabidopsis and tomato, overexpression of constitutive GTP- or GDP-bound mutant *Rabe1b* genes, and a T-DNA insertion mutant in Arabidopsis. As the only gene encoding an important chloroplast translation factor in Arabidopsis, *Rabe1b* is likely to be essential for plant growth based on the failure to obtain a homozygous T-DNA knockout mutant. Cosuppression and VIGS of *Rabe1b* in Arabidopsis and tomato, respectively, led to the photobleaching of green tissues, strikingly similar to the phenotype caused by the silencing of plant *PHYTOENE DESATURASE* (*PDS*). Plant *PDS* encodes an enzyme in the biosynthesis of carotenoids, which act as antioxidants in photooxidation by quenching singlet-oxygen and triple sensitizers generated during photosynthesis (Wang et al., 2005; Romero et al., 2011). The bleaching of green tissues associated with the silencing of chloroplast EF-Tu indicates that a high capacity of chloroplast protein biosynthesis is required for photoprotection during photosynthesis. Upon triggering of silencing of the *Rabe1b* gene and inhibition of chloroplast protein translation, photooxidation could be induced due to increased production of ROS as a result of the imbalance between light harvesting and subsequent photochemical reactions caused by the reduced synthesis of specific components in chloroplasts. In addition, increased photooxidation in *Rabe1b*-silenced green tissues could be caused by reduced levels of antioxidants and, consequently, reduced capacity of photoprotection in chloroplasts.

The comprehensive functional analysis through molecular genetic approaches supported the critical role of plastid EF-Tu in plant heat tolerance. The Arabidopsis *rabe1b-1* knockdown mutant was highly compromised in heat tolerance (Figs. 8 and 9). Likewise, tomato plants with silenced *Rabe1b* genes also became highly sensitive to high temperature (Fig. 10). The reduced expression of *Rabe1b* in the *rabe1b-1* knockdown mutant and in *Rabe1b*-silenced tomato plants was associated with other phenotypes, including pale greening, bleaching, and reduced growth (Figs. 7 and 10; Supplemental Fig. S2). Therefore, it could be argued that the role of *Rabe1b* in plant heat tolerance might be through its general effects on photosynthesis, chloroplast functions, and, ultimately, plant growth. The evidence against such an argument comes from the transgenic Arabidopsis plants overexpressing the constitutive

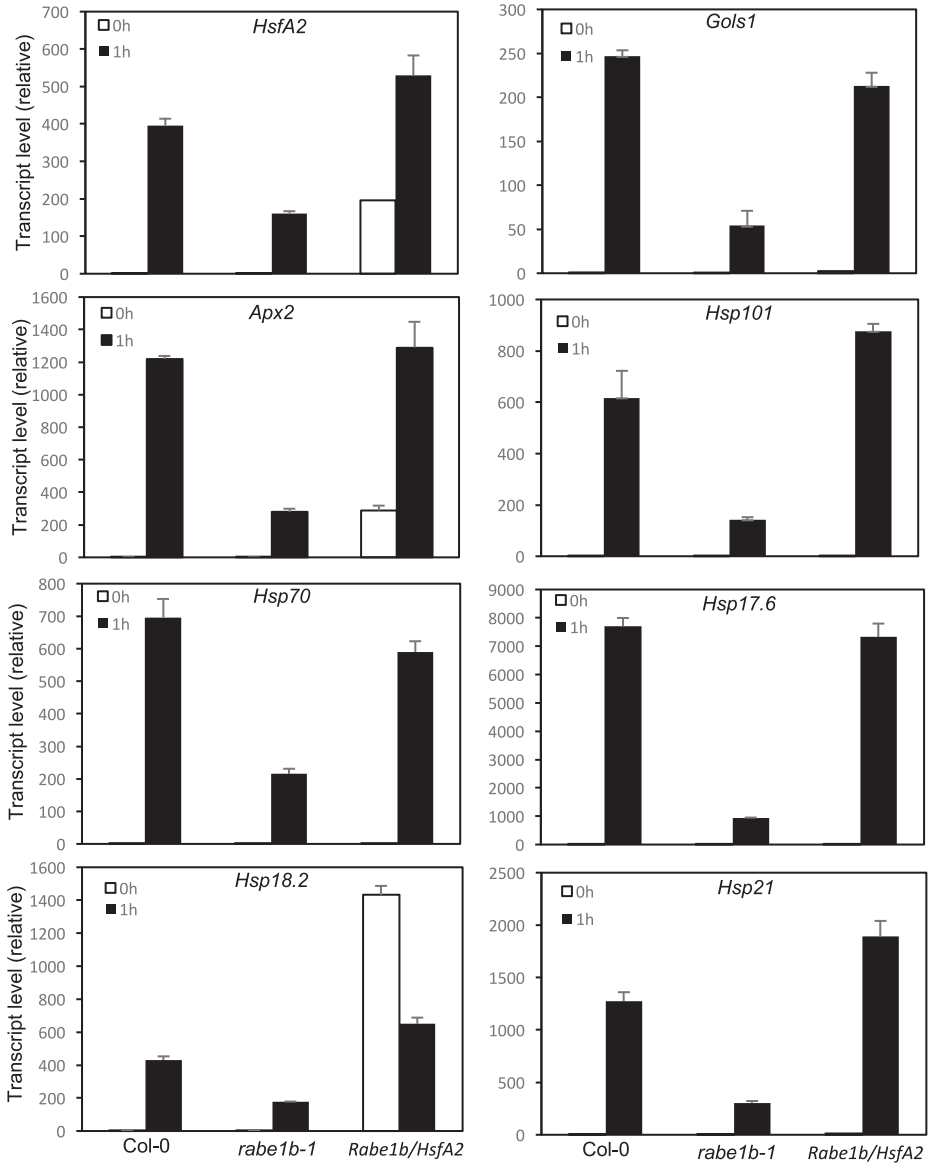

Figure 13. Effects of the constitutive expression of *HsfA2* on *HsfA2* and *HsfA2* target genes. Five-week-old Arabidopsis wild-type Col-0, the *rabe1b-1* mutant, and the *rabe1b* mutant constitutively expressing *HsfA2* were treated by heat stress at 45°C for 0 and 1 h. Total RNA was isolated from leaf samples, and transcript levels of the indicated genes were determined using RT-qPCR. Means and SE were calculated from three replicates.

GTP- or GDP-bound mutant Rabe1b form. Overexpressed GTP- and GDP-bound mutant Rabe1b forms can potentially interfere with the endogenous Rabe1b, thereby inhibiting plastid protein translation. Surprisingly, these transgenic plants were indistinguishable from wild-type plants in morphology and growth (Fig. 6A). They also were normal in photosynthetic efficiency of PSII when grown at normal temperatures (Fig. 6, B and C). The small effect of overexpressed GTP- and GDP-bound mutant Rabe1b forms on plant growth under normal conditions could be due to two reasons. First, plastid EF-Tu is a relatively abundant protein, based on our comparison of transgenic plants expressing myc-tagged *AtRabe1b* under the control of the strong CaMV 35S promoter versus its native promoter. Second, the dominant negative activity of the GTP- and GDP-bound mutant forms of Rabe1b may be relatively weak. Thus, the relative abundance of native Rabe1b proteins in these transgenic plants under normal conditions would make Rabe1b very unlikely to be a rate-limiting factor, with a relatively weak dominant effect from the overexpressed GTP- and GDP-bound mutant forms of Rabe1b. This interpretation is consistent with the finding that overexpression of GTP- and GDP-bound forms does not significantly affect plastid protein translation in transgenic plants under normal conditions (Fig. 14). However, transgenic plants overexpressing the GTP- and GDP-bound mutant forms displayed reduced heat tolerance at both their seedling and mature stages (Figs. 5 and 6). This effect of the GTP- and GDP-bound mutant forms on plant heat tolerance also could result from two possible reasons. First, due to heat-induced aggregation at high temperatures, the soluble, active form of the endogenous Rabe1b is

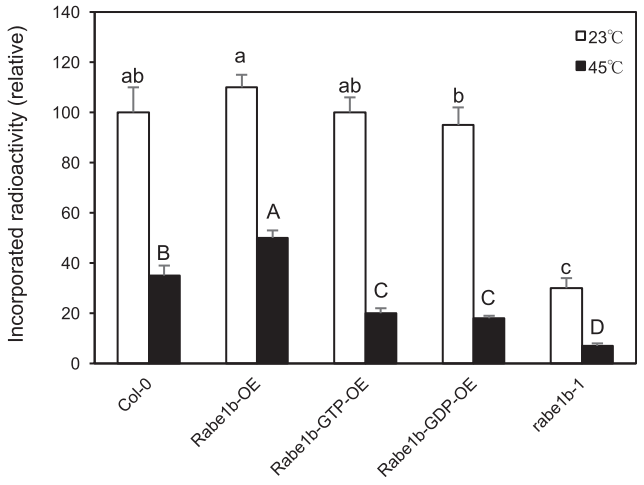

Figure 14. Effects of Rabe1b proteins and heat stress on plastid protein translation. Plastids were isolated from leaves harvested from plants grown under normal conditions (23°C) or after 6 h of heat treatment (45°C). Protein synthesis was assayed in isolated plastids with [^{35}S]Met. The relative levels of protein synthesis were represented by the relative amount of incorporated [^{35}S]Met radioactivity on an equal number of isolated plastids. Means and SE were calculated from three replicates. According to Duncan's multiple range test ($P < 0.05$), means of the relative amount of incorporated radioactivity in plastid protein synthesis from plants grown at normal conditions (23°C) do not differ significantly if they are indicated with the same lowercase letter; means of incorporated radioactivity in plastids isolated from heat-treated plants (45°C) do not differ significantly if they are indicated with the same uppercase letter.

reduced substantially, making it more likely to be a rate-limiting factor in plastid translation. Second, at high temperatures, the genes encoding the GTP- and GDP-bound mutant forms are under the control of a strong constitutive promoter; therefore, they are continuously synthesized, become misfolded, aggregate, and eventually degrade. Misfolded proteins are known to interact nonspecifically with other proteins, promote their misfolding, or interfere with their functions. Therefore, GTP- and GDP-bound Rabel1b mutant proteins could still interfere with the activity of endogenous Rabe1b or promote their aggregation through nonspecific interactions prior to or during their rapid aggregation. This interpretation is consistent with the finding that overexpression of the GTP- and GDP-bound mutant forms substantially reduces plastid protein translation at 45°C but not at room temperature (Fig. 14). Therefore, high levels of active chloroplast Rabe1b proteins appear to be particularly important for plant heat tolerance.

Both the Arabidopsis *rabe1b-1* knockdown mutant and *Rabe1b*-silenced tomato plants were severely compromised in heat tolerance, despite their residual levels of *Rabe1b* transcripts, which were approximately 15% to 25% of wild-type levels (Figs. 6 and 10). In vivo, a significant percentage of plastid Rabe1b proteins became insoluble at 41°C, and most aggregated within several hours at 43°C (Fig. 3). Thus, at a temperature above 43°C, the rapid aggregation of plastid Rabe1b proteins would lead to reduction of the soluble active form of the translation factor to a level that severely compromises plant heat tolerance. In other words, the high heat sensitivity of plastid EF-Tu likely makes it a critical limiting factor of plant heat stress responses. As a result, EF-Tu would likely be a target for evolutionary selection and genetic engineering for increased heat tolerance. In prokaryotes, the EF-Tu proteins from thermophiles have evolved to be more heat stable than their counterparts in mesophiles (Katava et al., 2016). In plants, genes encoding plastid EF-Tu are induced by heat and other stresses for the elevation of protein levels, and manipulation of the Rabe1b protein levels through molecular and genetic means could lead to altered heat tolerance in plants (Bhadula et al., 2001; Ristic et al., 2004, 2007, 2008; Momcilovic and Ristic, 2007; Fu et al., 2008). In light of the heat-sensitive nature of the plastid translation factor, a more effective way to improve plant heat tolerance would be to enhance not only the protein levels but also the heat stability of plastid EF-Tu.

Possible Mechanisms by Which Chloroplast EF-Tu Promotes Plant Heat Tolerance

Prokaryotic, mitochondrial, and plastid EF-Tu proteins not only function as essential translation factors in protein biosynthesis but also act as protein chaperones that promote the renaturation of denatured proteins and prevent their aggregation. Studies with EF-Tu proteins from prokaryotes, mammalian mitochondria, and plant plastids have concluded that EF-Tu proteins can promote the refolding of denatured proteins. The chaperone activities of EF-Tu proteins are widely believed to be primarily responsible for their positive role in plant heat tolerance (Fu et al., 2012). Unlike their activity as a protein translation elongation factor, which is dependent on dynamic GTP and GDP binding, the chaperone activity of EF-TU is not dependent on GTP or GDP (Malki et al., 2002; Rao et al., 2004; Ristic et al., 2007; Suzuki et al., 2007). In this study, we generated transgenic Arabidopsis plants that overexpressed plastid EF-Tu and its constitutive GTP- and GDP-bound mutant forms, which had similar levels of chaperone activity (Supplemental Fig. S8). If the chaperone activity of EF-Tu is primarily responsible for its positive role in plant heat tolerance, we expected that overexpression of Rabe1b and its GTP- and GDP-bound mutant forms would have similar positive effects on the heat tolerance of the transgenic plants. Importantly, we observed significantly increased heat tolerance only in transgenic plants overexpressing the wild-type Rabe1b proteins but not the constitutive GTP- or GDP-bound mutant forms (Figs. 5 and 6). In fact, the constitutive GTP- or GDP-bound forms of Rabe1b substantially reduced plant heat tolerance when overexpressed (Figs. 5 and 6), most likely by acting as

dominant negative proteins that interfere with wild-type Rabe1b in protein translation. Therefore, while the chaperone activities of plastid EF-Tu could play a role in heat tolerance, the high activity of EF-Tu as a protein translation factor is crucial for the growth and survival of plants under heat stress. It is noteworthy that the transgenic plants overexpressing the constitutive GTP- and GDP-bound mutant forms of plastid EF-Tu were normal in growth and photosynthesis under normal growth conditions but were highly compromised in plant heat tolerance (Figs. 5 and 6). Furthermore, the levels of plastid protein synthesis were strongly associated with the levels of heat tolerance among the wild type, the *rabe1b* mutant, and transgenic plants overexpressing wild-type Rabe1b or its constitutive GTP- and GDP-bound mutant forms (Fig. 14). These findings again indicated that sufficiently high levels of plastid EF-Tu activity as a protein translation factor are particularly important for plants under heat stress. This could arise from the need for increased synthesis of heat-induced plastid proteins with important roles in heat stress responses. Increased synthesis also would be necessary for heat-labile proteins important for chloroplast and other cellular functions under heat stress.

The compromised heat tolerance of the *rabe1b* knockdown mutant was associated with inhibited induction of HsfA2 and its target genes under heat stress (Fig. 11). Thus, plastid EF-Tu also acts as a positive regulator of chloroplast retrograde signaling regulating the heat-induced expression of nuclear heat-responsive genes. It has been reported previously that an Arabidopsis knockdown mutant for *CHLOROPLAST RIBOSOMAL PROTEIN S1* (*RPS1*) also exhibited reduced growth, pale green leaves, and compromised heat tolerance associated with reduced induction of HsfA2 and its target genes under heat stress (Yu et al., 2012). Importantly, constitutive overexpression of HsfA2 in the *rps1* knockdown mutant could restore not only the induction of the HsfA2 target genes but also the growth and heat tolerance of the mutant (Yu et al., 2012). The authors proposed that the reduced growth, destabilized thylakoid membranes, and compromised heat tolerance are caused by reduced production of ROS-scavenging enzymes and other protective mechanisms as a result of inhibited HsfA2 expression in the *rps1* mutant (Yu et al., 2012). Intriguingly, constitutive overexpression of HsfA2 in the *rabe1b* knockdown mutant led to elevated expression of some but not all of its target genes at normal conditions, as has been found previously in the *rps1* mutant (Yu et al., 2012). This could be due to the possibility that HsfA2 is inactive or not fully active in the absence of heat stress or other cofactors that are not overexpressed in the transgenic plants or activated at normal conditions. Constitutive overexpression of HsfA2 activated the expression of HsfA2 target genes at high temperature but only partially restored the heat tolerance of the mutant (Figs. 12 and 13). More importantly, the reduced growth and pale green leaves of the *rabe1b* mutant were not rescued by the constitutive overexpression of HsfA2 (Fig. 12). Likewise, the reduced size and pale green leaf phenotype of the *rabe1b-1* mutant were not rescued by the GTP- or GDP-bound mutant of Rabe1b (Fig. 7). Thus, inhibited HsfA2 only contributed in part to the compromised heat tolerance of the *rabe1b* mutant but was not responsible for the growth phenotype of the mutant. More likely, the reduced growth phenotype of the *rabe1b* mutant is due to the reduced protein level of the plastid translation factor and, consequently, the reduced plastid protein translation. These findings further highlight the importance of the levels of active Rabe1b proteins as a core plastid translation factor in determining the extent of plant growth, photoprotection, and heat tolerance.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

All Arabidopsis (*Arabidopsis thaliana*) materials were in the Col-0 background. The homozygous Arabidopsis *rabe1b-1* (SAIL_659_G09) mutant was identified by PCR using primers flanking the insertion site (5′-GATTTCCTCCACGGCATTAG-3′/5′-CGGGACAGAGAGAAGGAGAA-3′). Arabidopsis plants were grown in growth chambers at 23°C on a photoperiod of 12 h of light and 12 h of dark. Tomato (*Solanum lycopersicum* 'Alisa Craig') seeds were germinated in a growth medium filled with a mixture of peat and vermiculite (7:3, v/v) in trays. When the first true leaf was fully expanded, seedlings were transplanted into plastic pots containing the same medium.

Generation of *Rabe1b* and *HsfA2* Constructs

To generate the myc-tagged wild-type Arabidopsis *Rabe1b* gene for overexpression in Arabidopsis, we amplified its coding sequence using a pair of *AtRabe1b*-specific primers (5′-AGCCTCGAGATGGCGATTTCGGCTCCA-3′ and 5′-AGCTTAATTAATTCGAGGATCGTCCCAATAA-3′). Coding sequences for the constitutive GTP- and GDP-bound forms of AtRabe1b were generated by overlapping PCR using substitution-specific primers (5′-CCTGGTCTCGCTGATTACGTTAA-3′/5′-TTAACGTAATCAGCGAGACCAGG-3′ for Rabe1bH152L and 5′-GGTTGTGTTTCTTATCAAAGAGGATCAAGT-3′/5′-ACTTGATCCTCTTTGATAAGAAACACAACC-3′ for Rabe1bN203I). The amplified *AtRabe1b* fragments were fused with a 4×myc tag sequence in a binary vector. The coding sequence of *HsfA2* was PCR amplified using a pair of gene-specific primers (5′-AGCCTCGAGATGGAAGAACTGAAAGTGGAAATGG-3′ and 5′-AGCTTAATTAAAGGTTCCGAACCAAGAAAACCCAT-3′) and cloned into a binary vector behind the *CaMV 35S* promoter. The *AtRabe1b* gene also was cloned behind its native promoter of ~2 kb, which was PCR amplified from Arabidopsis genomic DNA using the following primers: 5′-AGCGGATCCGGGAGAAACCTGGTTTTTAT-3′ and 5′-AGCGTCGACGGGAAGATGGAATTGGAGAG-3′. The resulting constructs were introduced into *Agrobacterium tumefaciens* and transformed into Arabidopsis by the floral dip method (Clough and Bent, 1998). Transformants were identified by glufosinate resistance, and expression of the transgenes was analyzed by RT-qPCR or immunoblotting using an anti-myc monoclonal antibody (Sigma-Aldrich).

Yeast Two-Hybrid Assays

For assays of AtRabe1b protein interaction with plastid EF-Ts (AT4G29060) in yeast, the plastid EF-Ts coding sequence was PCR amplified using gene-specific primers (5′-AGCGGATCCGCTGAAAGCATCAAAGGCA-3′/5′-AGCGTCGACTCAGTTATCTTCTCCAAGAGTAAACTTC-3′) and cloned into a pBD-Gal4 vector. Full-length coding sequences of *AtRabe1b* and its GTP- and GDP-bound mutant genes were cloned into the pAD-Gal4 vector. Various combinations of bait and prey constructs were cotransformed into yeast cells, and interactions were analyzed by plating onto selection medium lacking Trp, Leu, and His.

RT-qPCR

Total plant RNA isolation and reverse transcription were performed as described previously (Zhou et al., 2013, 2014a). RT-qPCR was performed using the iCycler iQ real-time PCR detection system (Bio-Rad), and relative gene expression was calculated as described previously (Livak and Schmittgen,

2001). *Actin* genes from Arabidopsis and tomato were used as internal controls. Gene-specific primers for RT-qPCR are listed in Supplemental Table S1.

Separation of Soluble and Insoluble Proteins and Western Blotting

Separation and analysis of soluble and insoluble proteins were performed as described previously (Zhou et al., 2013, 2014b). Briefly, Arabidopsis leaves were collected before and after heat treatment, ground in liquid nitrogen, and homogenized in a detergent containing extraction buffer (100 mM Tris-HCl, pH 8, 10 mM NaCl, 1 mM EDTA, 1% Triton X-100 (v/v), and 0.2% β-mercaptoethanol (v/v)). The homogenates were filtered through a 300- and a 100-μm nylon mesh to obtain the total proteins. The total proteins were clarified by centrifugation at 2,200*g* for 5 min, and the supernatants were kept as soluble proteins. The pellets were resuspended in the same buffer and clarified by the same low-speed centrifugation. The process was repeated twice, and the final pellets were resuspended in the extraction buffer as insoluble proteins. Protein fractionation by SDS-PAGE and immunoblotting for the detection of myc-tagged Rabe1b proteins were performed using an anti-myc antibody. The antigen-antibody complexes were detected by enhanced chemiluminescence using luminal as a substrate.

Tomato VIGS

The TRV VIGS constructs for the silencing of tomato *Rabe1b* genes were generated by PCR amplification using gene-specific primers (5′-AGCGAATTCCGCCCCAGAAGAGCGTGCTC-3′/5′-AGCCTCGAGGAATATCATCACCAGGGAAC-3′), digested with *Eco*RI and *Xho*I restriction enzymes, and ligated into the same sites of pTRV2 (Liu et al., 2002). The resulting plasmids were transformed into *A. tumefaciens* strain GV3101. *A. tumefaciens*-mediated virus infection was performed as described previously (Ekengren et al., 2003). Plants were then kept at 23°C for 4 weeks before they were used for the experiments (Kandoth et al., 2007). Terminal leaflets of the fifth fully expanded leaves, which showed 15% to 20% transcript levels of the control plants, were used.

Analysis of F_v/F_m and Electrolyte Leakage

Chlorophyll fluorescence was measured using an Imaging-PAM Chlorophyll Fluorometer equipped with a computer-operated PAM-control unit (IMAG-MAXI; Heinz Walz). The plants were maintained in the dark for more than 30 min before the measurements were performed. The intensities of the actinic light and saturating light were 280 and 2,500 μmol m^{-2} s^{-1} photosynthetic photon flux density, respectively. F_v/F_m was measured and calculated as described previously (Zhou et al., 2013). Three replicates for each treatment were used with 12 plants for each replicate. For the determination of electrolyte leakage caused by high temperature, the terminal leaflets of the fifth fully expanded leaves were measured after heat stress as described previously (Huang et al., 2010).

Plastid Protein Translation

Six-week-old Arabidopsis plants were treated with heat stress at 45°C for 0 and 6 h. Approximately 20 g of leaves was used for plastid isolation by Percoll gradient centrifugation as described previously (Klein and Mullet, 1986). The number of plastids was determined using a hemocytometer with a photomicroscope. Assays of protein synthesis in isolated plastids were performed largely as described previously (Klein and Mullet, 1986). Briefly, the protein synthesis mixture (50 mL) contained 0.33 M sorbitol, 50 mM HEPES-KOH (pH 8), 40 μM of each amino acid (minus Met), 10 mM DTT, 25 μCi of [^{35}S]Met (Perkin Elmer), 5 mM ATP, and 5 mM $MgCl_2$. Isolated plastids were added to a final concentration of 5×10^6 per assay. The mixture was incubated at 23°C for 20 min before adding unlabeled Met to a final concentration of 8.5 mM. Sorbitol-HEPES was then added to terminate the reaction. Extraction and TCA precipitation of plastid proteins were performed, and TCA-insoluble radioactivity was determined as described previously (Klein and Mullet, 1986).

Assays of GTPase and Chaperone Activities of Recombinant Arabidopsis Rabe1b Proteins

The full-length coding sequences for Arabidopsis wild-type Rabe1b and the constitutive GTP- and GDP-bound mutant proteins were cloned into the expression vector pET-32a (Novagen) and transformed into *Escherichia coli* strain BL21(DE3). Induced expression and purification of recombinant proteins were performed according to the manual provided by Novagen. GTPase activity of the Rabel1b proteins was determined with a GTPase assay kit (BioLegend) following the manufacturer's instructions. Assays of the chaperone activity of the recombinant proteins were performed as described previously (Rao et al., 2004). Briefly, the assays (1 mL) containing 0.5 μM MDH (Sigma-Aldrich), 2 μM recombinant Rabe1b proteins, or BSA and other appropriate components were incubated at 43°C for 30 min. The MDH activities before and after incubation were measured as described (Rao et al., 2004).

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL data library under the following accession numbers: AT4G20360 (AtRabe1b), AT2G26150 (AtHsfA2), AT1G74310 (AtHsp101), AT1G16030 (AtHsp70b), AT4G27670 (AtHsp21), AT5G59720 (AtHsp18.2), AT5G12030 (AtHsp17.6), AT2G47180 (AtGolS1), AT3G09640 (AtAPX2), Solyc03g112150 and Solyc06g071790 (SlRabe1b), and AT4G29060 (AtEF-Ts).

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Assays of AtRabe1b GTPase activity and interaction with plastid EF-Ts.

Supplemental Figure S2. Cosuppression of Arabidopsis Rabe1b in transgenic plants.

Supplemental Figure S3. RT-qPCR analysis of *Rabe1b* transgene transcripts in transgenic *Rabe1b*, *Rabe1b-GTP*, and *Rabe1b-GDP* Arabidopsis plants.

Supplemental Figure S4. Effect of GTP on heat-induced aggregation of Arabidopsis plastid EF-Tu in vitro.

Supplemental Figure S5. Heat-induced rapid aggregation of Arabidopsis plastid EF-Tu in vitro.

Supplemental Figure S6. Restoration of growth and heat tolerance of the *rabe1b-1* mutant by the *Rabe1b* gene under the control of its native promoter.

Supplemental Figure S7. Sequence comparison between Arabidopsis and tomato mature chloroplast Rabe1b proteins.

Supplemental Figure S8. Effect of recombinant Arabidopsis Rabe1b proteins on the activity of MDH before and after incubation at 43°C.

Supplemental Table S1. Primers for RT-qPCR.

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