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Ectopic expression of Arabidopsis glutaredoxin *AtGRXS17* enhances thermotolerance in tomato

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Summary

While various signalling networks regulate plant responses to heat stress, the mechanisms regulating and unifying these diverse biological processes are largely unknown. Our previous studies indicate that the Arabidopsis monothiol glutaredoxin, AtGRXS17, is crucial for temperature-dependent postembryonic growth in *Arabidopsis*. In the present study, we further demonstrate that AtGRXS17 has conserved functions in anti-oxidative stress and thermotolerance in both yeast and plants. In yeast, AtGRXS17 co-localized with yeast ScGrx3 in the nucleus and suppressed the sensitivity of yeast *grx3grx4* double-mutant cells to oxidative stress and heat shock. In plants, GFP-AtGRXS17 fusion proteins initially localized in the cytoplasm and the nuclear envelope but migrated to the nucleus during heat stress. Ectopic expression of *AtGRXS17* in tomato plants minimized photo-oxidation of chlorophyll and reduced oxidative damage of cell membrane systems under heat stress. This enhanced thermotolerance correlated with increased catalase (CAT) enzyme activity and reduced H_2O_2 accumulation in *AtGRXS17*-expressing tomatoes. Furthermore, during heat stress, expression of the heat shock transcription factor (HSF) and heat shock protein (HSP) genes was up-regulated in *AtGRXS17*-expressing transgenic plants compared with wild-type controls. Thus, these findings suggest a specific protective role of a redox protein against temperature stress and provide a genetic engineering strategy to improve crop thermotolerance.

Introduction

Agricultural production is encountering multifaceted challenges from global climate change, and extensive agricultural losses are attributed to heat (Battisti and Naylor, 2009; Burke *et al.*, 2009). The majority of research to date using the Arabidopsis model has advanced our understanding of plant temperature perception and response to heat stress; however, further application of this knowledge towards crops to improve their thermotolerance still remains to be explored.

Heat stress is known to induce oxidative stress (Volkov *et al.*, 2006; Suzuki *et al.*, 2011). Protection against oxidative stress, therefore, is an important component in the thermotolerance of a plant under heat stress (Wahid *et al.*, 2007; Adachi *et al.*, 2009). However, the biological processes involved in controlling plant heat tolerance are multidimensional. For example, heat shock transcription factors (HSFs), heat shock proteins (HSPs), reactive oxygen species (ROS)-mediated signalling and enzymatic and nonenzymatic ROS scavenging mechanisms have been implicated in plant responses to heat stress (Kotak *et al.*, 2007; Larkindale and Vierling, 2008; Miller *et al.*, 2008; Penfield, 2008). Reactive oxygen species are toxic by-products of aerobic metabolism that accumulate in cells during abiotic stresses (Miller *et al.*, 2008; Jaspers and Kangasjarvi, 2010). During heat stress, ROS levels increase dramatically, resulting in significant damage to plant macromolecules and cell structures, which lead to inhibition of plant growth and development (Gill and Tuteja, 2010). Thus, toxic ROS must be rapidly detoxified by various cellular enzymatic or nonenzymatic mechanisms. However, the levels of ROS need to be judiciously regulated by plants, as ROS can also act as signals to help plants adapt to stress responses including heat stress (Miller *et al.*, 2008; Penfield, 2008; Gill and Tuteja, 2010; Miller *et al.*, 2010). To manage oxidative damage and simultaneously regulate signalling events, plants have orchestrated an elaborate antioxidant network system (Foyer and Noctor, 2005; Rouhier *et al.*, 2008).

As part of this network, glutaredoxins (Grxs) are small ubiquitous proteins of the thioredoxin (Trx) family and mediate reversible reduction of disulphide bonds of their substrate proteins in the presence of glutathione (GSH) via a dithiol or monothiol mechanism (Rouhier *et al.*, 2008). These enzymes have emerged as key regulators in diverse cellular processes and oxidative stress responses by regulating cellular redox state and redox-dependent signalling pathways and are conserved in both prokaryotes and eukaryotes (Shelton *et al.*, 2005; Lillig *et al.*, 2008; Rouhier *et al.*, 2008; Cheng *et al.*, 2011). Heterologous expression in yeast *Saccharomyces cerevisiae*-mutant cells has been used to establish some conserved functions among Grxs (Cheng *et al.*, 2006; Rouhier *et al.*, 2006; Cheng, 2008). Grxs appear to be ubiquitous in plants (Rouhier *et al.*, 2006; Garg *et al.*, 2010), but only a few have been characterized (Guo *et al.*, 2010; Sundaram and Rathinasabapathi, 2010; Cheng *et al.*, 2011).

Enhancement of anti-oxidative activity through the overexpression of antioxidant enzymes (proteins) has been implicated in thermotolerance in plant species (Larkindale and Knight, 2002; Wang *et al.*, 2006). In addition, transgenic approaches in Arabidopsis to increase thermotolerance by overexpression of redox proteins, such as superoxide reductase (SOR) and rubisco-activase, have been previously studied and demonstrated to enhance heat tolerance (Kurek *et al.*, 2007; Im *et al.*, 2009). Moreover, transgenic Arabidopsis lines expressing a Grx of the fern *Pteris vittata,* PvGRX5, were more tolerant to high temperature stress than control lines (Sundaram and Rathinasabapathi, 2010). Despite these advances, little work has focused on engineering these proteins to alter heat tolerance in crop species.

Our recent study demonstrates that an Arabidopsis Grx, AtGRXS17, is a critical component involved in ROS metabolism and essential for high temperature-dependent plant growth (Cheng *et al.*, 2011). Therefore, we hypothesize that ectopic expression of *AtGRXS17* enhances thermotolerance in tomatoes. In this study, we used yeast and plant heterologous expression systems to characterize AtGRX17 functions. Furthermore, we generated *AtGRXS17*-expressing tomato plants and determined how this gene influenced heat stress response and tolerance in tomato. The sustained growth of *AtGRXS17*-expressing tomatoes during prolonged periods of elevated temperatures demonstrates the potential of this biotechnology in crop improvement.

Results

Protective role of AtGRXS17 in oxidative and heat stress in yeast

AtGRXS17 has a conserved Trx-HD and three tandem Grx-HDs, which are similar to yeast and mammalian monothiol Grxs, while yeast ScGrx3/ScGrx4 have one Grx-HD, and a mammalian Grx3 has two repeated Grx-HDs at their C-termini (Cheng and Hirschi, 2003). In yeast, *ScGrx3* or *ScGrx4* deletions do not affect cell growth; however, deletion of both *ScGrx3* and *ScGrx4* significantly reduce cell growth (Figure 1a). This impaired growth could be rescued by overexpression of *ScGrx3* or *ScGrx4* (Figure 1a). To examine whether AtGRXS17 could complement ScGrx3/ScGrx4 functions in yeast *grx3grx4* double-mutant cells, *AtGRXS17* and C-terminal green fluorescent protein (GFP)-fused *AtGRXS17* (*AtGRXS17-GFP*) were expressed in the double-mutant strain, respectively. Both AtGRXS17 and AtGRXS17-GFP could restore mutant cell growth (Figure 1a).

Yeast ScGrx3 and ScGrx4 are required for cell survival under oxidative stress (Pujol-Carrion *et al.*, 2006). To determine whether AtGRXS17 could suppress the sensitivity of *grx3grx4* cells to external oxidants, both *AtGRXS17* and *AtGRXS17-GFP* were expressed in *grx3grx4*-mutant cells and grown in media with or without 1 mM H_2O_2 or0.5 mM diamide. In these conditions, both AtGRXS17 and AtGRXS17-GFP, like yeast ScGrx3, were able to rescue the growth of mutant cells (Figure 1b,c).

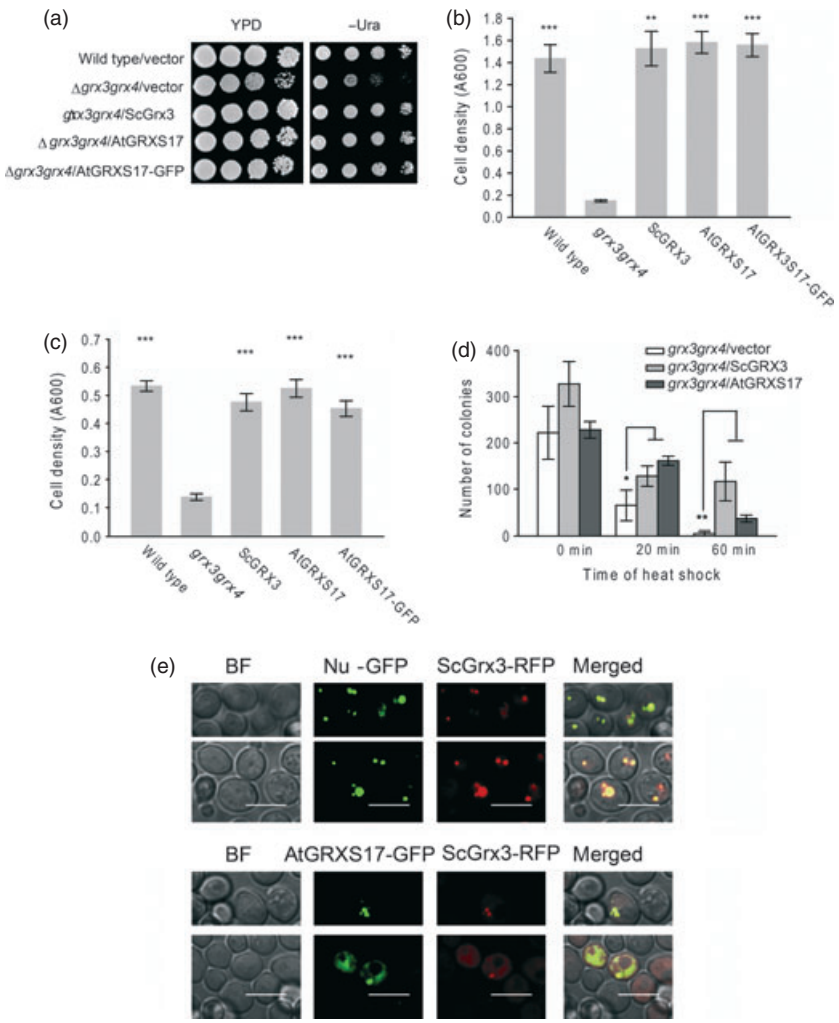

Figure 1 Arabidopsis AtGRXS17 suppresses yeast *grx3/grx4* mutant phenotypes. (a) AtGRXS17 rescues the cell growth of yeast *grx3/grx4* mutant. Vector-expressing wild-type cells and vector-, yeast *ScGrx3*-, *AtGRXS17*-, and *AtGRXS17-GFP*-expressing *grx3grx4* cells were grown on YPD and SC-Ura media. The photographs were taken after 3 days of growth at 30 °C. AtGRXS17 is able to suppress the sensitivity of *grx3grx4* cells to oxidative stress. Yeast strains carrying various plasmid DNAs, as indicated previously, were grown in SC-Ura liquid media with 1 mM H_2O_2 (b) or 0.5 mM Diamide (c). (d) AtGRXS17 suppresses the sensitivity of yeast *grx3/grx4* mutants to heat shock. Vector-, *ScGRX3*-, and *AtGRXS17*-expressing *grx3grx4* cells were grown in SC–Ura selection media overnight. 10^7 cells from each culture were treated at 50 °C for 0 min, 20 min, and 60 min and the cell cultures were diluted 10^4 fold and plated on YPD media for growth of 3 days at 30 °C, respectively. The numbers of colonies were counted. The bars indicate S.D. (n = 3). Student t test, $*P < 0.05$; $**P < 0.01$; $***P < 0.001$. (e) Co-localization of AtGRXS17-GFP and ScGrx3-RFP with nuclear markers in yeast cells. BF: bright field. Scale bars = 10 μm.

Moreover, AtGRXS17-GFP co-localized with yeast ScGrx3-RFP in the nuclei when expressed in *grx3grx4* cells (Figure 1e).

Given the conserved function of monothiol AtGRXS17 in counteracting oxidative stress in yeast (Figure 1b,c), we sought to determine whether the effects of AtGRXS17 on temperature stress responses are also conserved in yeast. In comparison with normal yeast growth conditions (30 °C), yeast *grx3grx4*-mutant cells grown at 50 °C for 20 min displayed reduced survival rate, and treatment at 50 °C for 60 min further limited their growth (Figure 1d), indicating that heat stress impaired the growth of yeast *grx3grx4*-mutant cells. When yeast *ScGrx3* or *AtGRXS17* were expressed in the mutant cells, the cells were more tolerant to the temperature stress (Figure 1d), indicating AtGRXS17 may also play a crucial role in the protective effects against elevated temperatures. These data suggest that monothiol AtGRXS17 participates in a conserved defence pathway against high temperature and oxidative stress.

Generation of *AtGRXS17*-expressing tomato plants

Based on the yeast results and our Arabidopsis data that *atgrxs17* alleles are hypersensitive to high temperature and display dramatic growth defects under heat stress (Cheng *et al.*, 2011), we hypothesize that enhancing *AtGRXS17* expression can improve thermotolerance in plants. To test this idea, we introduced a construct containing *AtGRXS17* driven by the cauliflower mosaic virus (CaMV) 35S promoter into tomato (*Solanum lycopersicum* L. cv Rubion). More than 20 independent transgenic lines were generated and 4 morphologically normal lines appearing to contain single transgene insertions in Southern blot analysis were selected and subjected to further analysis of response and adaptation to heat stress (*AtGRXS17*-3, -5, -6 and -9; Figure 2e). Consistent with a single insertion, the T1 seeds of these four lines showed a segregation pattern of 3 : 1 for the kanamycin resistance marker gene. To obtain homozygous *AtGRXS17* lines, segregation analysis on T2 seeds from self-pollinated T1 *AtGRXS17*-expressing plants was carried out on 100 mg/L kanamycin selection medium, and the expression of *AtGRXS17* was examined by reverse transcriptase (RT)-PCR analysis (Figure 2f).

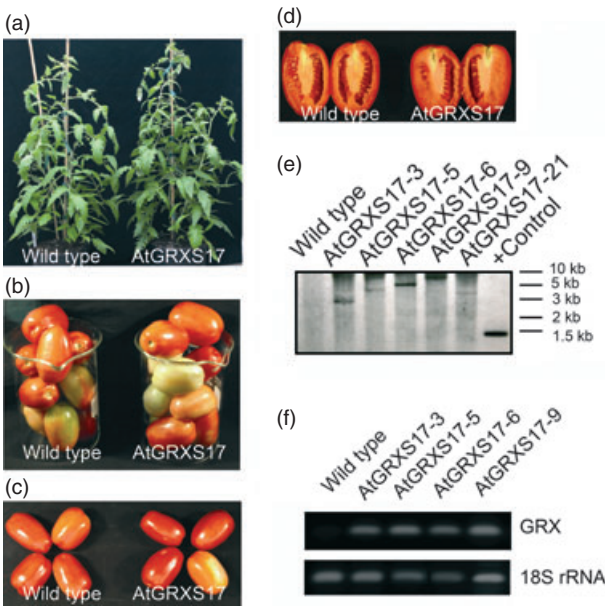

Figure 2 (a) The phenotypes of *AtGRXS17*-expressing and wild-type tomato plants are distinguishable. Expression of *AtGRXS17* does not affect the yield (b) and shape (c, d) of tomato fruits. (e) DNA gel blot analysis confirmed the stable integration of *AtGRXS17* into genome of tomato plants. (f) The expression of *AtGRXS17* was confirmed by RT-PCR in different lines.

The phenotype of the *AtGRXS17*-expressing tomatoes was indistinguishable from the wild-type plants under normal growth conditions (Figure 2a). Examination of plant height for the 2-month-old *AtGRXS17*-expressing tomato plants (plant height from soil surface to the upper leaf; 87 ± 14 cm; $n = 3$) found that they were not significantly different from that of wild-type plants (85 ± 12 cm; $n = 3$). Moreover, expression of *AtGRXS17* did not affect fruit shape and yield (Figure 2b–d).

Subcellular localization of AtGRXS17 with or without heat stress

To investigate the subcellular localization of AtGRXS17 in plant cells, N-terminal GFP-fused *AtGRXS17* (*GFP-AtGRXS17*) was transiently expressed in epidermal cells of tobacco leaves through *Agrobacterium*-infiltration (Sparkes *et al.*, 2006). The GFP-AtGRXS17 signals were primarily detected in the cytoplasm and the nuclear envelope (66 of 100 cells having signals detected in the cytoplasm with weaker signals in the nucleus, while 34 of 100 cells having stronger signals detected in the nucleus) under normal growth conditions (Figure 3a left panel, c). At increased temperature (90 min at 45 °C), GFP-AtGRXS17 signals were predominately nuclear in 85 of 100 cells examined (Figure 3a right panel, c). In contrast, free GFP targeted to nuclei independent of heat treatment (100 of 100 cells) (Figure 3b,c).

To further investigate the AtGRXS17 subcellular localization in tomato plants, we generated four stable *AtGRXS17-GFP* expression tomato plants driven by the *CaMV35S* promoter. Expression of *AtGRXS17-GFP* from all four transgenic lines could be detected by immunoblot using a GFP antibody (Figure 3e) and three lines (*AtGRXS17-GFP*-1, -6 and -9) were subjected to subcellular localization analysis of AtGRXS17 in the guard cells of stomata owing to the much more opaque nucleus that guard cells have in comparison with the epidermal cells of tomato. During a heat stress, we observed that the majority of GFP signals were localized in the nuclei from all three lines, while no GFP signals in the nuclei could be detected under normal growth conditions (Figure 2f). Together, these data indicate that AtGRXS17 translocate from the cytoplasm into the nucleus under heat stress.

Response and adaptation to heat stress in *AtGRXS17*-expressing tomato

For heat tolerance experiments, 35 different homozygous T2 *AtGRXS17*-expressing plants derived from each of the four T1 transgenic lines (*AtGRXS17*-3, *AtGRXS17*-5, *AtGRXS17*-6 and *AtGRXS17*-9) were analysed. The growth and development of *AtGRXS17*-expressing tomato plants were indistinguishable from those of wild-type plants before heat treatment (Figure S2). *AtGRXS17*-expressing tomatoes displayed robust growth and heat tolerance after 3 days at 38/28 °C (day/night, 16-h photoperiod) followed by 3 days at 42/32 °C (day/night, 16-h photoperiod), whereas the wild-type plants were severely damaged during these growth conditions (Figure 4a).

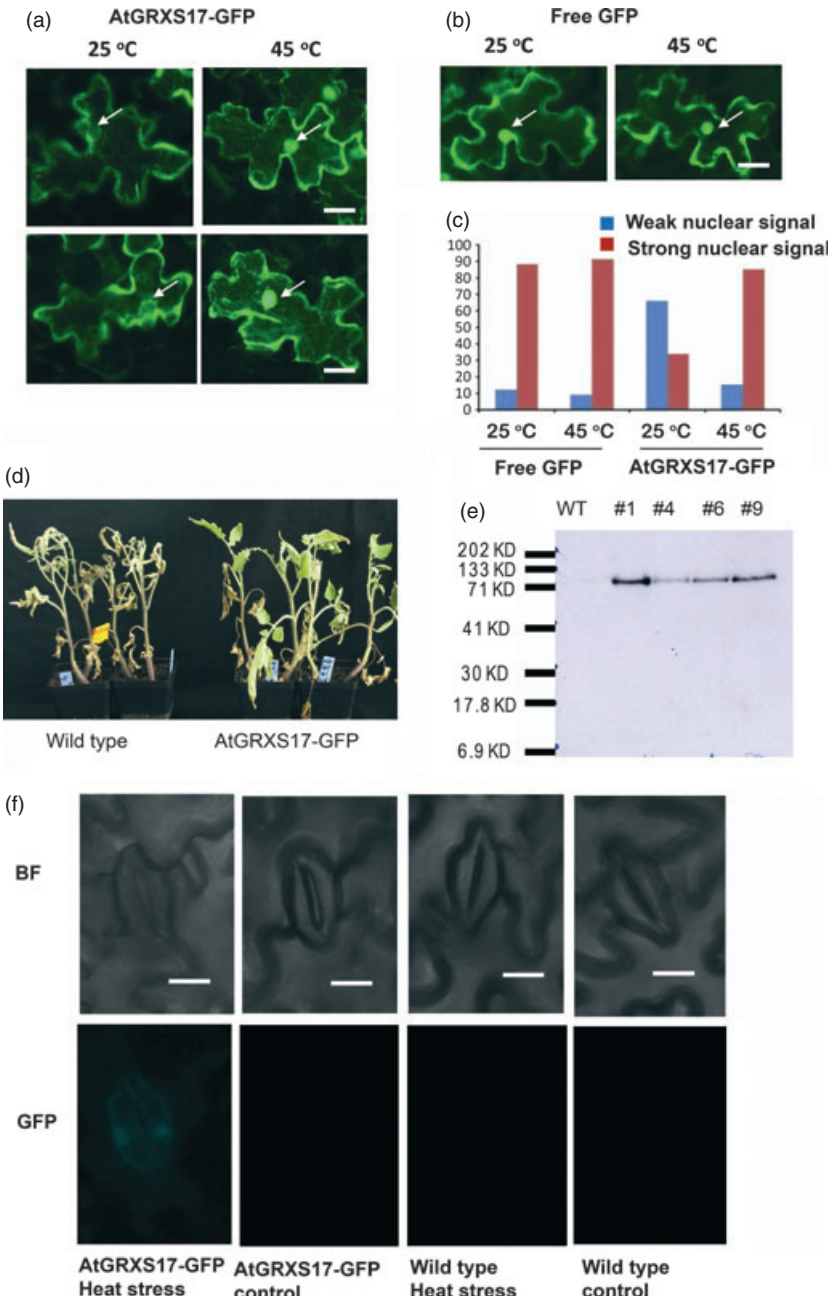

Figure 3 Subcellular localization of AtGRXS17. Transient expression of *GFP-AtGRXS17* (a) and free GFP (b) in tobacco epiderminal cells were imaged after being treated at 25 and 45 °C for 90 min, respectively. Scale bars = 25μm. The arrows highlight the nuclei. (c) The numbers of cells with weak nuclear signals or strong nuclear signals. (d) The phenotype of wild-type and *AtGRXS17-GFP*-expressing tomato plants after being treated at 38/28 °C (day/night, under a 16-h photoperiod) for 3 days, followed by treated under 42/32 °C (day/night, under a 16-h photoperiod) for additional 3 days. (e) Immunoblot detection of GFP-tagged recombinant proteins extracted from wild-type and *AtGRXS17-GFP*-expressing tomato plants. (f) GFP signal was imaged in the nucleus of stomatal cells of *AtGRXS17*-expressing tomato plants after being treated at 38/28 °C (day/night) for 3 days, followed by treated under 42/32 °C (day/night) for additional 2 days.

AtGRXS17-GFP lines also displayed heat tolerance under the same growth conditions as compared to wild-type plants (Figure 3d). The enhanced heat stress tolerance of the transgenic plants was further verified by measuring changes in chlorophyll fluorescence. The chlorophyll fluorescence Fv/Fm ratio, indicating the maximum quantum efficiency of Photosystem II, were similar in the *AtGRXS17*-expressing transgenic plants and the wild-type plants during the initial 3 days at 38/28 °C; however, following 3 days at 42/32 °C, the Fv/Fm ratio of wild-type plants was significantly decreased compared with *AtGRXS17*-expressing transgenic plants (Figure 4b). Moreover, heat stress caused severe electrolyte leakage in the wild-type plants, whereas the *AtGRXS17*-expressing transgenic plants were significantly less damaged, indicating increased plasma membrane integrity and reduced disruption of cell membranes in the transgenic lines (Figure 4c). The enhanced heat stress tolerance of the *AtGRXS17*-expressing transgenic plants compared with wild-type plants was also confirmed by measuring above-ground fresh and dry weight of the plants. The fresh and dry weight of wild-type and *AtGRXS17*-expressing transgenic plants did not show significant difference when grown under normal temperature for 2 months. However, after being treated by heat stress, the *AtGRXS17*-expressing transgenic plants displayed significantly higher fresh and dry weight yields than those of the wild-type plants (Figure 4d,e). The transgenic plants demonstrated more robust recovery when transferred to 25/22 °C (day/night, 16-h photoperiod), and the yield of the *AtGRXS17*-expressing tomatoes was indistinguishable from that of wild-type controls under normal growth conditions (Figure 4f).

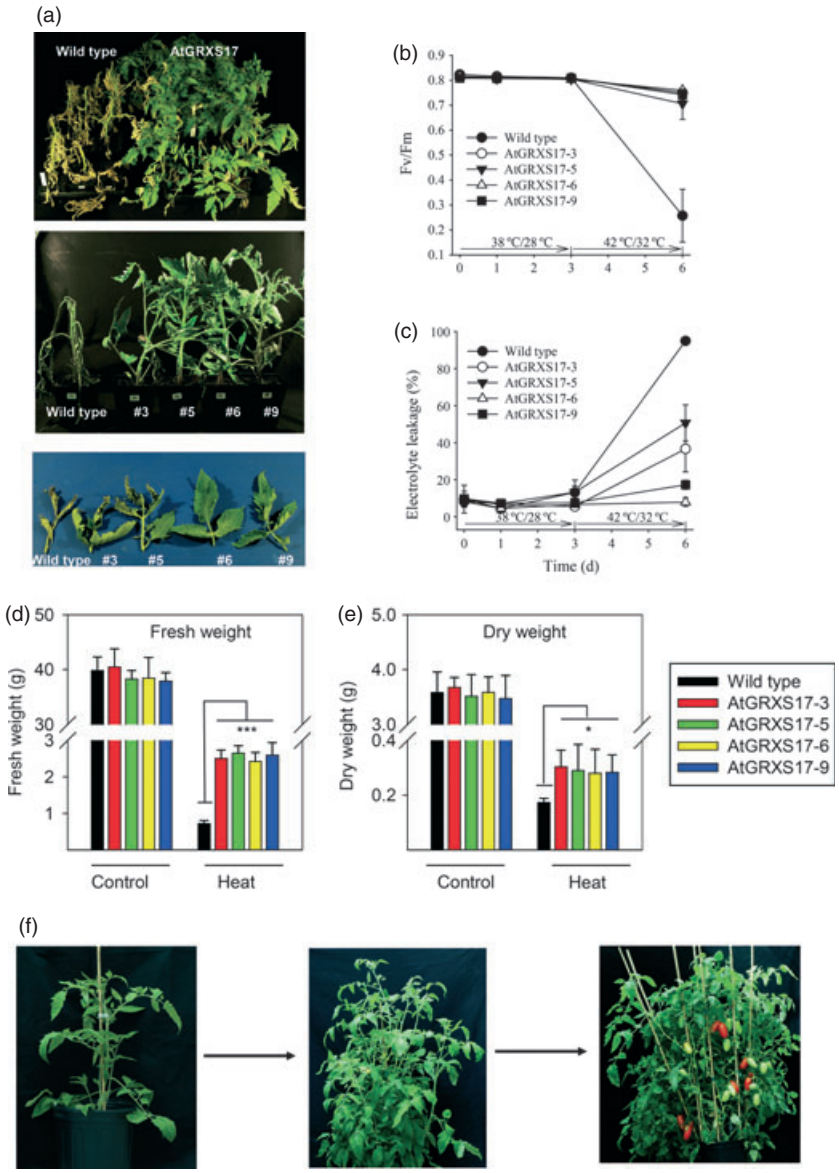

Figure 4 *AtGRXS17*-expressing tomato plants (T2) shown more tolerance to heat stress compared with wild-type controls. (a) After 3 days of treatment at 38/28 °C (day/night) followed by additional 3 days of treatment at 42/32 °C (day/night) from 4-week-old wild-type and *AtGRXS17*-expressing tomato plants, the leaves of wild-type plants were severely wilt and damaged; in contrast, the leaves of *AtGRXS17*-expressing tomato plants were still green and healthy. Top panel, an overview picture shows the phenotype of wild-type and *AtGRXS17*-expressing tomato plants; Middle panel, individual plant from each line; Bottom panel, close up picture of the leaves of tomato plants from different lines. (b) Chlorophyll fluorescence of wild-type and *AtGRXS17*-expressing plants. Error bars represent the means ± SD (n = 5). (c) Electrolyte leakage of wild-type and *AtGRXS17*-expressing plants. Error bars represent the means ± SD (n = 5). Above-ground fresh weights (d) and dry weights (e) of 8-week-old wild-type and *AtGRXS17*-expressing tomato plants with 3 days of treatment at 38/28 °C (day/night) followed by additional 25 days of treatment at 42/32 °C (day/night) (Heat) or grown at normal temperature for 28 more days (Control) with 4-week-old seedlings. The bars indicate ± SD (n = 3). Student t test, $*P < 0.05$; $**P < 0.01$; $***P < 0.001$. (f) After 6-d heat treatment, the *AtGRXS17*-expressing plants could completely recover from heat stress. The *AtGRXS17*-expressing tomato plants showed robust growth and were indistinguishable from that of wild-type plants. The *AtGRXS17*-expressing tomato plants do not appear to have adverse effects on fruit shape and size.

Catalase enzyme activity and accumulation of H_2O_2 in *AtGRXS17*-expressing tomato

Heat stress is accompanied by the formation of ROS such as $^{\bullet}O_2^-$, H_2O_2 and OH radical, causing damage to membranes and macromolecules (Wang *et al.*, 2003). To investigate the potential mechanism by which *AtGRXS17* improved thermotolerance in tomato, we measured H_2O_2 accumulation by using 3,3′-diaminobenzidine (DAB) staining of leaves from wild-type plants and three different homozygous T2 *AtGRXS17*-expressing plants derived from each of 2 T1 transgenic lines (*AtGRXS17*-6 and *AtGRXS17*-9). In both *in vitro* and *in vivo* tests, the leaves of *AtGRXS17*-expressing plants displayed less brown spotting and diffuse staining than those of the wild-type plants under the heat stress regime, indicating less H_2O_2 accumulation in the transgenic lines (Figure 5a). The intensity and quantity of brown spots caused by H_2O_2 accumulation in the leaves was analysed by image J after transforming the pictures to 256 grey scale images (Figure S1). The statistical analysis further confirmed significantly less H_2O_2 accumulation in the transgenic lines compared with the wild-type plants after the heat treatment (Figure 5b) (Analysing details were described in the Supporting information Figure S1).

We then investigated the effects of ectopic expression of *AtGRXS17* on the activities of other antioxidant components. We measured peroxidase (POD), catalase (CAT) and glutathione reductase (GR) activities in both wild-type controls and transgenic lines. The activities of CAT, but not those of GR and POD, were significantly increased in all *AtGRXS17*-expressing lines when compared to wild-type controls under both normal and heat stress conditions (Figures 5c and S3). Together, these results suggest that the tolerance of the *AtGRXS17*-overexpressing tomato plants against heat stress, at least in part, results from reducing ROS accumulation through the enhancement of the antioxidant activity of CAT.

Expression analysis of HSPs and HSFs in *AtGRXS17*-expressing tomato

The induction of HSPs is one of the predominant responses to temperature stress, and these HSPs perform important

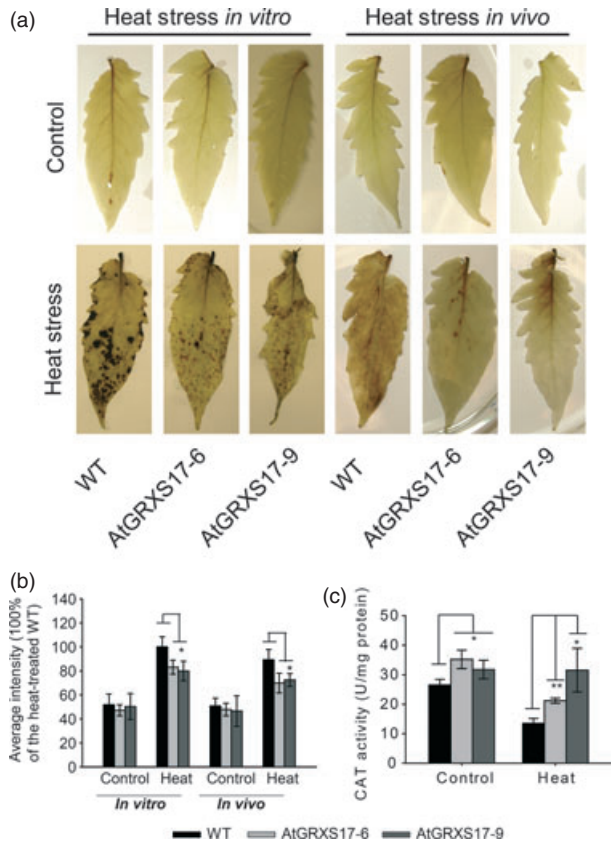

Figure 5 Effects of AtGRXS17 on H_2O_2 accumulation and CAT activity of tomato plants. (a) Effects of heat stress on the accumulation of H_2O_2 in leaves of 30-day-old wild-type (WT) and *AtGRXS17*-expressing (GRXS17-6 and GRXS17-9) tomato plants. Treatment details were described in Experimental procedures. (b) The average intensity of DAB staining leaves after being transformed to 256 grey scale images. The bars indicate ± SD ($n = 3$). Student t test, $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. (c) Effects of *AtGRXS17*-expression on CAT activity under normal or heat treatments. Control, the 33-day-old tomato plants were grown under normal growth condition. Heat, the 30-day-old tomato plants were grown under normal growth condition and then treated at 42/32 °C (day/night) for 3 days. The whole shoot parts were harvested for enzymatic assays. Error bars represent ± SD of three biological replicates, and each biological replicate is the average of two technical replicates.

physiological functions as molecular chaperones for protein quality control, such as preventing the aggregation of denatured proteins and promoting the renaturation of aggregated protein molecules caused by high temperature regimes (Wahid *et al.*, 2007; Liberek *et al.*, 2008). Given that *AtGRXS17*-expressing tomatoes are highly tolerant to heat stress, a possible interaction between AtGRXS17 and HSPs was further investigated.

To evaluate how the expression of *AtGRXS17* in heat stressed tomatoes influences the expression levels of HSPs, HSP21 (nuclear-coding protein located in the chloroplast) (Neta-Sharir *et al.*, 2005), LeERHSP21.5 (endoplasmic reticulum-located small heat shock protein) (Frank *et al.*, 2009) and LeMTSHP (mitochondrial small heat shock protein) (Liu and Shono, 1999) were selected for real-time qRT-PCR analysis (Table S1). After the initial 3 days at 38/28 °C, both wild-type and *AtGRXS17*-expressing tomato plants (*AtGRXS17*-6 and -9) showed increased expression of all three HSPs (Figure 6a). Moreover, after 3 days of treatment at 38/28 °C plus 1 days of treatment at 42/32 °C, both wild-type and *AtGRXS17*-expressing tomato plants also showed significantly increased expression of all three HSPs, exhibiting more than 100-fold elevated expression, as compared to the expression after only 3 days of treatment at 38/28 °C. Most importantly, expression levels of all HSPs in the *AtGRXS17*-expressing tomato plants were significantly higher than those of the wild-type plants after 1 day of treatment at extreme high temperatures (42/32 °C), exhibiting more than 2.5- to 4-fold elevated expression (Figure 6a).

Heat shock transcription factors (HSFs) are the components of signal transduction regulating the expression of HSPs and play an important role in heat stress responses and thermotolerance in plants (Kotak *et al.*, 2007). Given that *AtGRXS17*-expressing tomatoes showed highly elevated expression levels of HSPs under heat stress, a possible interaction between AtGRXS17 and HSFs was also further investigated. We examined the expression of two tomato HSFs, LeHSFA1a and LeHSFA2 using qRT-PCR analysis (Table S1). In tomato, HSFA1a has been defined as a master regulator of heat stress response (Mishra *et al.*, 2002), whereas HSFA2 is an important HSF for thermotolerance (Kotak *et al.*, 2007). After 3 days of treatment at 38/28 °C, *AtGRXS17*-expressing tomato (*AtGRXS17*-9) showed significantly increased expression of both *LeHSFA1a* and *LeHSFA2* as compared to wild-type controls (Figure 6b). Furthermore, after 3 days of treatment at 38/28 °C plus 1 day of treatment at 42/32 °C, *AtGRXS17*-expressing tomato (*AtGRXS17*-6) displayed significantly increased expression of both *LeHSFA1a* and *LeHSFA2* as compared to wild-type controls and *AtGRXS17*-9 lines (Figure 6b). These data suggest that up-regulation of HSPs/HSFs may account for thermotolerance in *AtGRXS17*-expressing tomato plants.

Discussion

Manipulation of cellular redox states directly affects plant growth and responses to environmental stress, such as high temperature (Potters *et al.*, 2009; Bashandy *et al.*, 2010; Suzuki *et al.*, 2011). Our previous genetic analysis of an Arabidopsis *atgrxs17* knockout mutant indicates that AtGRXS17 has crucial functions in regulating cellular ROS metabolism and postembryonic growth under high temperature (Cheng *et al.*, 2011). Here we have further characterized AtGRXS17 through ectopic expression studies and demonstrated that AtGRXS17 is a critical mediator of heat stress-related genes and enhances heat stress tolerance in tomatoes by protecting against oxidative damage.

AtGRXS17 belongs to a large *Grx* gene family (Garg *et al.*, 2010) and consists of a low abundance of transcripts under normal growth condition but is highly induced upon heat stress (Cheng *et al.*, 2011). Previous studies indicate that members of this group of *Grx* genes share some conserved functions across species (Herrero and de la Torre-Ruiz, 2007). Indeed, Arabidopsis AtGRXcp and AtGRX4 were able to suppress the sensitivity of yeast *grx5*-mutant cells to oxidative stress (Cheng *et al.*, 2006; Cheng, 2008). Poplar GRXS17 could complement yeast ScGrx5 function in counteracting external oxidants (Bandyopadhyay *et al.*, 2008). Yeast ScGrx3 and ScGrx4 are critical for iron regulation and antioxidative stress response (Ojeda *et al.*, 2006; Pujol-Carrion *et al.*, 2006). Interestingly, our study showed that yeast ScGrx3 and ScGrx4, like AtGRXS17, play an important role in heat shock response (Figure 1d). Furthermore, expression

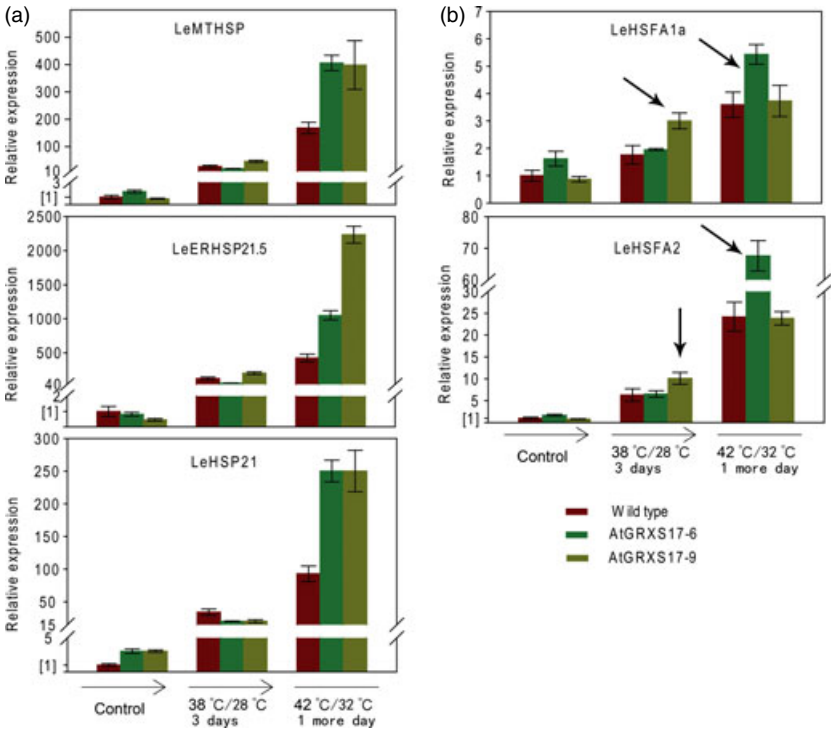

Figure 6 Expression analysis of heat shock proteins (HSPs) (a) and heat shock factors (HSFs) (b) in the wild-type control and *AtGRXS17*-expressing tomato plants by qRT-PCR. The plants were grown under control condition for 30 days [25/22 °C (day/night)], and then treated under 38/28 °C (day/night) for 3 days, followed by treated under 42/32 °C (day/night) for additional 1 days. The top fresh leaves of tomato plants were sampled for RNA extraction and qRT-PCR analysis. Error bars represent ± SD of three biological replicates. Data are expressed as relative values based on wild-type plants grown under control condition as reference sample set to 1.0. The primers and accession numbers of the genes were listed in Table S1.

of *AtGRXS17* in yeast *grx3gxr4*-mutant cells could suppress these sensitivities as did yeast endogenous ScGrx3 (Figure 1a–e), suggesting that both Grxs share some common pathways/functions in regulating heat stress responses. Given AtGRXS17 has multiple Grx domains compared with its yeast homologues, their interchangeability will help us to identify the functional domains in those Grxs that contribute to heat tolerance and facilitate our attempt to engineer better version of proteins that minimize heat damage in the future.

AtGRXS17-expressing tomato plants showed greater tolerance to heat stress compared with wild-type plants, possibly by protecting against the photo-oxidation of chlorophyll and reducing the oxidative damage of cell membrane systems under heat stress (Figure 4a–d). Indeed, we found that ectopic expression of *AtGRXS17* dramatically reduced the H_2O_2 accumulation in both *in vitro* and *in vivo* tests of tomato leaves (Figure 5a), suggesting that AtGRXS17 functions in mediating ROS scavenging. Interestingly, among ROS scavengers, the activity of CAT, but not those of GR and POD, was significantly increased in the *AtGRXS17*-expressing plants when compared with wild-type plants after heat treatment (Figure 5c and Figure S3). In fact, CAT is indispensable for oxidative stress tolerance (Willekens *et al.*, 1997; Rizhsky *et al.*, 2002) and thermotolerance in plants (Dat *et al.*, 1998). Therefore, increased CAT activity could be essential for suppressing toxic ROS levels and controlling ROS-dependent signals for thermotolerance in *AtGRXS17*-expressing plants. There are two possible ways to protect the CAT function by ectopic expression of *AtGRXS17* in tomato. Firstly, AtGRXS17 could protect CAT activity by direct physical interaction with CAT. In support of this, previous proteomic analysis identified CAT as one of the Grx-interacting proteins targeted by Poplar Grx C1 (Rouhier *et al.*, 2005). Secondly, AtGRXS17 may stabilize CAT indirectly by increasing the expression of HSPs under heat stress as it has been reported that overexpression of sHSP17.7 in rice protects CAT from precipitation under heat stress and further improves thermotolerance in transgenic rice (Murakami *et al.*, 2004). Indeed, we found that AtGRXS17 up-regulated the expression of HSPs and HSFs (Figure 6a,b), leading the regulatory interplay among heat stress response pathways and protecting against oxidative damage in plants under heat stress. As CAT activity is necessary for the recovery from heat stress (Noventa-Jordao *et al.*, 1999), our results suggest that CAT activity harnessed by overexpression of *AtGRXS17*, at least in part, contributes to thermotolerance in transgenic tomato plants.

HSFA1a and HSFA2 are crucial for thermotolerance in tomato (Mishra *et al.*, 2002), and cross-talk between HSFs and HSPs plays a pivotal role in heat stress response in plants (von Koskull-Doring *et al.*, 2007; Hahn *et al.*, 2011). In *AtGRXS17*-expressing tomato plants, both HSFs and HSPs were up-regulated (Figure 6a,b), suggesting that activation of HSF/HSP pathways attributes to heat tolerance in transgenic plants. Interestingly, several small heat shock genes (*HSP21*, *LeERHSP21.5* and *LeMTSHP*), rather than cytosolic *HSP70*, were significantly induced in *AtGRXS17*-expressing tomato plants under heat stress treatment (Figure 6a). One explanation is that HSP70 is induced more rapidly in response to acute heat shock, whereas small HSPs are up-regulated under heat acclimation as treated in this study (Sung *et al.*, 2001; Hahn *et al.*, 2011). This induction pattern of HSFs and HSPs is consistent with enhanced

AtGXRS17 expression after 24-h heat stress (Cheng *et al.*, 2011). Furthermore, up-regulation of HSPs controlled by HSFs suggests an increased HSF activity in *AtGRXS17*-expressing tomato plants compared with wild-type controls.

Even though HSPs are mainly regulated by HSFs at the transcriptional level (Kotak *et al.*, 2007), previous studies also suggest that the accumulation of maize HSP101 protein under heat stress is not correlated with its induction of transcripts (Young *et al.*, 2001), indicating post-transcriptional regulation of HSPs also plays a key role in heat stress response. Therefore, further studies to address how ectopic expression of *AtGRXS17* affects the accumulation of HSPs proteins at the post-transcriptional level are needed.

Activation of HSFs involves protein nuclear translocation and effective DNA binding (Nover *et al.*, 1996). Given that the accumulated nuclear retention of AtGRXS17 under heat stress correlated with activation of HSFs (Figures 3 and 6), we posit that the increased nuclear pool of AtGRXS17 under heat stress presumably facilitates the transcriptional activation of HSFs that enhance thermotolerance. Previous studies have demonstrated that activation of HSF and HSP activities are regulated through redox-dependent mechanisms (Zhong *et al.*, 1998; Bijur *et al.*, 1999; Ozaki *et al.*, 2000; Graf and Jakob, 2002; Ahn and Thiele, 2003). Furthermore, recent studies reveal that Grx target proteins are involved in many processes, including oxidative and heat stress responses, and protein folding as specific partners of Grx (Rouhier *et al.*, 2005). Nonetheless, it remains to be determined which partner proteins interact with AtGRXS17 and how AtGRXS17 specifically regulates HSF/HSP expression and activity in the future.

In conclusion, we demonstrate here that expression of a member of the Grx family enhances thermotolerance in an agriculturally important crop by mediating the anti-oxidative and HSP/HSF systems. This technology could ultimately be applied to other crop species, such as heat-susceptible wheat or potato, to improve their thermotolerance.

Experimental procedures

Yeast assays

The full-length cDNA of *AtGRXS17* was amplified using an *AtGRXS17* forward primer: 5′-ATG AGC GGT ACG GTG AAG GAT-3′ and an *AtGRXS17* reverse primer: 5′-TAG CTC GGA TAG AGT TGC TTT-3′. The fidelity of all clones was confirmed by sequencing. To express *AtGRXS17* in yeast cells, the *AtGRXS17* was subcloned into yeast expression vector piUGpd. *Saccharomyces cerevisiae* wild-type strain CML235 (*MAT*a *ura3-52 leu2*Δ*1 his3*Δ*200*) and *grx3grx4* double-mutant (*MAT*a *ura3-52 leu2*Δ*1 his3*Δ*200 grx3::kanMX4 grx4::kanMX4*) were provided by Dr. Enrique Herrero (Universitat de Lleida, Lleida, Spain) and used in all yeast experiments. Yeast growth assays were followed the published protocol (Cheng *et al.*, 2006). For AtGRXS17 subcellular localization in yeast assays, full-length *AtGRXS17* was fused to the N-terminus of green fluorescent protein (GFP) using a procedure described previously (Cheng *et al.*, 2006). The *AtGRXS17-GFP* fusion was then subcloned into the yeast vector as described previously. In yeast, AtGRXS17-GFP was imaged in colocalization with yeast Grx3-RFP. The fluorescence signals were detected at 510 nm (excitation at 488 nm) for GFP, at 582 nm (excitation at 543 nm) for DsRed.

Tomato plants transformation and growth condition

Seeds of tomato *Solanum lycopersicum* L. (cv Rubion) were surface sterilized and germinated on the Murashige and Skoog (1962) inorganic salt medium. Tomato transformation was performed via *Agrobacterium*-mediated transformation method using cotyledon and hypocotyls explants as described (Park *et al.*, 2003). *Agrobacterium tumefaciens* LBA 4404 was used for generating stable transgenic plants. The plasmid *pCaMV35S::AtGRXS17* and *pCaMV35S::AtGRXS17-GFP* were introduced into *A. tumefaciens* using the freeze–thaw method (Holsters *et al.*, 1978), respectively. After inoculatinge with *A. tumefaciens*, the plant cultures were maintained at 25 °C under a 16-h photoperiod. After 6 to 8 weeks, regenerated shoots were transferred to rooting medium for 6 more weeks. The temperature of the greenhouse was maintained within a range of 25–30 °C.

Subcellular localization of AtGRXS17 in plant cells

For agroinfiltration transient expression in tobacco leaves (*Nicotiana benthamiana*), full-length *AtGRXS17* was fused to the C-terminus of green fluorescent protein (GFP) using a procedure described previously (Cheng *et al.*, 2006). The *GFP-AtGRXS17* construct was made by LR reaction (Invitrogen, Carlsbad, CA) between the binary vector pB7WGF2 (Karimi *et al.*, 2002) and the entry vector carrying *AtGRXS17* (pENTER-4, Invitrogen, Carlsbad, CA). *pB7WGF2/GFP-AtGRXS17* was introduced into *Agrobacterium* strain GV3101 as described and then used for agro-infiltration as previously described (Liu *et al.*, 2005). For heat shock treatment, at 1.5–2 days postinfiltration (DPI), the infiltrated leaves were detached from tobacco plants, kept in Petri dishes with the moistened filter paper and incubated at 25 or 42 °C for 90 min, respectively. For AtGRXS17-GFP localization in tomato stomatal cells, 30-day-old stable *AtGRXS17-GFP*-expressing plants were treated by either heat or control treatments before microscopic observation. Images were captured with a confocal laser scanning system (Leica, SP5 X, Leica Microsystems Inc., Buffalo Grove, IL) and fluorescence microscope (Zeiss Axio-Plan, Carl Zeiss Microscopy, Thornwood, NY). The fluorescence signals were detected at 510 nm (excitation at 488 nm) for GFP and at 582 nm (excitation at 543 nm) for DsRed.

DNA isolation and Southern blot analysis

Tomato genomic DNA was extracted from leaf tissue using Qiagen Plant DNA extraction kit. DNA (10 μg) was digested with *Xba*I and separated by electrophoresis and blotted onto a nylon membrane (Zeta-probe GT membrane, Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions. The probe for the *AtGRXS17* gene was isolated from a pGEM-T-easy vector contained *AtGRXS17* by enzyme digestion. The membranes were prehybridized overnight at 65 °C in 7% SDS and 0.25 M Na_2HPO_4 and then hybridized overnight at 65 °C in the same solution containing the probe labelled by NEBlot Phototope Kit (New England Biolabs, Ipswich, MA). Membranes were washed twice for 40 min each with 20 mM Na_2HPO_4 and 5% SDS at 65 °C and then washed twice again for 30 min each with 20 mM Na_2HPO_4 and 1% SDS at 65 °C. The signal was detected by Phototope-Star Detection Kit (New England Biolabs) according to the manufacture's instruction.

RNA extraction and qRT-PCR

Total RNA was isolated using the Qiagen Plant RNeasy kit from leaves of tomato plants according to the manufacturer's

instructions. RNA for real-time PCR was treated with RNase-free DNase prior to the synthesis of first-strand cDNA by oligo (dT) priming using moloney murine leukaemia virus-reverse transciptase (BD Biosciences Clontech, Palo Alto, CA). One microlitre of the reverse transcription reaction solution was used as a template in a 25-μL PCR solution. Real-time PCR was performed in 25-μL reactions containing 10.5 μL of cDNA, 1 μL of 10 mM of each primer and 12.5 μL of SYBR Green PCR Master Mix (Bio-Rad Laboratories). Analysis was performed using the Bio-Rad IQ3 (Bio-Rad Laboratories). Primer efficiencies were measured, and relative expression level was calculated using the comparative C_t method. The primers for PCR were listed in the supplementary data (Table S1).

Western blot analysis

Total leaf protein was extracted from 100 mg of fresh leaf tissue using the Plant Total Protein Extraction Kit (Sigma-Aldrich, St. Louis, MO). The protein concentration was determined by the Pierce BCA Protein Assay Kit (Thermo Scientific, Rockford, IL). Total protein that was separated by 10% SDS/PAGE was electroblotted to PVDF membrane (Millipore, Billerica, MA) and immunoblotted with Rabbit anti-GFP antibody (Invitrogen) and goat anti-rabbit secondary antibody (Bio-rad) using standard methods. The signal was detected with Amersham ECL kit (GE Healthcare, Pittsburgh, PA).

Electrolyte leakage and Fv/Fm ratio

Injury to tomato plants was characterized by measuring chlorophyll fluorescence and electrolyte leakage of leaves as described by Oh *et al.* (2009). Chlorophyll fluorescence from the adaxial side of the leaf was monitored using a portable chlorophyll fluorometer (PEA, Hansatech Instruments, Ltd., UK). Photochemical efficiency of leaves, as determined by chlorophyll fluorescence ratios (Fv/Fm), was monitored during and after heat treatment. Measurements were taken during the light cycle on the leaves using the saturation pulse method after 30 min of dark adaptation. For electrolyte leakage, leaf samples were incubated in 15 mL of distilled water for 10 h to measure the initial electrolyte leakage using an YSI conductance meter (Model 32, YSI, Inc., Yellow Springs, OH). The samples were subjected to 80 °C for 2 h to release the total electrolytes and then held at room temperature for 10 h. The final conductivity on the leachate was measured to determine the per cent percentage of electrolyte leakage from the leaf samples.

Measurement of the CAT activities

Four-week-old seedlings were treated by 42 °C/32 °C (day/-night) for 4 days, and then the shoots were harvested and homogenized in liquid nitrogen. The resulting powder was suspended (1:5 m/v) in an ice-cold protein extraction buffer made by 50 mM PBS, 1% PVP and 1 mM PMSF, and centrifuged at 13 000 ***g*** for 15 min at 4 °C. The protein concentration was measured by BCA protein assay kit (Pierce, Rockford, IL). The activity of CAT was assayed by measuring the rate of disappearance of H_2O_2 at 240 nm, following the method of Ouyang *et al.* (2010). One unit of the CAT activity was defined as 0.01 absorbance decrease per minute at 240 nm. There were 6 biological replicates for each data points.

Heat tolerance experiment

T2-generation *AtGRXS17*-expressing transgenic or wild-type tomato seeds were surface sterilized and germinated on MS medium. Positive candidates were selected after 15 days and transferred to pots containing Miracle Gro (700) soil growing medium and grown in a growth chamber with a temperature of 25/22 °C (day/ night) under a 16-h photoperiod. The plants were regularly watered and fertilized on a weekly basis with 20 : 20 : 20 fertilizer (Scotts). The 4-week-old seedlings were treated under 38/28 °C (day/night) for 3 days and then 42/32 °C (day/night) for additional 3 days.

DAB staining

Leaves from 4-week-old wild-type and *AtGRXS17*-expressing tomato plants were used for the DAB (3, 3′-diaminobenzidine) staining. For *in vivo* heat stress test, wild-type and *AtGRXS17*-expressing plants grown under normal condition were incubated at 25/22 °C (day/night) (control, upper row) and 42/32 °C (day/night) (heat stress, lower row) for 48 h, respectively. After treatments, leaves were picked and steeped in solutions containing 1 mg/mL DAB (pH 3.8) at 25 °C in the dark for 1 h to take up the stain. H_2O_2 accumulation was detected as brown spots after DAB staining. After staining by DAB, all tomato leaves were bleached by incubating in boiled 95% ethanol for 5 min. For *in vitro* detection, tomato leaves were cut and immediately immersed in water containing 1 mg/mL DAB solution (pH 3.8) and kept at 25 °C (for control) or 42 °C (for heat treatment) in the dark for 3 h. Then, the leaves were bleached by immersion in boiling 95% ethanol to visualize the brown spots.

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Supporting information

Additional Supporting information may be found in the online version of this article:

Figure S1 Quantitative analysis of H_2O_2 accumulation.

Figure S2 The phenotype of 4-week-old *AtGRXS17*-expressing and wild-type tomato plants was indistinguishable before heat treatment.

Figure S3 Effects of *AtGRXS17* expression on the GR (upper panel) and POD (lower panel) activity under control or heat treatments.

Table S1 Primers used for qPCR.

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