IMPROVEMENT OF ABIOTIC STRESS TOLERANCE AND CALCIUM-DEFICIENCY DISORDER RESISTANCE OF TOMATO PLANTS

by

QINGYU WU

B. E., Northeast Agricultural University, Harbin, China, 2005
M. S., Chinese Academy of Agricultural Sciences, Beijing, China, 2008

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

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Department of Horticulture, Forestry and Recreation Resources
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Abstract

Plants are continuously exposed to numerous abiotic stresses, which adversely affect plant growth, development, and yield. Plants have developed different signaling pathways to cope with abiotic stresses, and some of the pathways converge to help plants tolerate simultaneous stresses. Here, we report ectopic expression of an Arabidopsis glutaredoxin *AtGRXS17* that confers tolerance to multiple abiotic stresses in tomato plants. In yeast assays, AtGRXS17 co-localized with yeast ScGrx3 in the nucleus and suppressed the sensitivity of yeast *grx3grx4* double mutants to oxidative stress and heat shock. In plants, GFP-AtGRXS17 fusion proteins initially localized in the cytoplasm 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. Furthermore, expression of the heat shock transcription factor (HSF) and heat shock protein (HSP) genes was up-regulated in *AtGRXS17*-expressing tomato plants during heat stress when compared to wild-type controls. Under cold, drought, and oxidative stress conditions, *AtGRXS17*-expressing tomato plants also displayed more vigorous growth and less physiological damage than those of the wild-type control plants. Quantitative real-time PCR (qRT-PCR) analysis indicated that expression of *AtGRXS17* alters multiple stress defense signaling pathways, including the Abscisic Acid (ABA) and C-Repeat Binding Factors (CBF) pathways. The results revealed a conserved function for a glutaredoxin protein in abiotic stress adaptation, and manipulation of AtGRXS17 may be a useful approach to improve crop stress tolerance and understand plant signaling under abiotic stress conditions.

Deregulated expression of an Arabidopsis H^+/Ca^{2+} antiporter (sCAX1) in agricultural crops increases total calcium (Ca^{2+}) but may result in yield loses due to calcium-deficiency like symptoms. Here we demonstrate that co-expression of a maize calreticulin (CRT, a Ca^{2+} binding protein located at endoplasmic reticulum) in *sCAX1*-expressing plants mitigated these adverse effects while maintaining enhanced

Ca^{2+} content. Co-expression of *CRT* and *sCAX1* could alleviate the hypersensitivity to ion imbalance in tobacco plants. Furthermore, blossom-end rot (BER) in tomato may be linked to changes in CAX activity and enhanced *CRT* expression mitigated BER in *sCAX1* expressing lines. These findings suggest that co-expressing Ca^{2+} transporters and binding protein at different intracellular compartments can alter the content and distribution of calcium within the plant matrix.

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Dedication

I would like to dedicate this dissertation to my wife, Xiaofei Wang and my parents for their never ending love.

Chapter 1 - Introduction

Abiotic stress conditions such as drought, salinity, cold, or heat adversely affect plant growth, development, and seed development, causing extensive losses to agricultural production world wide (Mittler & Blumwald, 2010). Previous studies suggest that abiotic stresses result in a reduction of average yields of most major crops by more than 50% and annual losses estimated at billions of dollars (Wang *et al.*, 2003; Mittler, 2006). Therefore, improvement of tolerance of plants against abiotic stresses remains a major focus of agricultural research. Understanding the mechanisms related to how plants adapt to abiotic stress will be the first step to help plants survive under extreme conditions.

Because plants are sessile organisms that cannot escape abiotic stresses, they have developed sophisticated strategies to regulate their metabolism and signaling molecules to prevent damage caused by various abiotic stresses. One of the changes of plants under abiotic stress conditions is producing reactive oxygen species (ROS). ROS, which were initially recognized as toxic by-products of aerobic metabolism, recently have drawn attention for their important signaling roles in abiotic stress response (Bailey-Serres & Mittler, 2006).

It is generally observed that ROS production and ROS-induced damage increase during abiotic stress, but ROS are also important signaling molecules (Moller *et al.*, 2007). The production of ROS, such as superoxide ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), hydroxyl radicals ($HO^{\bullet}$), and singlet oxygen ($^1O_2$), is an unavoidable consequence of aerobic metabolism (Moller *et al.*, 2007), and can be produced continuously as byproducts of various metabolic pathways that are localized in different cellular compartments such as chloroplasts, mitochondria and peroxisomes (del Rio *et al.*, 2006; Navrot *et al.*, 2007). Under normal growth conditions, ROS are produced at a low level. However, during stress, their production rate is dramatically elevated (Miller *et al.*, 2010). Over-accumulation of ROS results in oxidative stress, which damages plant macromolecules and cell structures and leads to inhibition of plant growth and development (Gill & Tuteja, 2010; Jaspers & Kangasjarvi, 2010; Suzuki *et al.*, 2011). Despite the negative effects of ROS, recent studies have shown that ROS play a key role in plants as signal transduction molecules involved in mediating responses to abiotic stress (Mittler *et al.*, 2004). The rapid increase in ROS production, referred to as ‘the oxidative burst’, has been shown to be essential

for abiotic stress adaptation. The 'oxidative burst' is sensed by unknown receptors, and further passes the signal to downstream signaling events, such as Ca^{2+} signaling, then activates the expression of abiotic stress-responsive genes (Mittler *et al.*, 2004). The two opposing functions of ROS, that is, on the one hand, the damaging toxic molecules, and on the other hand, the beneficial signal transduction molecules, require delicate regulation of the ROS production and scavenging in plants. Therefore, elucidating the mechanisms that regulate ROS signaling in plants during abiotic stresses could provide a powerful strategy to enhance the tolerance of plants to these environmental stress conditions.

Emerging evidence suggests that there is a tight interaction between Ca^{2+} signaling and ROS signaling. For instance, it has been known that downstream signaling events associated with ROS sensing involve Ca^{2+} and Ca^{2+}-binding proteins, such as calmodulin (Mittler *et al.*, 2004). In addition, ROS mediate ABA-induced $[Ca^{2+}]_{cyt}$ elevation by regulating plasma membrane Ca^{2+}-permeable (I_{Ca}) channels (Kwak *et al.*, 2003). Monshausen and colleagues' study (2009) further suggests that Ca^{2+}-dependent activation of NADPH oxidase RBOH C results in ROS production, and ROS production appears to be coordinated with intra- and extracellular pH changes to induce cytosolic Ca^{2+} transient. However, a considerable gap still exists between the ROS signaling and Ca^{2+} signaling.

Calcium is another versatile signaling molecule that controls many aspects of plant growth, development, and stress adaptation (White & Broadley, 2003; Dodd *et al.*, 2010). The Ca^{2+} signals take the form of transient increase in cytosolic free Ca^{2+} ($[Ca^{2+}]_{cyt}$) from the outside of cells and/or subcellular compartments, such as the vacuole, apoplast, and ER, in which the concentration of Ca^{2+} is much higher compared with the cytosol (Dodd *et al.*, 2010). Elevated $[Ca^{2+}]_{cyt}$ in plant cells, also named "calcium signature", often occur as repetitive oscillations or spiking of $[Ca^{2+}]_{cyt}$ where the frequency, amplitude and shape of the Ca^{2+} increase are determined by the nature of the stimulus. The Ca^{2+} oscillation is sensed by Ca^{2+} sensor proteins that contain Ca^{2+} binding domains, such as EF hands and high affinity Ca^{2+}-binding sites in the C-domain of calmodulin. These Ca^{2+}-binding proteins further amplify the Ca^{2+} signal by targeting a series of downstream proteins, including cellular transporters, protein kinases, and transcription factors, regulating many aspects of plant growth, development, and signaling (Kudla *et al.*, 2010).

Besides the signaling roles of Ca^{2+}, it also functions in cell wall and membrane

stabilization. Calcium is a key element in the structure of primary cell wall and it links pectin, which is the most abundant class of macromolecule that interlocks the cross-linking glycans (Pilbeam & Morley, 2007). In addition, Ca^{2+} also stabilizes membrane with phosphatases and carboxylates, and helps protect against proton degradation of membranes (Pilbeam & Morley, 2007).

Due to the important signaling and structural roles of Ca^{2+}, Ca^{2+} is an essential plant nutrient. Thus, adequate supply of Ca^{2+} is a key factor to maintain a high quality of crops, fruits, and vegetables. Ca^{2+}-deficiency disorders adversely affect the growth and production of plants, further lead to yield losses and poor fruit quality. Calcium-deficiency symptoms are usually observed in young expanding tissues, such as tip burning in leaf and blossom-end rot (BER) in fruit (Hirschi, 1999; White & Broadley, 2003). Among different Ca^{2+}-deficiency symptoms, BER is one of the most significant impediments to produce high quality fruits, especially for tomatoes. This physiological disorder can be very damaging, with losses of up to 50% or more in some years for tomatoes. Although differences exist among varieties with respect to susceptibility to BER, no varieties as yet have commercially superior tolerance, and the mechanisms that cause BER are still not clear yet.

Because of the important roles of ROS and Ca^{2+} in plant productivity, in this dissertation, we manipulated a redox regulation enzyme *Arabidopsis* glutaredoxin S17 (AtGRXS17) to enhance the abiotic stress tolerance in the tomato plants. In addition, we manipulated a tonoplast Ca^{2+} antiporter, *Arabidopsis* cation exchanger (AtCAX1) and a maize Ca^{2+}-binding protein, calreticulin (CRT) in tomato plants. The results suggest that the Ca^{2+} cellular distribution is important for the tomato fruit quality.

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Chapter 2 - Ectopic expression of Arabidopsis glutaredoxin *AtGRXS17* enhances thermotolerance in tomato

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Abstract

While various signaling 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 post-embryonic growth in plants. 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 to 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 signaling, and enzymatic and non-enzymatic 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 signaling 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 disulfide 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 signaling 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 plants (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 tomato plants. 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 2.1a). This impaired growth could be rescued by overexpression of *ScGrx3* or *ScGrx4* (Figure 2.1a). To examine if 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 2.1a).

Yeast ScGrx3 and ScGrx4 are required for cell survival under oxidative stress

(Pujol-Carrion *et al.*, 2006). To determine if 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 or 0.5 mM diamide. In these conditions, both AtGRXS17 and AtGRXS17-GFP, like yeast ScGrx3, were able to rescue the growth of mutant cells (Figure 2.1b, c). Moreover, AtGRXS17-GFP co-localized with yeast ScGrx3-RFP in the nuclei when expressed in *grx3grx4* cells (Figure 2.1e).

Given the conserved function of monothiol AtGRXS17 in counteracting oxidative stress in yeast (Figure 2.1b, c), we sought to determine if the effects of AtGRXS17 on temperature stress responses are also conserved in yeast. In comparison to normal yeast growth conditions (30 ^{o}C), yeast *grx3grx4* mutant cells grown at 50 ^{o}C for 20 min displayed reduced survival rate, and treatment at 50 ^{o}C for 60 min further limited their growth (Figure 2.1d), 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 2.1d), 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 defense 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 2.2e). Consistent with a single insertion, the T1 seeds of these 4 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 2.2f).

The phenotype of the *AtGRXS17*-expressing tomatoes was indistinguishable from the wild-type plants under normal growth conditions (Figure 2.2a). 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 no significant 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 out of 100 cells having signals detected in the cytoplasm with weaker signals in the nucleus, while 34 out of 100 cells having stronger signals detected in the nucleus) under normal growth conditions (Figure 2.3a left panel, c). At increased temperature (90 min at 45 ^{o}C), GFP-AtGRXS17 signals were predominately detected in the nuclei in 85 out of 100 cells examined (Figure 2.3a right panel, c). In contrast, free GFP targeted to nuclei independent of heat treatment (100 out of 100 cells) (Figure 2.3b, c).

To further investigate the AtGRXS17 subcellular localization in tomato plants, we generated 4 stable *AtGRXS17-GFP* expression tomato plants driven by the *CaMV35S* promoter. Expression of *AtGRXS17-GFP* from all 4 transgenic lines could be detected by immunoblot using a GFP-antibody (Figure 2.3e) and 3 lines (*AtGRXS17-GFP*-1, -6 and -9) were subjected to subcellular localization analysis of AtGRXS17 in the guard cells of stomata due to the much more opaque nucleus that guard cells have in comparison to the epidermal cells of tomato. During a heat stress, we observed that the majority of GFP signals were localized in the nuclei from all 3 lines, while no GFP signals in the nucleus could be detected under normal growth conditions (Figure 2.3f). 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, -5, -6 and -9) were

analyzed. The growth and development of *AtGRXS17*-expressing tomato plants were indistinguishable from those of wild-type plants before heat treatment (Figure 2.S2). *AtGRXS17*-expressing tomatoes displayed robust growth and heat tolerance after 3 d at 38/28 ^{o}C (day/night, 16-h photoperiod) followed by 3 d at 42/32 ^{o}C (day/night, 16-h photoperiod), whereas the wild-type plants were severely damaged during these growth conditions (Figure 2.4a). *AtGRXS17-GFP* lines also displayed heat tolerance under the same growth conditions as compared to wild-type plants (Figure 2.3d). 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 d at 38/28 ^{o}C; however, following 3 d at 42/32 ^{o}C, the Fv/Fm ratio of wild-type plants was significantly decreased compared to *AtGRXS17*-expressing transgenic plants (Figure 2.4b). 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 2.4c). The enhanced heat stress tolerance of the *AtGRXS17*-expressing transgenic plants compared to 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 controls 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 2.4d,e). The transgenic plants demonstrated more robust recovery when transferred to 25/22 ^{o}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 2.4f).

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 3 different homozygous T2 *AtGRXS17*-expressing plants derived from each of 2 T1

transgenic lines (*AtGRXS17*-6 and -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 2.5a). The intensity and quantity of brown spots caused by H_2O_2 accumulation in the leaves was analyzed by Image J after transforming the pictures to 256 gray scale images. The statistical analysis further confirmed significantly less H_2O_2 accumulation in the transgenic lines compared to the wild-type plants after the heat treatment (Figure 2.5b) (Analyzing details were described in the Supporting information, Supporting Method).

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 (Figure 2.5c and Figure 2.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 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 tomato plants 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 2.S1). After the initial 3 d at 38/28 ^{o}C, both wild-type and *AtGRXS17*-expressing tomato plants (*AtGRXS17*-6 and -9) showed increased expression of all three HSPs (Figure 2.6a). Moreover, after 3 d of treatment at 38/28 ^{o}C

plus 1 d of treatment at 42/32 ^{o}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 d of treatment at 38/28 ^{o}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 d of treatment at extreme high temperatures (42/32 ^{o}C), exhibiting more than 2.5- to 4-fold elevated expression (Figure 2.6a).

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 tomato plants 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 2.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 d of treatment at 38/28 ^{o}C, *AtGRXS17*-expressing tomato plants (*AtGRXS17*-9) showed significantly increased expression of both *LeHSFA1a* and *LeHSFA2* as compared to wild-type controls (Figure 6b). Furthermore, after 3 d of treatment at 38/28 ^{o}C plus 1 d of treatment at 42/32 ^{o}C, *AtGRXS17*-expressing tomato plants (*AtGRXS17*-6) displayed significantly increased expression of both *LeHSFA1a* and *LeHSFA2* as compared to wild-type controls and *AtGRXS17*-9 lines (Figure 2.6b). 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* knock-out mutant indicates that AtGRXS17 has crucial functions in regulating cellular ROS metabolism and post-embryonic 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 2.1d). Furthermore, expression of *AtGRXS17* in yeast *grx3gxr4* mutant cells could suppress these sensitivities as did yeast endogenous ScGrx3 (Figure 2.1a-e), suggesting that both Grxs share some common pathways/functions in regulating heat stress responses. Given AtGRXS17 has multiple Grx domains compared to 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 to 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 2.4a-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 2.5a), 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 to wild-type plants after heat treatment (Figure 2.5c and Figure 2.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 over-expression 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 2.6a, b), leading the regulatory interplay among heat stress response pathways and protecting against oxidative damage in plants under heat stress. Since 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 crosstalk 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 2.6a, 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 2.6a). 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 hr 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 to 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 posttranscriptional 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 posttranscriptional 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 to activation of HSFs (Figure 2.3 and 2.6), 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 by 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. In order 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 leu2Δ1 his3Δ200*) and *grx3grx4* double mutant (*MAT*a *ura3-52 leu2Δ1 his3Δ200 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 above. 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 inorganic salt medium (Murashige and Skoog, 1962). 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 inoculating with *A. tumefaciens*, the plant cultures were maintained at 25 ^{o}C under a 16-h photoperiod. After 6 to 8 weeks, regenerated shoots were transferred to rooting medium, the MS medium contained 0.1 mg/l IAA and 2 mg/l Zeatin, for 6 more weeks. The temperature of the greenhouse was maintained within a range of 25 ^{o}C to 30 ^{o}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). *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 to 2 day post infiltration (DPI), the infiltrated leaves were detached from tobacco plants, kept in Petri dishes with the moistened filter paper, and incubated at 25 ^{o}C or 42 ^{o}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 AxioPlan, 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 by 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 ^{o}C in 7% SDS and 0.25 M Na_2HPO_4, and then hybridized overnight at 65 ^{o}C in the same solution containing the probe labeled by NEBlot Phototope Kit (New England Biolabs, Ipswich, MA). Membrane were washed twice for 40 min each with 20 mM Na_2HPO_4 and 5% SDS at 65 ^{o}C and then washed twice again for 30 min each with 20 mM Na_2HPO_4 and 1% SDS at 65 ^{o}C. The signal was detected by Phototope-Star Detection Kit (New England Biolabs, Ipswich, MA) according to the manufacture's instruction.

RNA extraction and qRT-PCR for HSP expression

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, USA). One microliter of the reverse transcription reaction solution was used as a template in a 25 μl PCR solution. Real-time PCR was performed in 25 ul reactions contain 10.5 μl cDNA, 1 μl 10mM of each primer, and 12.5 μl 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 Ct 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 separated by 10% SDS/PAGE was electroblotting to PVDF membrane (Millipore, Billerica, MA, USA) and immunoblotted with Rabbit anti-GFP antibody (Invitrogen) and goat anti-rabbit secondary antibody (Bio-rad) using standard methods. The Rabbit anti-GFP antibody was diluted 1:2000 prior to incubation with the blotand was detected, after washing, using 1: 30 000-diluted anti-rabbit secondary antibody. 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.* (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 made 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, USA). The samples were subjected to 80 ^{o}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 percent percentage of electrolyte leakage from the leaf samples.

Measurement of the CAT activities

Four-week-old seedlings were treated by 42 ^{o}C/32 ^{o}C (day/night) for 4 d, 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 centrifuge at 13000 g for 15 min at 4 ^{o}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 Quyang *et al.* (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 d and transferred to pots containing Miracle Gro (700) soil growing medium (Scotts: Marysville, Ohio) and grown in a growth chamber with a temperature of 25/22 ^{o}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 ^{o}C (day/night) for 3 d and then 42/32 ^{o}C (day/night) for additional 3 d.

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 ^{o}C (day/night) (control, upper row) and 42/32 ^{o}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 ^{o}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 bleaching 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 ^{o}C (for control) or 42 ^{o}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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Figures

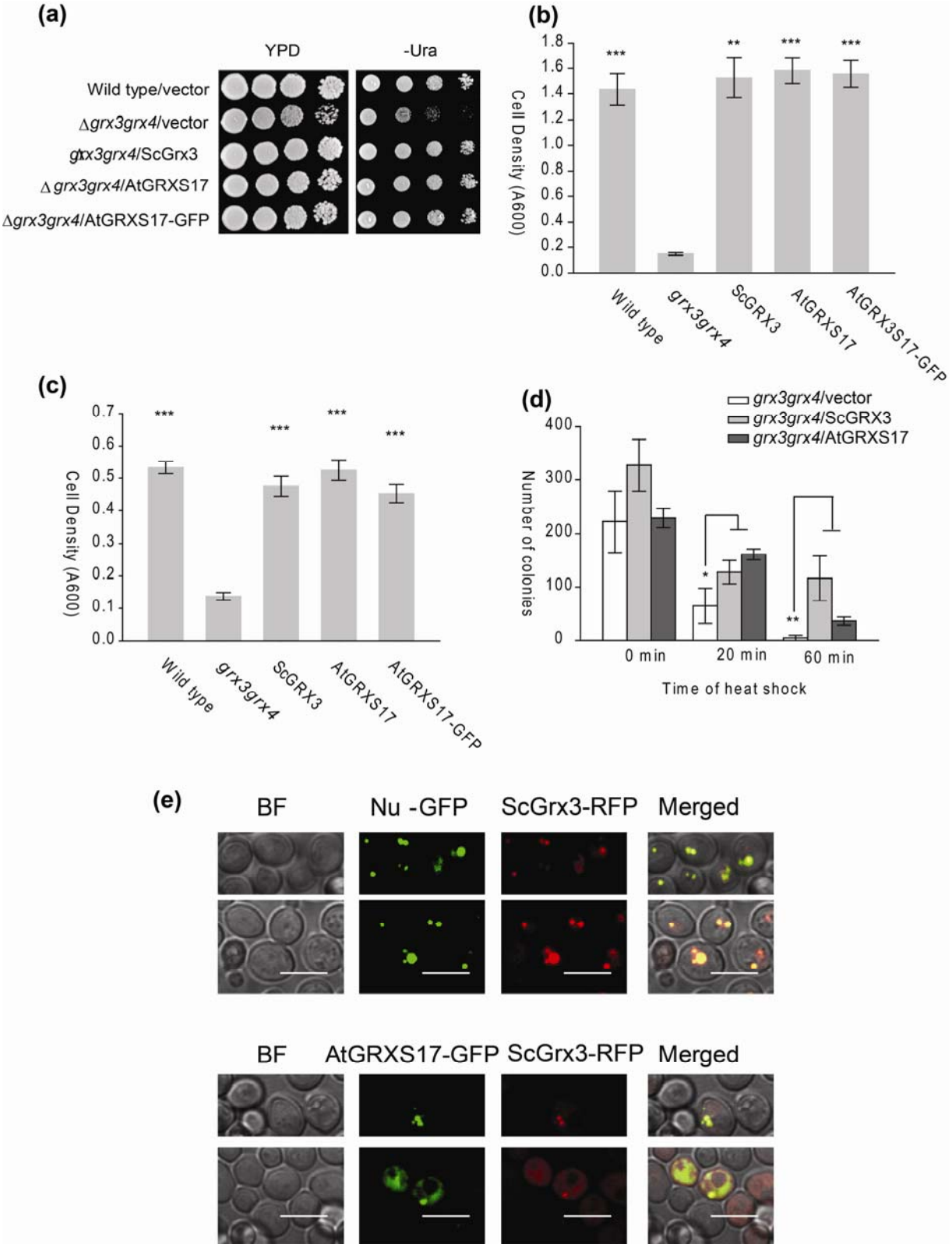
(a)
YPD
-Ura
Wild type/vector
Δgrx3grx4/vector
Δgrx3grx4/ScGrx3
Δgrx3grx4/AtGRXS17
Δgrx3grx4/AtGRXS17-GFP
(b)
Cell Density (A600)
Wild type
grx3grx4
ScGRX3
AtGRXS17
AtGRXS17-GFP
(c)
Cell Density (A600)
Wild type
grx3grx4
ScGRX3
AtGRXS17
AtGRXS17-GFP
(d)
grx3grx4/vector
grx3grx4/ScGRX3
grx3grx4/AtGRXS17
Number of colonies
0 min
20 min
60 min
Time of heat shock
(e)
BF
Nu -GFP
ScGrx3-RFP
Merged
BF
AtGRXS17-GFP
ScGrx3-RFP
Merged

Figure 2.1 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 d 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 above, 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 ^oC 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 d at 30 ^oC, 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=10μm.

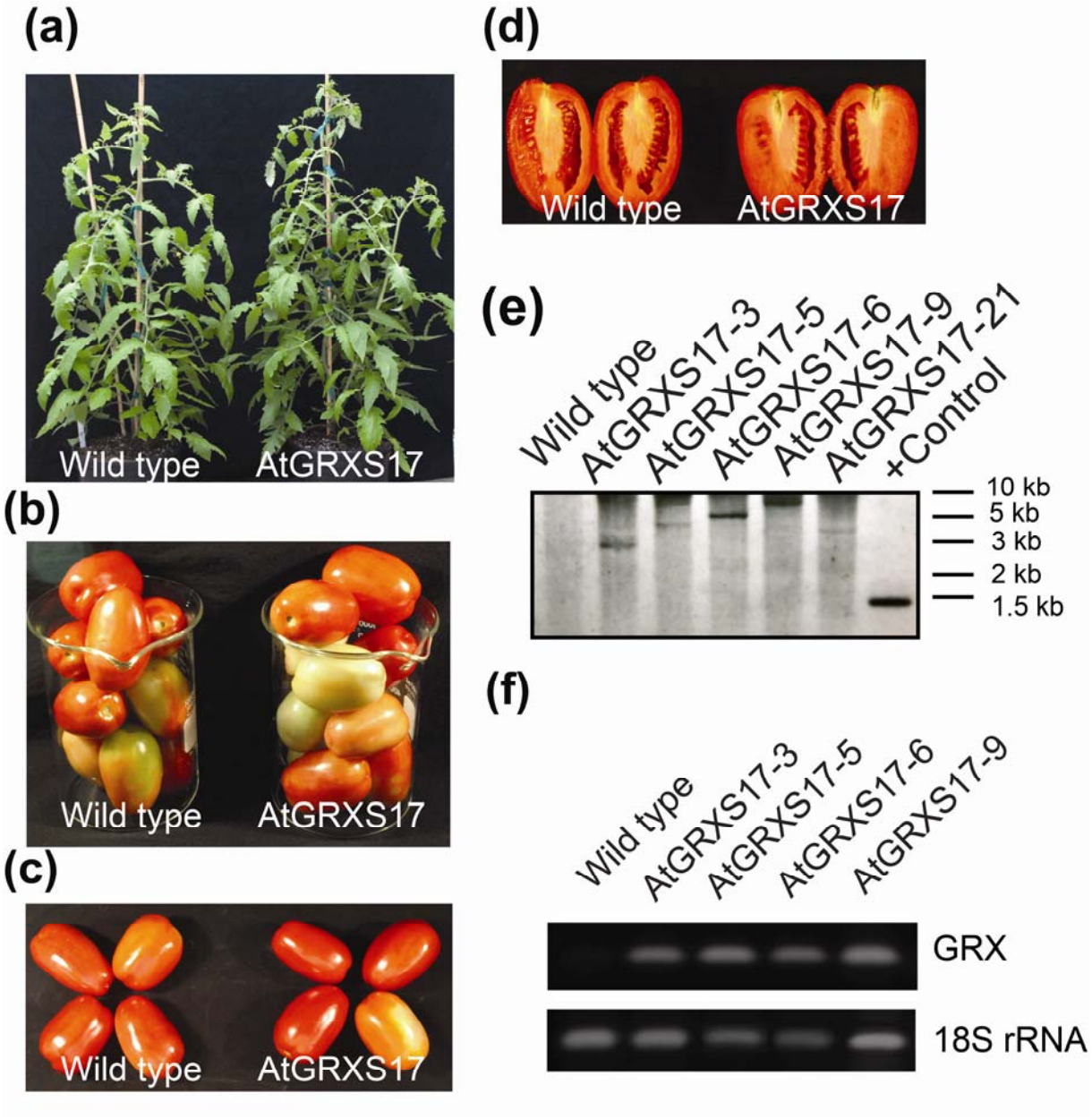

Figure 2.2 Phenotype of *AtGRXS17*-expressing tomato plants.

(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.

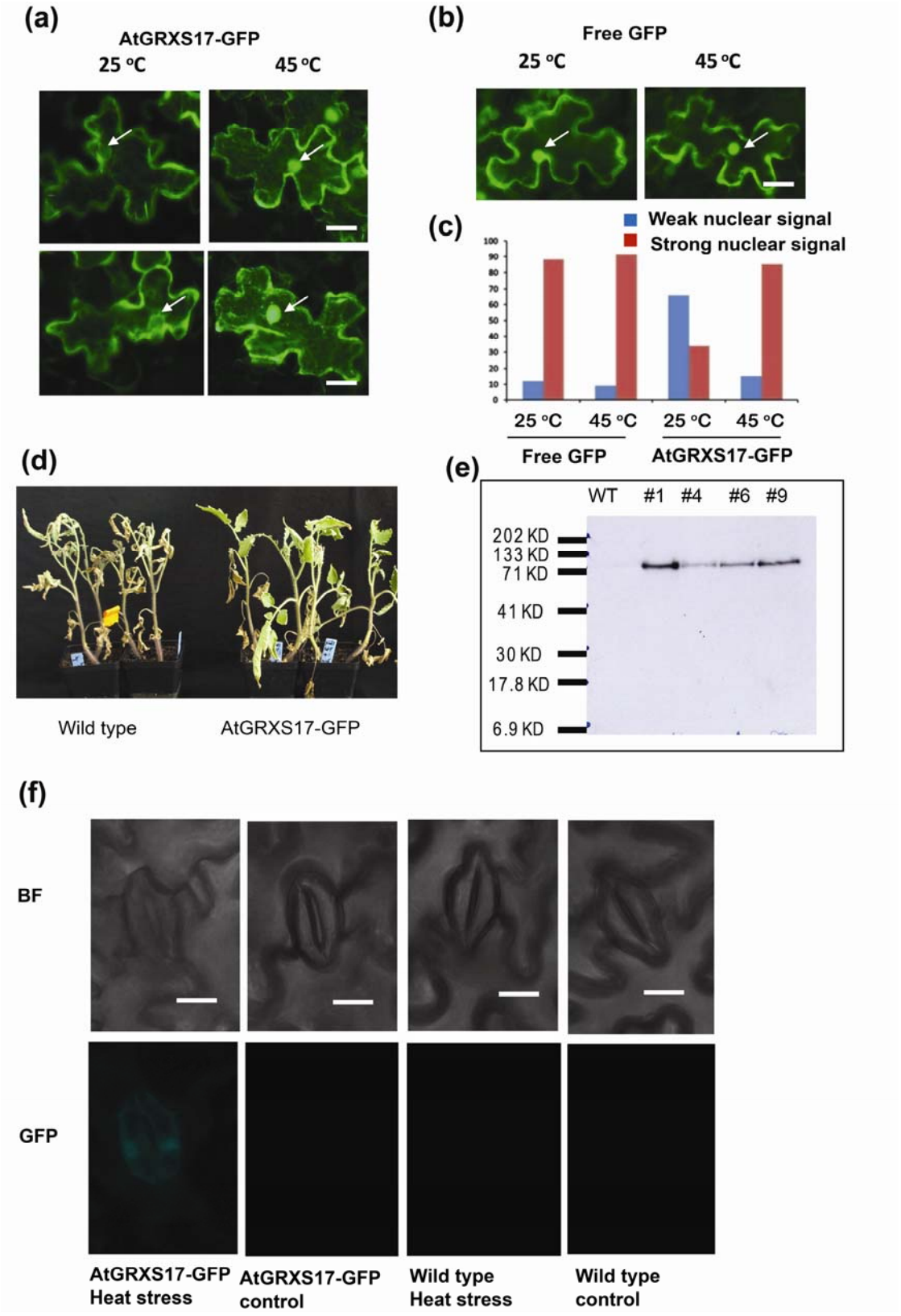
(a)
AtGRXS17-GFP
25 °C
45 °C
(b)
Free GFP
25 °C
45 °C
(c)
Weak nuclear signal
Strong nuclear signal
100
90
80
70
60
50
40
30
20
10
0
25 °C
45 °C
25 °C
45 °C
Free GFP
AtGRXS17-GFP
(d)
Wild type
AtGRXS17-GFP
(e)
WT
#1
#4
#6
#9
202 KD
133 KD
71 KD
41 KD
30 KD
17.8 KD
6.9 KD
(f)
BF
GFP
AtGRXS17-GFP
Heat stress
AtGRXS17-GFP
control
Wild type
Heat stress
Wild type
control

Figure 2.3 Subcellular localization of AtGRXS17.

Transient expression of *GFP-AtGRXS17* (a) and free GFP (b) in tobacco epidermal cells were imaged after being treated at 25 ^{o}C and 45 ^{o}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 ^{o}C (day/night, under a 16-h photoperiod) for 3 d, followed by treated under 42/32 ^{o}C (day/night, under a 16-h photoperiod) for additional 3 d. (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 ^{o}C (day/night) for 3 d, followed by treated under 42/32 ^{o}C (day/night) for additional 2 d.

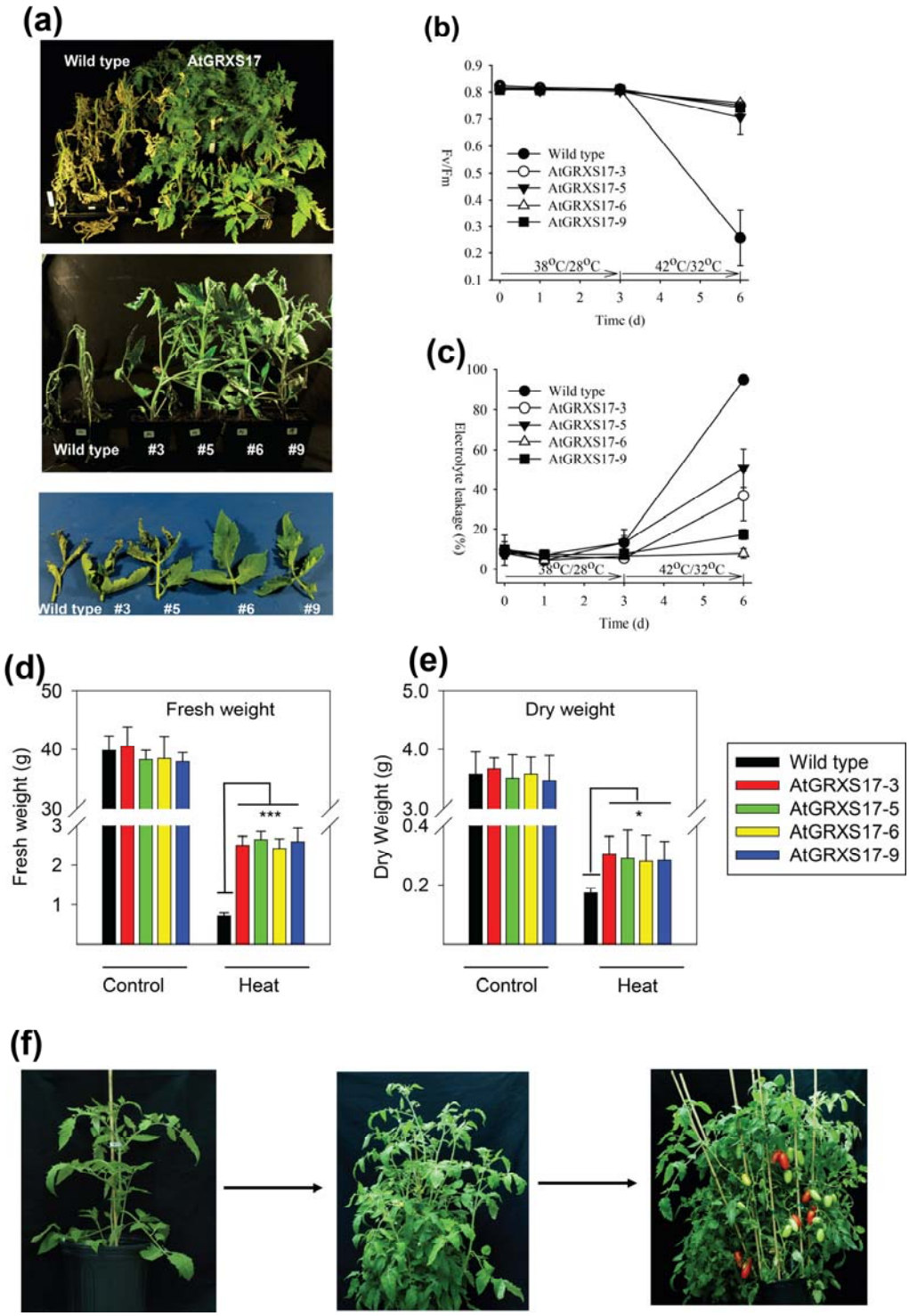

Figure 2.4 ***AtGRXS17*-expressing tomato plants (T2) shown more tolerance to heat stress compared with wild-type controls.**

(a) After 3 d of treatment at 38/28 ^{o}C (day/night) followed by additional 3 d of treatment at 42/32 ^{o}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 d of treatment at 38/28 ^{o}C (day/night) followed by additional 25 d of treatment at 42/32 ^{o}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.

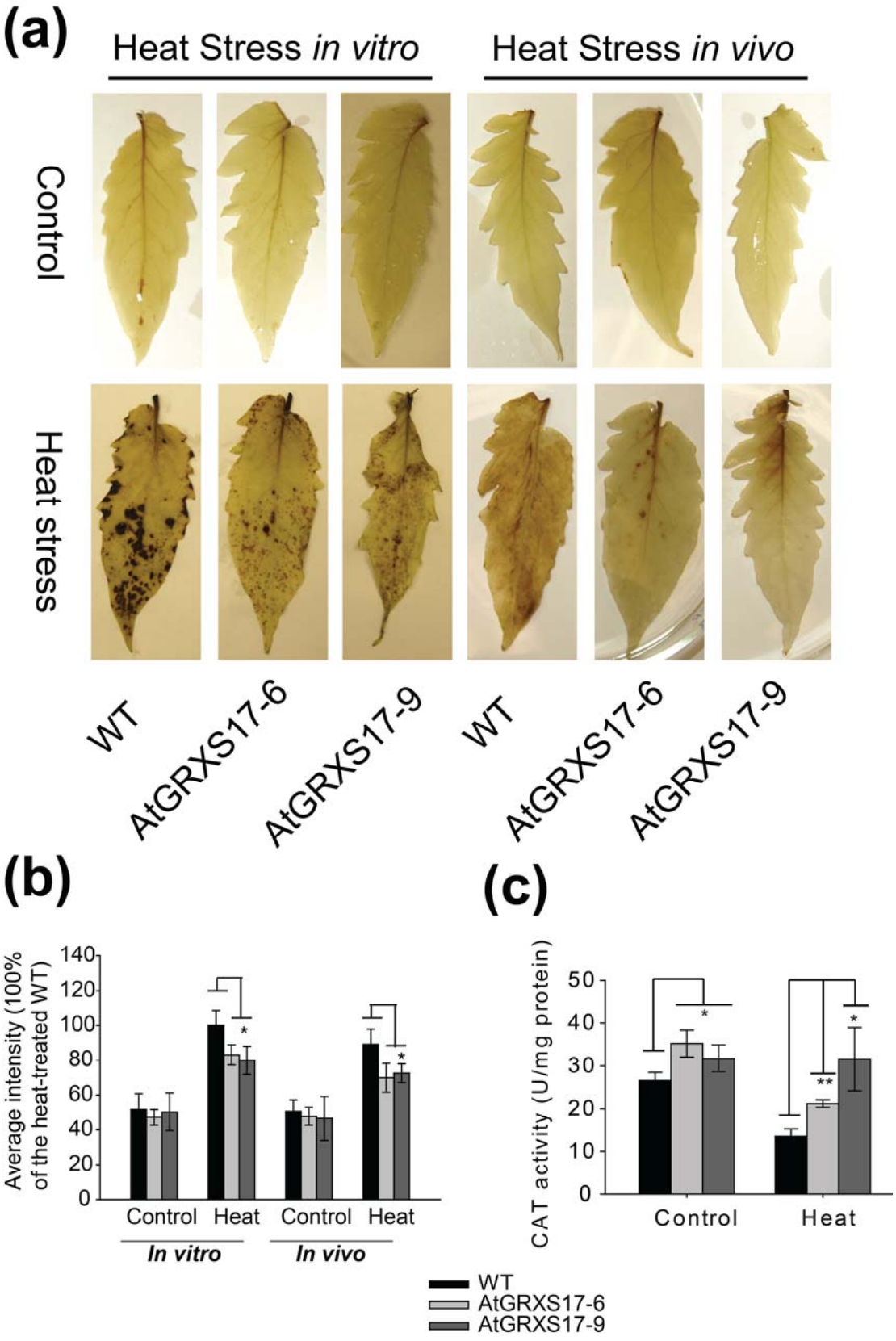
(a)
Heat Stress in vitro
Heat Stress in vivo
Control
Heat stress
WT
AtGRXS17-6
AtGRXS17-9
WT
AtGRXS17-6
AtGRXS17-9
(b)
Average intensity (100% of the heat-treated WT)
140
120
100
80
60
40
20
0
Control
Heat
Control
Heat
In vitro
In vivo
(c)
CAT activity (U/mg protein)
50
40
30
20
10
0
Control
Heat
WT
AtGRXS17-6
AtGRXS17-9

Figure 2.5 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 gray 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 ^{o}C (day/night) for 3 d. The whole shoot parts were harvested for enzymatic assays. Error bars represent ±SD of 3 biological replicates, and each biological replicate is the average of two technical replicates. Each technical replicate is the same plant sample that was determined once using the spectrophotometer.

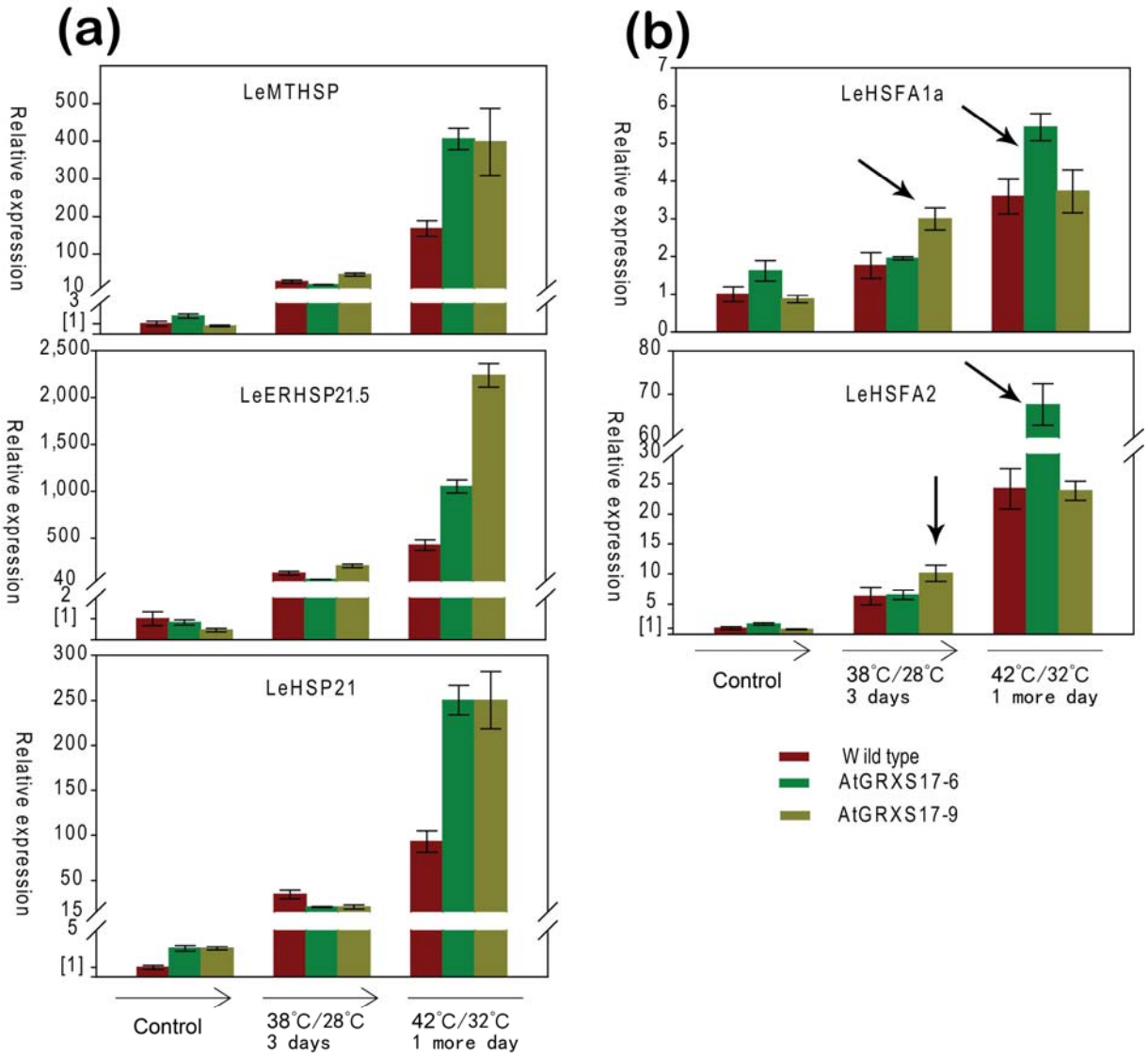

Figure 2.6 Expression analysis of heat shock proteins (HSPs).

(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 d [25/22 ^{o}C (day/night, 16/8 h)], and then treated under 38/28 ^{o}C (day/night) for 3 d, followed by treated a 42/32 ^{o}C (day/night) treatment for additional 1 d. The top fresh leaves of tomato plants were sampled for RNA extraction and qRT-PCR analysis. Error bars represent ±SD of 3 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 SI Table 1.

Supporting information

Supporting method-statistical analysis of DAB staining leaves

We converted the color pictures to gray scale images (0-256) as shown in Figure S1a. Then, we used Histogram function of Image J to analyze the average intensity of the leaf area. Brighter image give higher value, indicating higher H_2O_2 accumulation (Figure S1b). Student's t-test analysis was conducted using the mean value of the histogram distribution (Figure S1c).

Supporting figures

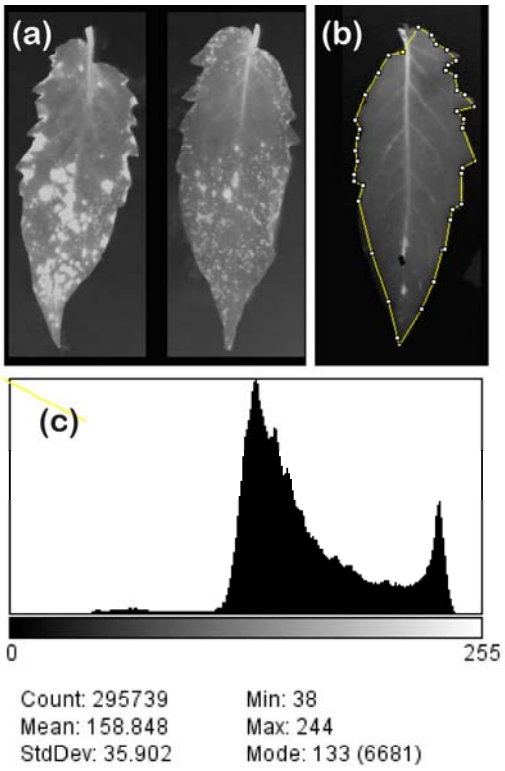

Figure 2.S1 Quantitative analysis of H_2O_2 accumulation.

(a) Gray scale pictures of DAB-stained leaves. (b) The selected area was analyzed by Image J. (c) One example of the Histogram output. The mean value was used for statistical analysis.

Figure 2.S2 The phenotype of 4-week-old *AtGRXS17*-expressing and wild-type tomato plants was indistinguishable before heat treatment.

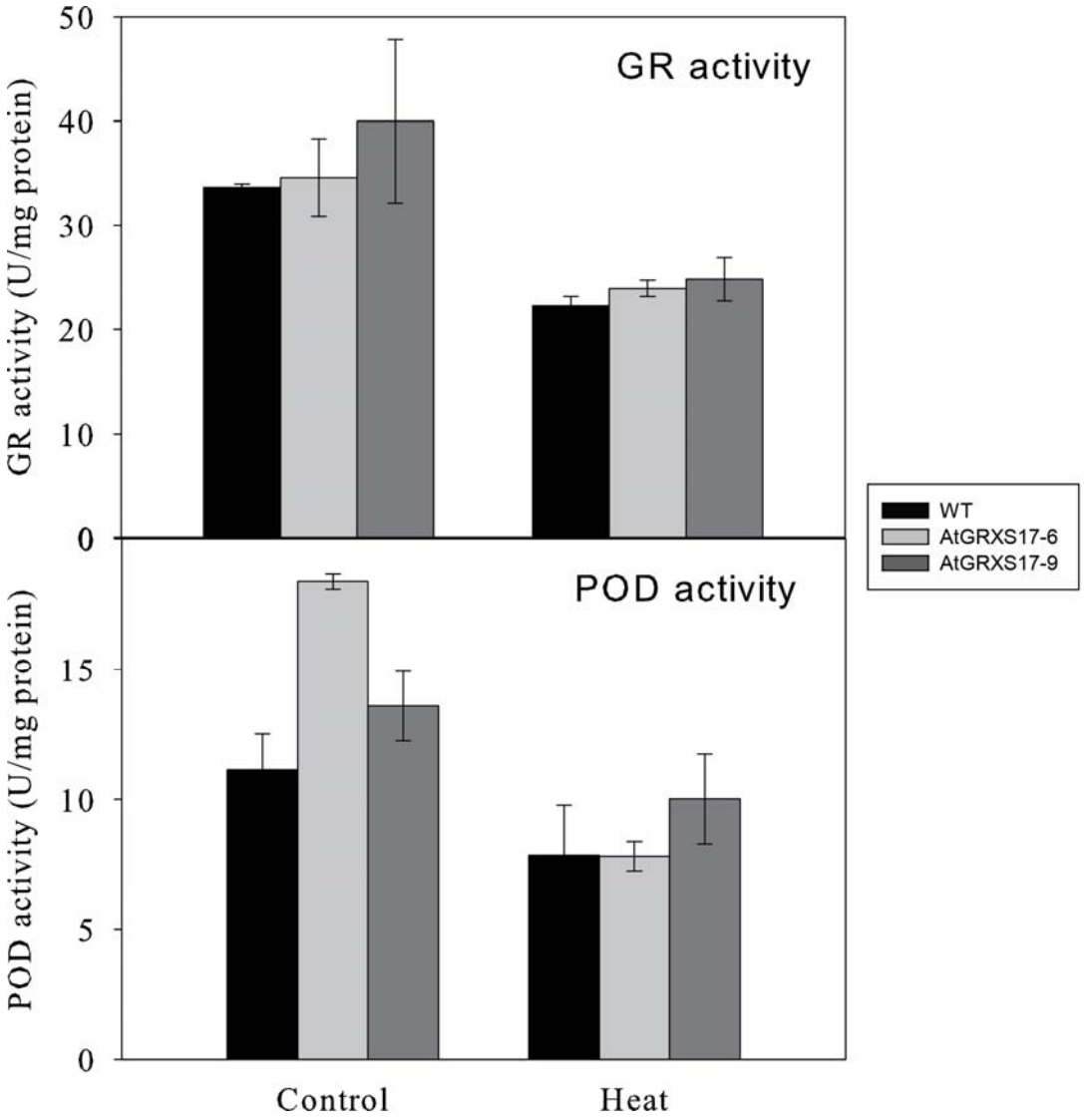

Figure 2.S3 Effects of AtGRXS17 expression on the GR (upper panel) and POD (lower panel) activity under control or heat treatments.

Control, the 33-day-old tomato plants grown under normal growth condition. Heat, the 30-day-old tomato plants were grown under normal growth condition and then treated by 42 ^{o}C/32 ^{o}C (day/night) for 3 days. Error bars represent ±SD of 3 biological replicates, and each biological replicate represents the average of two technical replicates.

Table 2.S1 Primers used for qPCR

Gene name	Accession #	Primer sequence
LeHSP21	U66300	Forward: TGCGTTTTGACATGCCGGGA Reverse: TCTGGGAGACTTAAACGAGTGTCG
LeERHSP21.5	Ab026983	Forward: GGCATTGTGTTGAAAGGAGTCATGGA Reverse: CCACAGAGAGAAACAGAGGATGAAC
LesHSPMT	AB017134	Forward: GCGGTGGAGGAGAACACGCT Reverse: TCTCCGCCTTGATTCCATCCA
LeHSFA1a	AW223123	Forward: GGCAGCAAAGGCAATGTTGAGGGA Reverse: TGGGAACATGTGCCAAGATGAGATGA
LeHSFA2	AK325085	Forward: GGCGGTCTCTAGTAGCGCATGT Reverse: TGGTTGAGGAAAGCCGAGTCCA
LePP2Acs (housekeeping gene)	AY325818	Forward: CGATGTGTGATCTCCTATGGTC Reverse: AAGCTGATGGGCTCTAGAAATC

Chapter 3 - Ectopic expression of Arabidopsis glutaredoxin *AtGRXS17* in tomato (*Solanum lycopersicum*) enhances tolerance to abiotic stress

Abstract

Crop plants are continuously exposed to changing environmental conditions and different signaling pathways converge to help plants counteract numerous stresses. Our previous studies suggest that the *Arabidopsis* monothiol glutaredoxin, AtGRXS17, has conserved function in thermotolerance in yeast, *Arabidopsis*, and tomato plants. In the present study, we report introducing the *Arabidopsis* glutaredoxin *AtGRXS17* into tomato plants also enhances tolerance to drought, chilling, and oxidative stress. After drought stress treatment, the *AtGRXS17*-expressing tomato plants retained twice the shoot water content as compared to wild-type plants, and this enhanced tolerance tightly correlated with maintaining abundance of endogenous *SlAREB1 (Solanum lycopersicum* ABA-responsive element binding protein). When the tolerance of *AtGRXS17*-expressing tomato plants against chilling stress was compared with that of wild-type and *AtCBF3 (Arabidopsis C-repeat Binding Factor-3)*-expressing tomato plants, ectopic expression of both *AtGRXS17* and *AtCBF3* could enhance chilling tolerance in tomato plants; however, the *AtGRXS17*-expressing tomato plants showed more vigorous recovery compared to *AtCBF3*-expressing tomato plants after chilling stress. In addition, expression of *AtGRXS17* in tomato plants appeared to compromise a role of its endogenous *SlCBF1* during chilling stress. After oxidative stress treatment, *AtGRXS17*-expressing tomato plants stimulated up-regulation of *SlCAT1* (Catalase 1) and displayed significantly longer root length as compared to wild-type plants, indicating that AtGRXS17 plays an important role that helps ameliorate severe growth defects caused by oxidative damage. Therefore, these findings suggest that ectopic expression of *AtGRXS17* may impact multiple traits involved in various abiotic stresses, and provide a useful approach to improve tolerance to abiotic stresses in agriculturally important crop species.

Introduction

Various abiotic stresses (drought, cold, heat, salt) adversely affect the growth,

development, and seed development of crop species (Yamaguchi-Shinozaki and Shinozaki 2006; Munns and Tester 2008; Mittler and Blumwald 2010). These abiotic stresses often occur simultaneously, such as heat and drought, drought and salt, and pose a serious challenge for agricultural productivity worldwide, causing annual losses estimated at billions of dollars per year (Mittler and Blumwald 2010). Therefore, improvement of tolerance of plants against abiotic stresses remains a major focus of agricultural research.

Drought is one of the major abiotic stresses affecting plant production; more than 50% of the Earth's surface area, including the vast majority of agricultural lands, is vulnerable to drought (Kogan 1997). Tremendous efforts have been made to understand the molecular, biochemical, and physiological basis of plant adaptation to drought stress. Previous studies have indicated that the phytohormone abscisic acid (ABA) is a key mediator that coordinates a complex regulatory network enabling plants to cope with decreased water availability by regulation of stomatal aperture and downstream transcription factors, such as ABA-responsive element binding protein (AREB) (Cutler et al. 2010; Kim et al. 2010). Two proteins of AREB family in tomato, SlAREB1 and SlAREB2, have been characterized (Yanez et al. 2009; Orellana et al. 2010), and SlAREB1 confers drought tolerance when overexpression in tomato, while *SlAREB1* antisense lines have displayed hypersensitivity to drought stress, indicating SlAREB1 is an important protein that mediates ABA-dependent drought tolerance (Orellana et al. 2010).

Cold stress adversely affects plant growth and development and thus limits crop productivity (Chinnusamy et al. 2007). Plants have developed sophisticated response pathways, such that CBF (C-repeat binding factor) pathway contributes to chilling resistance and cold acclimation (Chinnusamy et al. 2007). Tomato is one of the species unable to tolerate freezing and suffer chilling injury when exposed to low temperatures (Zhang et al. 2004). Efforts have been made to improve tomato chilling tolerance by ectopic expression of the *Arabidopsis CBF1*. The transgenic tomato plants have displayed significant chilling tolerance (Hsieh et al. 2002).

Under drought or cold stresses, plants dramatically increase the production of reactive oxygen species (ROS) as part of their responses to those stresses. However, although ROS can act as signals to help plants adapt to stress responses ((Miller et al. 2008; Penfield 2008; Gill and Tuteja 2010; Miller et al. 2010), excess ROS cause oxidative damages to plant macromolecules and cell structures, leading to inhibition of plant growth and development (Suzuki and Mittler 2006; Gill and Tuteja 2010; Jaspers and Kangasjarvi 2010). Therefore, the level of ROS should

be judiciously regulated in plants through the coordinate of ROS production system and ROS scavenging system to manage oxidative damage and simultaneously regulate signaling events (Foyer and Noctor 2005; Rouhier et al. 2008), but the underlying mechanisms are still largely unknown.

As a part of the ROS scavenging system, Glutaredoxins (Grxs) are small ubiquitous proteins of the thioredoxin (Trx) family and mediate reversible reduction of disulfide 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 signaling pathways, and are conserved in both prokaryotes and eukaryotes (Rouhier et al. 2005; Shelton et al. 2005; Lillig et al. 2008; Rouhier et al. 2008; Cheng et al. 2011). In plants, Grxs are a big protein family not only necessary for redox buffering, but also play roles in heavy metal detoxification, plant development, plant-pathogen interactions, iron homeostasis, and abiotic stress response (Rouhier et al. 2008). Recent studies suggest that even a single glutaredoxin protein may have multiple functions in plants. For instance, the *Pteris vittata* glutaredoxin PvGRX5 plays roles in both arsenic tolerance and thermotolerance (Sundaram et al. 2009; Sundaram and Rathinasabapathi 2010). In addition, the *Arabidopsis* glutaredoxin AtGRXS13 functions in both pathogen and photooxidative stress response (La Camera et al. 2011; Laporte et al. 2012). Our recent study also demonstrates that ectopic expression of an *Arabidopsis* Grx, *AtGRXS17*, in tomato plants elevates thermotolerance through reducing oxidative damage caused by excess ROS accumulation in transgenic plants (Wu et al. 2012). Thus, it is imperative to examine how ectopic expression of *AtGRXS17* in tomato plants responds to other abiotic stresses.

In this study, we initially characterized the ion leakage of wild-type and *atgrxs*17 knock-out (KO) *Arabidopsis* plants under chilling stress conditions to determine how AtGRXS17 influences chilling tolerance in *Arabidopsis*. Furthermore, we treated the *AtGRXS17*-expressing and wild-type tomato plants with chilling, drought, and oxidative stress, respectively, to test if ectopic expression of *AtGRXS17* in tomato plants can also enhance tolerance to these abiotic stresses. Finally, we investigated how introducing of *AtGRXS17* affects the expression of endogenous stress-responsive genes in tomato plants.

Materials and Methods

Bacterial strains for plant transformation

AtGRXS17 coding region was cloned into pBICaMV vector as described (Wu et al. 2012). The CBF3 coding region was also cloned into pMDC99 vector as described (Feng et al. 2011), and binary plasmids pMDC-CBF3 and pBICaMV-AtGRXS17 were introduced into *Agrobacterium tumefaciens* strain LBA4404 using the freeze-thaw method, respectively (Holsters et al. 1978). Briefly, five mill liter LBA4404 Agrobacterium was cultured in YEP medium with 100 mg/l streptomycin at 28 ^{o}C for 36 h. Five hundred micro liter Agrobacterium culture was transferred to 4.5 ml fresh YEP medium contained 100 mg/l streptomycin and was shaking at 250 rpm to OD of 1.0. The culture was chilled on ice for 5 min. The cell culture was centrifuged at 3000 rpm for 5 min at 4 ^{o}C, and the supernatant was discarded. The pellet will be resuspended with 1 ml 20 mM $CaCl_2$. One micro gram DNA will be incubated with 100 μl cell culture on ice for 30 min. We freezed the cells in liquid nitrogen for 1 min, and thawed cells by incubating in 37 ^{o}C water bath for 5 min. We added 1 ml YEP media and incubated the transformants for 2-4 hat 28 ^{o}C with gentle shaking. After centrifuging the cells for 30 sec, we spread the cells on YEP agar medium containing proper antibiotics. The transformed colonies will appear in 2-3 d.

Tomato transformation

Seeds of tomato *Solanum lycopersicum* L. (cv Rubion) were surface sterilized and germinated on the Murashige and Skoog inorganic salt medium (Murashige and Skoog 1962). Tomato transformation was performed via *Agrobacterium*-mediated transformation method using cotyledon and hypocotyls explants as described (Park et al. 2003). *A. tumefaciens* LBA 4404 strains containing pBICaMV-*AtGRXS17* or pMDC-CBF3 were used for generating stable transgenic lines.

Growth conditions and tolerance analyses of Arabidopsis and tomato

Wild-type (ecotype Columbia, Col-0) and *atgrxs17* KO seeds of *Arabidopsis* plants were surface-sterilized, germinated, and grown in soil. Twenty-day-old seedlings grown at 22 ^{o}C were moved to a growth chamber set at 0 ^{o}C. The leaves were sampled after 0-, 5-, 10-, and 20-h chilling treatment and subjected to electrolyte leakage analysis.

T2 generation of *AtGRXS17*-expressing (Wu et al. 2012), *AtCBF3*-expressing or wild-type tomato seeds were surface-sterilized, germinated, and grown on pots containing Micracle Gro (700) soil growing medium in growth chamber. The temperature of growth chamber was maintained at 24 ^{o}C/20 ^{o}C (day/night) under a 16-h photoperiod, and the light intensity was maintained at 300 μmol/m^2/sec. The plants were regularly watered and fertilized on a weekly basis with N : P_2O_5 : K_2O 20: 20: 20 (Scotts: Marysville, Ohio). For the drought treatment, 4-week-old *AtGRXS17*-expressing and wild-type seedlings were withheld from watering for 12 days, and then re-watered. The phenotype and the chlorophyll fluorescence were tracked during the drought treatment. For the chilling treatment, 4-week-old *AtGRXS17*-expressing, *CBF3*-expressing or wild-type seedlings were treated at 4 ^{o}C (day/night) for 3 weeks in a walk-in growth chamber, and then recovered in normal growth conditions as mentioned above for 5 days. The chlorophyll fluorescence was tracked during the first 7-d chilling treatment. For oxidative stress treatment, 7-day-old *AtGRXS17*-expressing and wild-type seedlings grown on the MS media were transferred into the MS medium with or without 20 μM methyl viologen (MV) in magenta boxes and incubated for 14 days. After being harvested, the primary root length was measured, and total RNA was extracted from the leaves.

Time-course analysis of chilling and drought stress-responsive genes

To evaluate the effects of AtGRXS17 on the expression of chilling and drought tolerance related genes, chilling and drought treatments were applied using 14-day-old *AtGRXS17*-expressing and wild-type seedlings grown in Petri dishes with MS medium. The treatment and sampling were designed according to previous reports of *SlAREB1* and *SlCBF1* expression study with slightly modification (Zhang et al. 2004; Orellana et al. 2010). For chilling treatment, the seedlings were moved to a growth chamber set at 4 ^{o}C. The seedlings were harvested after being treated for 0, 0.5, 2, 4, 8, 24, and 48 h, respectively. For drought treatment, the seedlings were transferred and incubated in Petri dishes containing two layers of dry filter paper for 0, 0.5, 2, 4, 8, and 24 h, respectively. The seedlings after chilling or drought treatments were harvested, and the RNA was extracted using the method described below.

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 qRT-PCR was treated with

RNase-free DNase prior to the synthesis of first-strand cDNA by oligo (dT) priming using moloney murine leukemia virus-reverse transcriptase (BD Biosciences Clontech, Palo Alto, CA, USA). One microliter of the reverse transcription reaction solution was used as a template in a 25 μl PCR solution. Real-time PCR was performed in 25 ul reactions contain 10.5 μl cDNA, 1 μl 10mM of each primer, and 12.5 μl 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 Ct method (Wu *et al*., 2012). *SlPP2ACS* was uses as a normalization control (Lovdal and Lillo 2009). The primers for PCR were listed in the supplementary data (Table S1).

Electrolyte leakage and Fv/Fm ratio

Injury to plants was characterized by measuring chlorophyll fluorescence and electrolyte leakage of leaves as described by Oh et al. (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 the chilling or drought treatment. Measurements were made during the light cycle on the leaves using the saturation pulse method after 30 min of dark adaption. For electrolyte leakage, *Arabidopsis* or tomato 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, USA). The samples were subjected to 80 ^{o}C for 2h to release the total electrolytes and then held at room temperature for 10h. The final conductivity on the leachate was measured to determine the percent electrolyte leakage from the leaf samples.

Shoot water content

Shoot water content (SWC) was expressed as the difference between leaf fresh weight and dry weight, and calculated as follows:

SWC = (fresh weight- dry weight)/dry weight

Results

Response and adaptation to drought stress in AtGRXS17-expressing tomato

Drought and heat stress often come together and threaten the agricultural production, and both are tightly related to ROS stress (Barnabas et al. 2008); thus, we tested whether AtGRXS17 confers drought tolerance as well. The *AtGRXS17* driven by the cauliflower mosaic virus (CaMV) 35S promoter was introduced into tomato plants as previously described, and the lines (*AtGRXS17*-3, -5, -6 and -9) that appeared to contain single transgene insertions and displayed strong thermotolerance were used in this study (Wu *et al*., 2012). Four-week-old wild-type and *AtGRXS17*-expressing tomato plants were subjected to drought treatment by withholding the water. Despite both wild-type and *AtGRXS17*-expressing tomato plants were adversely affected by the drought stress, the damaging of wild-type plants was more severe than that of *AtGRXS17*-expressing tomato plants (Fig. 3.1A). In addition, all the *AtGRXS17*-expressing lines showed significantly higher shoot water content (SWC) compared to wild-type plants after 10-day drought treatment (Fig. 3.1B). We also tracked changes in chlorophyll fluorescence during the drought treatment, and the data indicate that the *AtGRXS17*-expressing tomato plants showed higher Fv/Fm ratio compared to wild-type plants (Fig. 3.1C). These results suggest that ectopic expression of *AtGRXS17* in tomato plants improves drought tolerance.

Given the evidence that *AtGRXS17*-expressing tomato plants are drought tolerant, we also further investigated if AtGRXS17 affects ABA signal pathway during drought stress by measuring the time-course abundance of *SlAREB1*. *SlAREB1* is an ABA-dependent transcription factor that confers drought tolerance in tomato (Orellana et al. 2010). The *SlAREB1* transcripts peaked at 4 h and then started to decrease in both wild-type and transgenic lines. However, the abundance of *SlAREB1* in *AtGRXS17*-expressing lines remained at a higher level than that of wild-type plants (Fig. 3.2). This result suggests that there may be some interactions between GRXs and ABA signaling during drought stress.

atgrxs17 KO line was more sensitive to chilling stress as compared with wild-type Arabidopsis

Since our previous study suggests that T-DNA insertion *atgrxs17* KO mutant plants are sensitive to elevated temperatures (Wu et al., 2012), we tested if the *atgrxs17* KO plants were also sensitive to reduced temperatures. After being treated under 0 ^{o}C for varying times (0, 5, 10,

and 20 h), the electrolyte leakage of KO plants and wild-type plants were determined. The data suggest that the electrolyte leakage of KO plants was higher than that of wild-type plants (Fig. 3.3), indicating more severe membrane damaging in KO plants after being treated under chilling stress.

Response and adaptation to chilling stress in AtGRXS17-expressing tomato

Based on the sensitivity of *atgrxs17* KO plants to chilling stress, we hypothesize that enhancing *AtGRXS17* expression can improve chilling tolerance in tomatoes. Since the CBF3 is a well characterized transcription factor that confers chilling tolerance (Kasuga et al. 1999), we generated the *AtCBF3*-expressing tomatoes as positive control lines and compared their chilling tolerance with *AtGRXS17*-expressing tomatoes generated from our previous studies (lines, *AtGRXS17*-3, -5, -6 and -9; Wu et al., 2012). The *AtCBF3*-expressing tomato plants (lines, *AtCBF3*-2 and -3) were confirmed by PCR (SI Fig. 3.S1), and 4-week-old *AtCBF3*-, *AtGRXS17*-expressing and wild-type tomato plants were treated at 4 ^{o}C for 3 weeks. The wild-type tomato plants showed more severe damage than *AtCBF3*- or *AtGRXS17*-expressing tomato plants (Fig. 3.4A, B); however, the *AtGRXS17*-expressing tomato plants showed more vigorous growth than that of *AtCBF3*-expressing transgenic plants after being moved into normal growth condition [24/20 ^{o}C (day/night)] and recovered for 5 days (Fig. 3.4C). The enhanced chilling tolerance of the plants was further verified by measuring changes in chlorophyll fluorescence and electrolyte leakage. The electrolyte leakage of *AtCBF3*- or *AtGRXS17*-expressing transgenic plants was significantly lower than that of wild-type after chilling treatment, indicating reduced disruption of cell membranes in the transgenic lines (Fig. 3.5A). In addition, the chlorophyll fluorescence of both *AtCBF3*- and *AtGRXS17*-expressing plants was higher than that of wild-type (Fig. 5B). These data indicate that AtGRXS17 confers chilling tolerance, and even plays a stronger protective role to plants than CBF3, especially during recovery. Moreover, *AtCBF3*-expressing tomato plants displayed stunted shape and reducing number of fruits as compared to wild-type plants under normal growth condition (SI Fig. 3.S2).

The induction of *CBFs* is one of the predominant responses to cold stress (Chinnusamy et al. 2007). In previous studies by Thomashow and colleagues, three tomato CBFs have been characterized, and only *SlCBF1* among those genes is strongly induced by chilling treatment

(Zhang et al. 2004). Therefore, we further investigated possible effects of AtGRXS17 on the dynamic change of abundance of endogenous tomato *CBFs*, particularly *SlCBF1* transcripts, under chilling stress. qRT-PCR results indicate that up-regulation of *SlCBF1* in both wild-type and *AtGRXS17*-expressing lines peaked at 4 h after chilling stress (Fig. 3.6). However, the up-regulated expression level of *SlCBF1* in *AtGRXS17*-expressing lines returned rapidly to resting level, while a slow return to resting level of *SlCBF1* in wild-type lines was observed (Fig. 3.6). These data suggest a potential direct correlation between AtGRXS17 and compromising a role of SlCBF1 in CBF signaling pathways under chilling stress.

AtGRXS17-expressing tomato plants improved tolerance to oxidative stress

Both drought and cold stress trigger accumulation of ROS, which results in cellular damage by oxidative stress (Jaspers and Kangasjarvi 2010). We hypothesized that *AtGRXS17*-expressing tomato plants may affect improved tolerance to oxidative stress as well. To examine this, we incubated 7-day-old wild-type and *AtGRXS17*-expressing tomato seedlings in MS media with or without 20 μM methyl viologen (MV) for 14 d, which stimulates formation of ROS within chloroplasts. The pro-oxidant herbicide methyl viologen (paraquat), which acts as a cycling catalyzer of electron transfer from PSI FeS centers to O_2, greatly enhances the superoxide production at PSI (Foyer and Noctor 2009). The results showed that *AtGRXS17*-expressing tomato seedlings displayed more vigorous growth and longer root length as compared to wild-type seedlings (Fig. 3.7), indicating that AtGRXS17 reduced oxidative damages caused by excess ROS accumulation.

Catalase (CAT) plays important roles in ROS scavenging by converting H_2O_2 to O_2 and H_2O. Our previous heat stress study suggests that ectopic expression of *AtGRXS17* in tomato increases CAT activity (Wu et al., 2012). Thus, we determined the *SlCAT1* expression level under oxidative stress. The data demonstrated that the *SlCAT1* displays more dramatic up-regulation in *AtGRXS17*-expressing lines as compared to wild-type after being treated by 20 μM MV for 14 d (Fig. 3.8).

Discussion

Regulation of cellular redox states affects plant development, growth and responses to biotic and abiotic stresses (Potters et al. 2009; Bashandy et al. 2010; Suzuki et al. 2011). Our previous studies have also suggested that *Arabidopsis* AtGRXS17 plays an important role in

temperature-dependent postembryonic growth in *Arabidopsis* and enhances heat stress tolerance in tomatoes by protecting against oxidative damage (Cheng et al. 2011; Wu et al. 2012). In this study, we further investigated the roles of AtGRXS17 in response and adaptation to various abiotic stresses and showed how ectopically expressed *AtGRXS17* conferred tolerance to chilling, drought, oxidative stress and affected endogenous stress-responsive genes in tomato plants.

C-repeat/dehydration-responsive element binding factor (CBFs/DREB1s pathway is a well characterized chilling response pathway, and extensive efforts have been made to improve cold tolerance by manipulating CBFs across different species (Morran et al. 2011; Xu et al. 2011; Yang et al. 2011). In tomato plants, ectopic expression of *Arabidopsis CBF1* cDNA driven by 35S CaMV promoter improves the chilling tolerance (Hsieh et al. 2002; Zhang et al. 2011). However, the CBF regulons from cold-tolerant species such as *Arabidopsis* and cold-sensitive species such as tomato are different (Zhang et al. 2004; Carvallo et al. 2011), limiting the effectiveness of CBFs for engineering cold tolerant crop species. For example, expression of *CBF3/DREB1a* or *SlCBF1* in *Arabidopsis* significantly improves freezing tolerance of transgenic plants while constitutive overexpression of either *SlCBF1* or *AtCBF3* in transgenic tomato plants does not increase freezing tolerance (Kasuga et al. 1999; Zhang et al. 2004). Indeed, when we introduced the same *AtCBF3* into tomato, it marginally improved chilling tolerance as compared to wild-type plants, and showed less protective against chilling stress than *AtGRXS17*-expressing tomato plants (Fig. 3.4).

Interestingly, our results demonstrated that introducing *AtGRXS17* into tomato plants alters the response of *SlCBF1* expression under chilling stress condition. The up-regulated expression level of *SlCBF1* in *AtGRXS17*-expressing lines returned rapidly to resting level compared to wild-type lines (Fig. 3.6), suggesting a potential direct correlation between AtGRXS17 and compromising a role of SlCBF1 in CBF signaling pathways under chilling stress. In fact, the accumulation of CBFs should be tightly regulated to minimize its negative effects on plants. In *Arabidopsis*, the expression of CBFs is maintained at a very low level, and induced within 15 min of exposing plants to low temperatures (Stockinger et al. 1997; Gilmour et al. 1998; Medina et al. 1999). Despite the pivotal role of CBFs in chilling tolerance, constitutive expression of either *AtCBF3* or *SlCBF1* in both *Arabidopsis* and tomato plants results in stunted growth (Kasuga et al. 1999; Zhang et al. 2004). In this study, the *AtCBF3*-expressing tomato plants also displayed stunted growth and reducing yield under normal growth condition (SI Fig.

3.S2) as demonstrated in previous report (Pino et al., 2007). Most of these studies indicate that over-accumulation of CBFs disrupts the regular biological processes of plants, and a negative regulation loop of CBFs should exist to control the abundance of CBFs under different environmental conditions. Additional results also suggest that self-regulation plays an important role in regulating CBFs during cold acclimation (Chinnusamy et al. 2007). For example, CBF2 is a negative regulator of *CBF1* and *CBF3* expression during cold acclimation, while CBF3 negatively regulates *CBF2* expression (Chinnusamy et al. 2003; Novillo et al. 2004). Furthermore, MBY15 and ZAT12 also appear to function as negative regulator of CBFs (Chinnusamy et al. 2007). Our results suggest ectopic expression of *AtGRXS17* reduces the ROS accumulation and membrane damage caused by cold stress to some extend; however, how this protective signal negatively regulates the CBFs pathway remains unknown. Thus, further research is needed to elucidate the direct or indirect interactions between AtGRXS17 and the negative regulation of CBFs. Nevertheless, manipulation of AtGRXS17 may be another effective way other than that of CBFs to improve chilling tolerance across different species.

Various strategies can be used to engineer plants with drought tolerance by promoting vigorous root system, inducing stomatal closure, adjusting osmolytes, and minimizing oxidative damages (Yoshiba et al. 1997; Park et al. 2005; Huang et al. 2009; Chu et al. 2010). Ectopic expression of *AtGRXS17* in tomato also significantly enhances drought tolerance of tomato plants (Fig. 3.1), presumably by improvement of ROS scavenging capability in plant cells. This strategy has been proved to be an effective way to enhance drought tolerance. For example, ectopic expression of *PtADC* (*Poncirus trifoliata* Arginine decarboxylase) enhances drought tolerance in tobacco and tomato by reducing the accumulation of ROS (Wang et al. 2011).

In addition, expression of *AtGRXS17* could alter the endogenous drought responsive pathways, such as ABA pathway, and it may therefore lead to improved drought tolerance in tomato plants. As part of the drought responsive signaling pathways, the phytohormone ABA plays a key role in regulating gene expression and stomatal aperture. Members of the ABA-responsive element binding protein (AREB) family have been implicated as essential components in ABA signaling pathway. Furthermore, SlAREB1 plays important roles in drought stress tolerance and regulates abiotic stress-related genes in tomato (Orellana *et al*. 2010). In fact, dramatic up-regulation of *SlAREB1* under drought stress was also observed in this study (Fig. 3.2). Interestingly, the *SlAREB1* transcripts start to decrease in both wild-type and transgenic

lines after reaching its highest peak at 4 h, but the abundance of *SlAREB1* in *AtGRXS17*-expressing lines remains at a higher level than that of wild-type plants (Fig. 3.2), suggesting that there may be a tight interaction between AtGRXS17 and ABA signaling during drought stress. We speculate that AtGRXS17 may physically interact with a key enzyme in ABA signaling pathway, and possibly regulates maintaining abundance of *SlAREB1* to contribute, at least in part, to the drought tolerance in *AtGRXS17*-expressing tomato plants. Expression of some stress-responsive genes in the signal pathways is regulated at the posttranscriptional or posttranslational level (Yang et al., 2010). Therefore, we also speculate that AtGRXS17 may regulate *SlAREB1* at the posttranscriptional level, leading to drought tolerance by maintaining abundance of *SlAREB1*. However, further research must be conducted to address these hypotheses.

Our oxidative stress data provide direct evidence that AtGRXS17 relives the defective growth of primary roots correlated with increased accumulation of ROS (Fig. 3.7). ROS are known to be accumulated during various abiotic stresses including both cold and drought stress. ROS also cause damage to cell membranes and other cellular components including DNA, proteins, and carbohydrates (Moller et al. 2007; Jaspers and Kangasjarvi 2010). On the other hand, ROS are key regulators of growth, development and defense pathways (Mittler et al. 2004). Thus, the ROS and redox state should be tightly regulated by ROS-scavenging and ROS-producing system. GRXs are oxidoreductase enzymes that are capable to mediate reversible reduction of their substrate proteins in the presence of glutathione (GSH) (Garg et al. 2010), and thus maintain and regulate the cellular redox state and redox-dependent signaling pathways (Lillig et al. 2008). Therefore, introducing *AtGRXS17* into tomato plants may improve the adjustment capability of redox status of plant cells, and balance the damaging and signaling of ROS, resulting in enhanced tolerance to multiple abiotic stresses.

Catalase (CAT), an H_2O_2 scavenger, is one of the most important enzymes in ROS-scavenging system (Mittler et al. 2004). Overexpression of *CAT* in tobacco plants has been reported to significantly enhance tolerance to methyl viologen (Miyagawa et al. 2000). Our results demonstrate that ectopic expression of *AtGRXS17* in tomato more sharply induces the response of *SlCAT1* to oxidative stress compared to wild-type plants (Fig. 3.8). Accordingly, our previous study suggests that the activity of CAT is significantly increased in the *AtGRXS17*-expressing plants when compared to wild-type plants after heat treatment (Wu et al.,

2012). Therefore, it may be possible for AtGRXS17 to tightly regulate the CAT at the transcriptional and/or posttranscriptional level under oxidative stress condition. However, which one is the major regulation step remains unknown. Previous studies suggest many antioxidant genes in plant are not strongly induced in transcriptional level by ROS because they are quite strongly expressed even in non-stress conditions, in readiness for impending environmental fluctuations (Foyer and Noctor 2009). In addition, previous proteomic analysis has identified CAT as one of the Grx interaction proteins targeted by Poplar Grx C1 (Rouhier et al. 2005). Therefore, we speculate that the major regulation of CAT by GRXs may be at the protein level; however, further research is needed to address this question.

In this study, we demonstrate that ectopic expression of a member of the Grx family, AtGRXS17, in tomato enhances tolerance to multiple abiotic stresses. A possible working model how *AtGRXS17*-expressing tomato enhances tolerance to drought, chilling and oxidative stress is summarized (Fig. 3.9). Expression of *AtGRXS17* inhibits CBF responsiveness during chilling stress, but induces AREB responsiveness during drought stress. Both cold and drought stress trigger the accumulation of ROS, causing oxidative stress. AtGRXS17 protects plant cells from oxidative stress. This is partly mediated through up-regulating *CAT* expression. Due to the universal existent of GRXs in plant species, manipulation of GRXs across different species may be a useful approach to improve tolerance to abiotic stresses and understand the plant signaling under abiotic stress conditions in many agriculturally important crop species.

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Figures

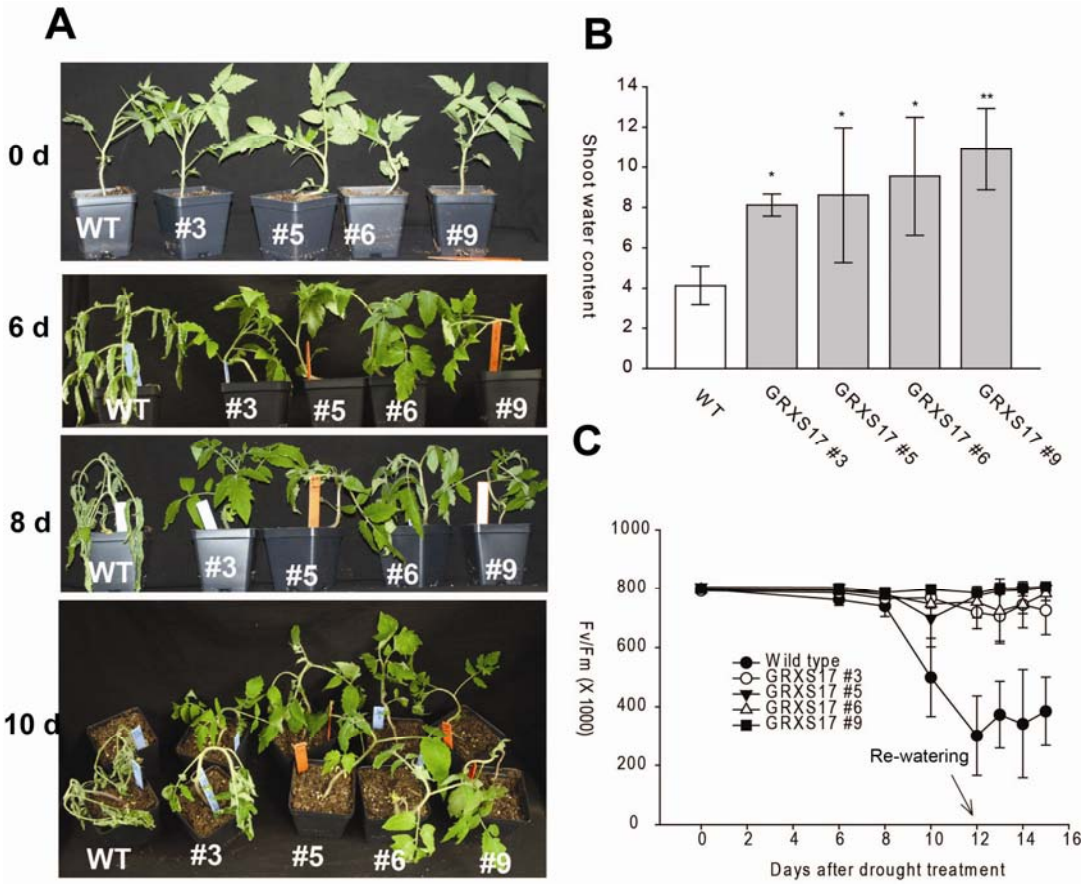

Figure 3.1 *AtGRXS17*-expressing tomato plants shown more resistant to drought stress compared to wild type plants.

(A) Morphology of *AtGRXS17*-expressing and wild-type plants before and after drought stress. (B) Shoot water content of *AtGRXS17*-expressing and wild-type plants after 10-d withholding water. (C) Chlorophyll fluorescence of *AtGRXS17*-expressing and wild-type plants during drought treatment. Data represent mean ± SD from three independent biological replicates (* $P < 0.05$, ** $P < 0.01$).

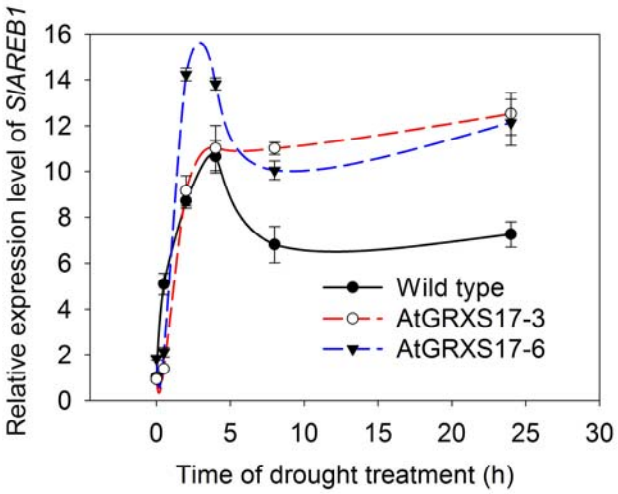

Figure 3.2 Dynamic changing of SlAREB1 transcripts affected by AtGRXS17 under drought stress.

Relative mRNA levels of *SlREB1* gene in 14-d-old *AtGRXS17*-expressing and wild-type tomato seedlings after being treated at 4 ^{o}C for 0, 0.5, 2, 4, 8, and 24 h, respectively. Data represent mean ± SD from three independent biological replicates.

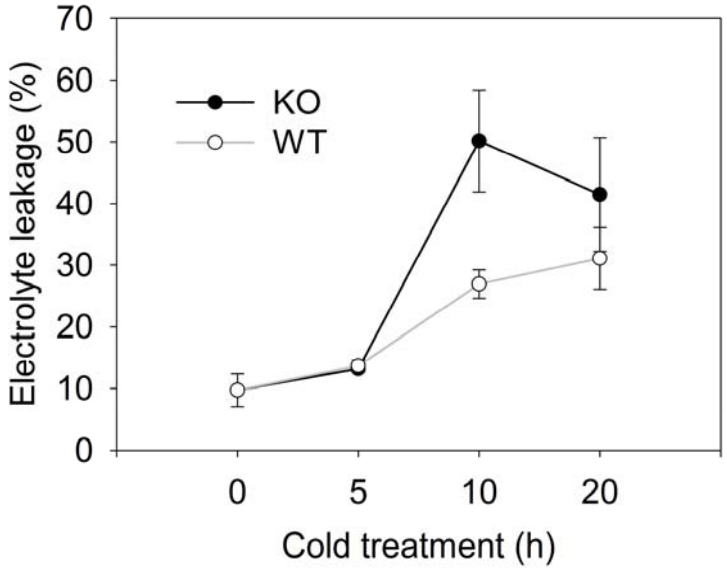

Figure 3.3 Electrolyte leakage of *Arabidopsis atgrxs17* KO and wild-type plants after cold treatment.

Twenty-day-old *Arabidopsis* seedlings grown at 22 ^{o}C were move to 4 ^{o}C. The leaves were sampled after 0-, 5-, 10-, and 20-h treatment, and electrolyte leakage was analyzed. Error bars

represent ±SD of 3 biological replicates.

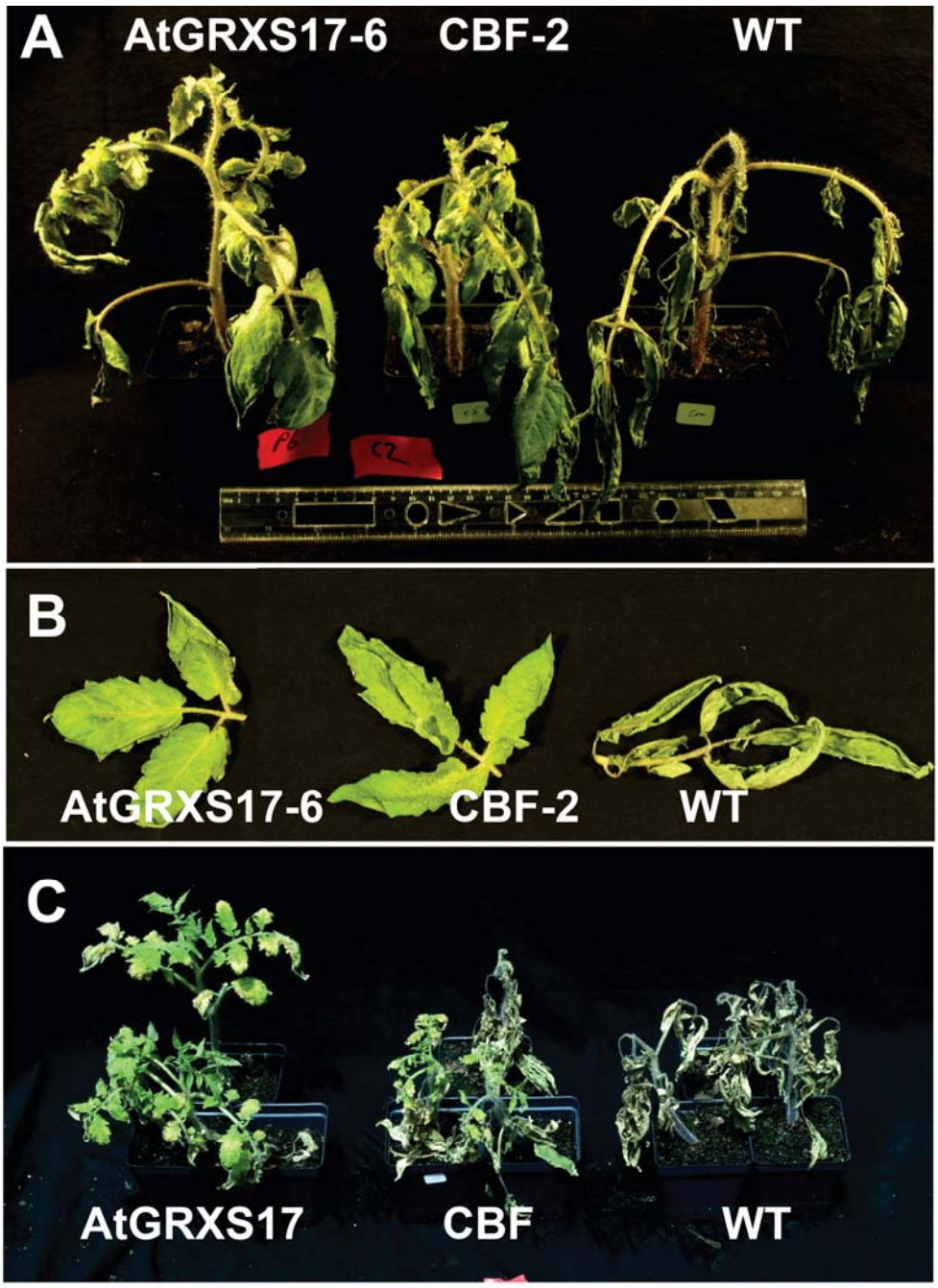

Figure 3.4 Phenotype of *AtGRXS17*-, *AtCBF3*- expressing and wild-type plants after cold treatment.

(A) Four-week-old *AtGRXS17*-expressing, *AtCBF3*-expressing or wild-type seedlings were being treated under 4 ^{o}C (day/night) for 3 more weeks. (B) *AtGRXS17*-, *AtCBF3*-expressing and wild-type tomato leaves after cold treatment. (C) Five-days recovery after 3-week-cold

treatment.

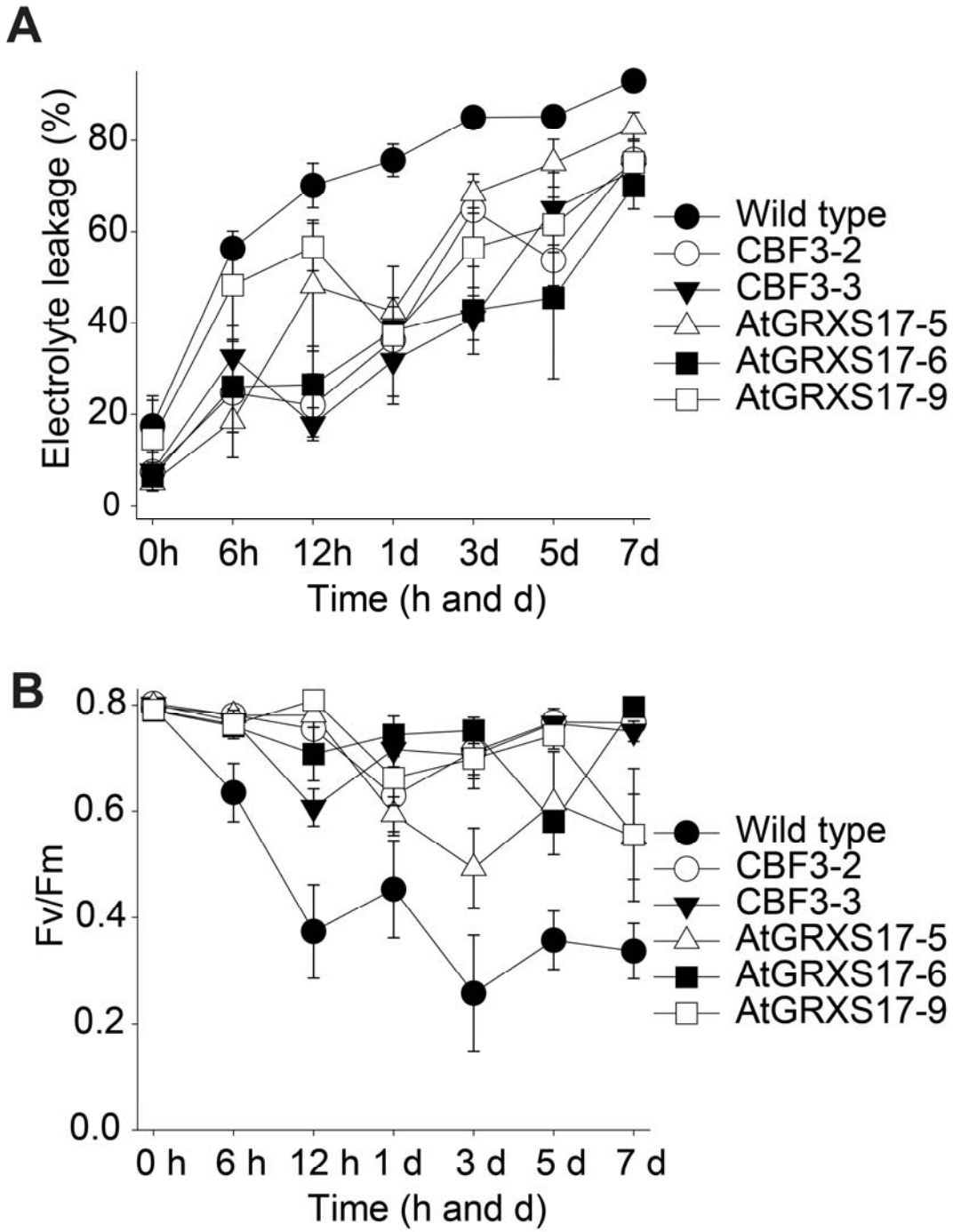

Figure 3.5 Electrolyte leakage and chlorophyll fluorescence.

(A) Electrolyte leakage of wild type, *AtCBF3*-, and *AtGRXS17*-expressing tomato plants during cold treatment. (B) Chlorophyll fluorescence of the bottom second leaves of wild type, *At*CBF3-, *AtGRXS17*-expressing tomato plants during cold treatment. The error bars indicates ±SD (n=3).

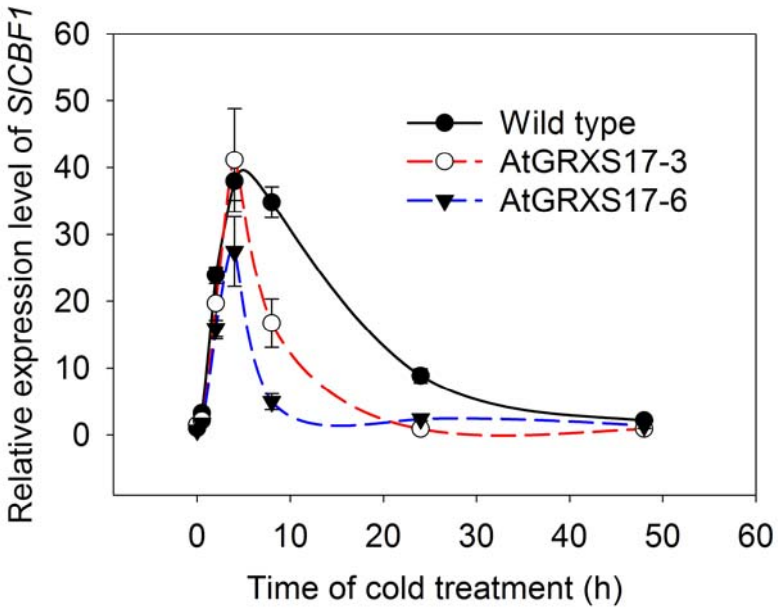

Figure 3.6 Dynamic changing of SlCBF1 transcripts affected by AtGRXS17 under cold stress.

Relative mRNA levels of *SlCBF1* gene in 14-d-old wild-type and *AtGRXS17*-expressing tomato seedlings after being treated at 4 ^{o}C for 0, 0.5, 2, 4, 8, 24, and 48 h, respectively. Data represent mean ± SD from three independent biological replicates.

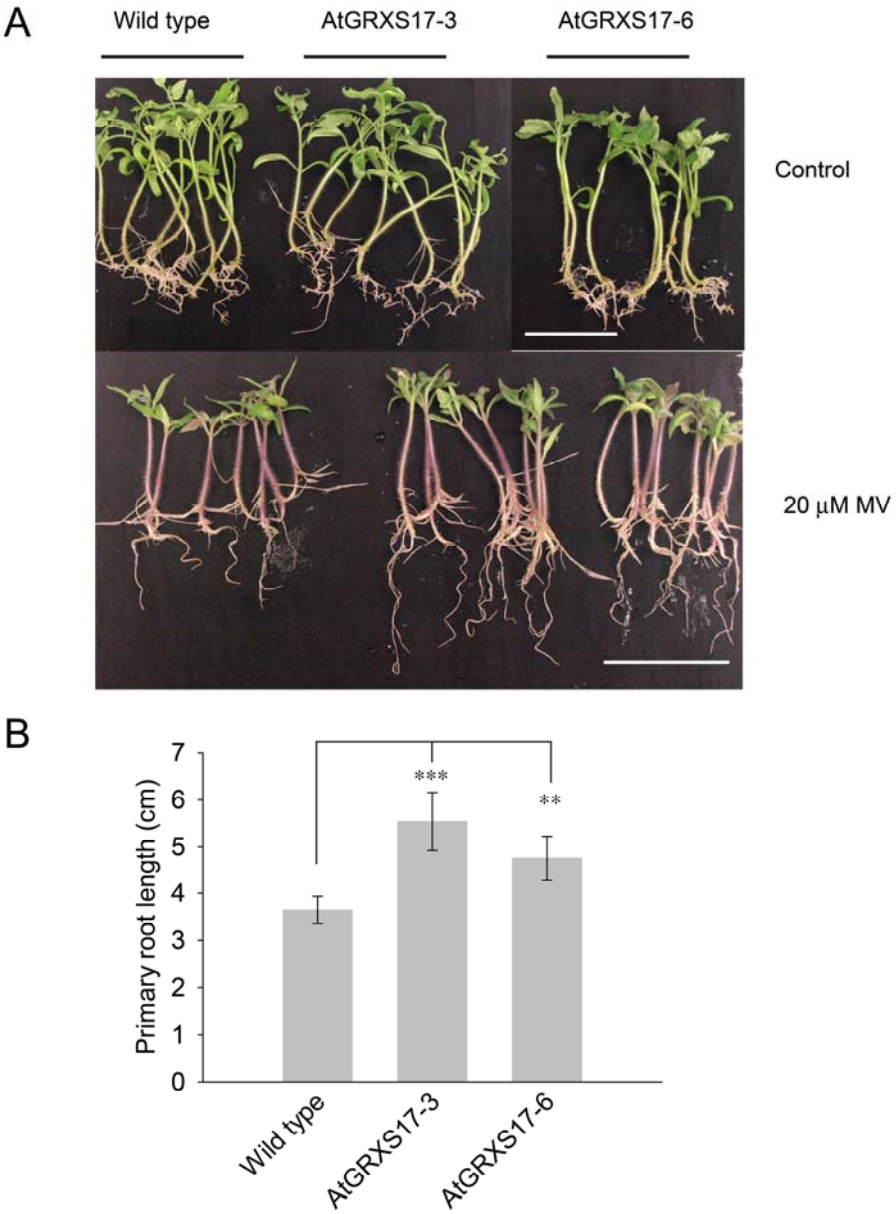

Figure 3.7 *AtGRXS17*-expressing tomato plants shown more resistant to oxidative stress compared with wild-type plants.

(A) Photos of *AtGRXS17*-expressing and wild-type tomato seedlings treated with oxidative herbicide. Seven-day-old *AtGRXS17*-expressing and wild-type tomato seedlings were transferred onto MS media with (lower panel) or without (upper panel) 20 μM MV and incubated for 14 d. Bars = 5 cm. (B) Root length of wild-type and *AtGRXS17*-expressing tomato seedlings that were

being treated by 20 μM MV. Data represent mean ± SD from eight independent biological replicates (** P < 0.01, *** P < 0.001).

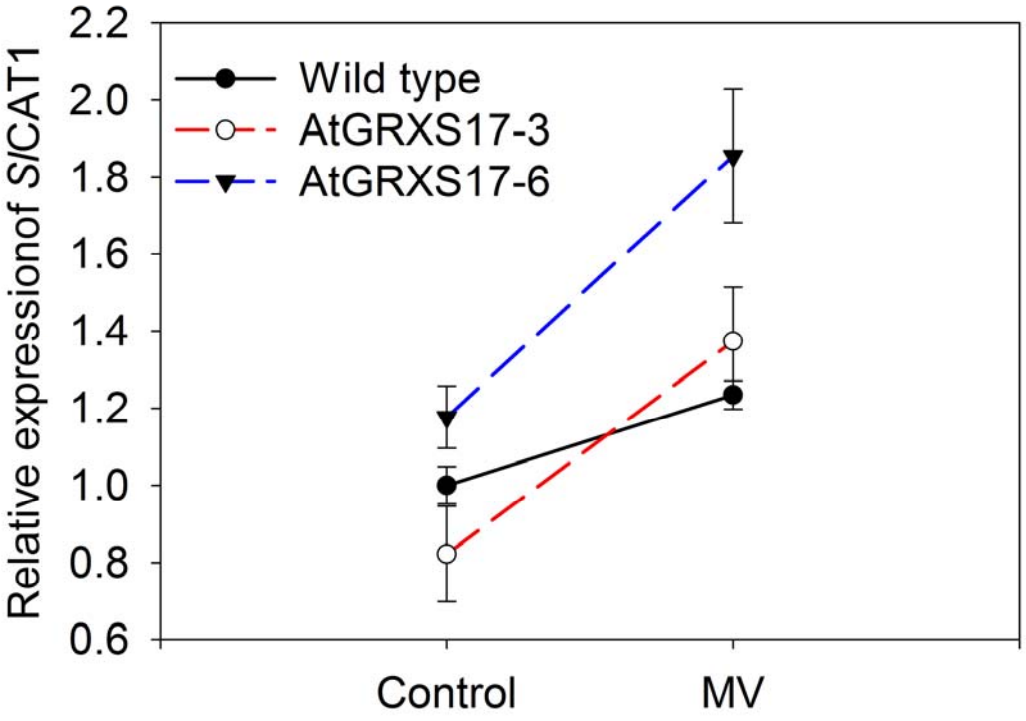

Figure 3.8 Relative mRNA levels of *SlCAT1* gene in leaf tissue of *AtGRXS17*-expressing and wild-type plants exposed to oxidative stress.

Data represent mean ± SD from three independent biological replicates.

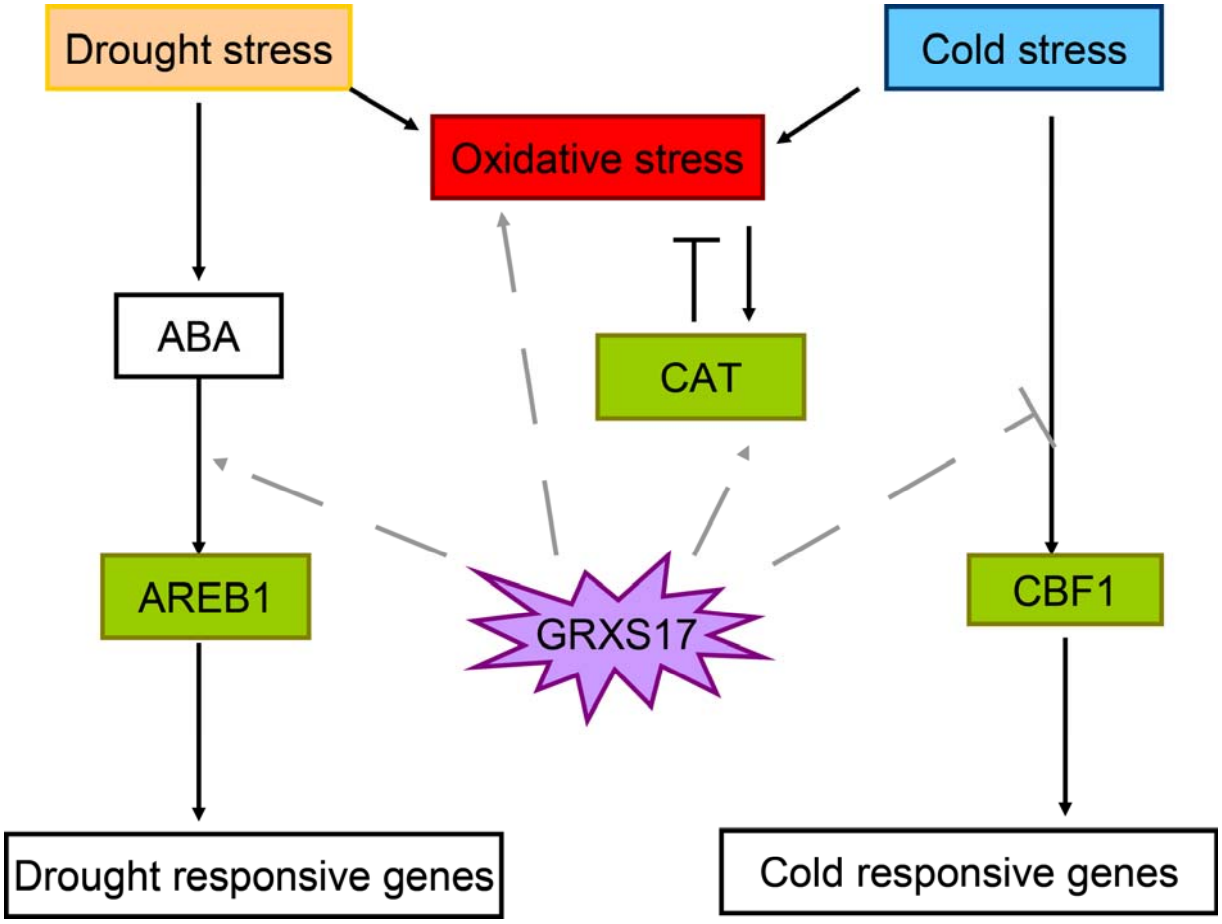

Figure 3.9 Model of AtGRXS17-mediated tolerance to cold, drought, and oxidative stresses.

AtGRXS17 expression inhibits CBF responsiveness during cold stress, but induces *AREB* responsiveness during drought stress. Both cold and drought stress trigger the accumulation of ROS, thus causing oxidative stress. AtGRXS17 protects plant cells from oxidative stress. This is partly mediated through up-regulating *CAT* expression.

Supporting Information

Table 3.S1 Primers used for PCR

Gene name	Accession #	Primer sequence
SlAREB1	AY530758	Forward: ACCAACAATCACAGCCACAG Reverse: TGCTCTTCCCAAGTCCATCT
SlCBF1	AY034473.1	Forward: GCTGGCAGGAAGAAGTTTCG Reverse: GAGTTGGAGGAAGCAGGGATAG
SlCAT1	M93719	Forward: ATTGCTGCTGGAAACTATCCTGAG Reverse: GGTCCAATACGGTGTCTCTGAGTA
SlPP2Acs (housekeeping gene)	AY325818	Forward: CGATGTGTGATCTCCTATGGTC Reverse: AAGCTGATGGGCTCTAGAAATC

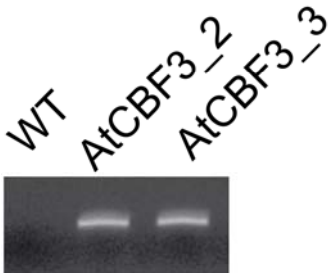

Figure 3.S1 The integration of *AtCBF3* into tomato genome has been confirmed by PCR using HYG primers.

HYG_F: 5’-GGCGACCTCGTATTGGGAATCC-3’; HYG_R: 5’-AAGTTCGACAGCGTCTCGGACC-3’

Figure 3.S2 The phenotype of wild-type and *AtCBF3*-expressing tomato plants under normal growth condition.

Chapter 4 - Ectopic expression of a maize calreticulin mitigates calcium deficiency like disorders in *sCAX*-expressing tobacco and tomato

Abstract

Deregulated expression of an *Arabidopsis* H^+/Ca^{2+} antiporter (sCAX1) in agricultural crops increases total calcium (Ca^{2+}) but may result in yield losses due to Ca^{2+} deficiency-like symptoms. Here we demonstrate that co-expression of a maize calreticulin (CRT, a Ca^{2+} binding protein located at endoplasmic reticulum) in *sCAX1*-expressing tobacco and tomato plants mitigated these adverse effects while maintaining enhanced Ca^{2+} content. Co-expression of *CRT* and *sCAX1* could alleviate the hypersensitivity to ion imbalance in tobacco plants. Furthermore, blossom-end rot (BER) in tomato may be linked to changes in CAX activity and enhanced *CRT* expression mitigated BER in *sCAX1* expressing lines. These findings suggest that co-expressing Ca^{2+} transporters and binding proteins at different intracellular compartments can alter the content and distribution of Ca^{2+} within the plant matrix.

Introduction

In vegetables and fruits, calcium (Ca^{2+}) deficiency is a critical factor reducing their quality and yield due to Ca^{2+}-related physiological disorders, such as blossom-end rot (BER) in tomato, pepper, eggplant and melon, tipburn in lettuce, celery and cabbage, and bitter pit in apple fruit (White & Broadley, 2003; Dayod *et al.*, 2010; de Freitas *et al.*, 2011). Moreover, low human dietary intake of Ca^{2+} has been associated with a disease, osteoporosis, which may lead to a bone fracture (Bachrach, 2001). Plant-based foods are excellent sources of dietary Ca^{2+}, and increased levels in vegetables and fruits may help ameliorate the incidence of osteoporosis caused by consumption of inadequate dietary Ca^{2+} (Hirschi, 2009; Park *et al.*, 2009). Therefore, better understanding of Ca^{2+} improvement in plant cells is required in order to positively impact human nutrition and high quality fruits and vegetables production.

Calcium is unique amongst the elements in plants and animals because it plays both a pivotal structural and an essential signaling role (White & Broadley, 2003; Hirschi, 2004). Consequently steep gradients for Ca^{2+} exist across cell membranes and cell endomembranes: the

plasma membrane (PM), tonoplast (TN), and the endoplasmic reticulum (ER). Gradients across these organelles are important for normal cellular function and for the regulation of metabolic processes that requires low but fluctuating levels of cytosolic Ca^{2+}. These gradients are established by a dynamic balance between influx and efflux of Ca^{2+} across each of the cellular membranes.

The concentration gradient of Ca^{2+} across the tonoplast is established partially by high-capacity H^+/Ca^{2+} antiporters (Zhao *et al.*, 2009). Among them, CAXs (Cation/H^+ exchangers), a group of high-capacity, low-affinity transporters that export cations out of the cytosol to maintain ion homeostasis across biological membranes (Pittman & Hirschi, 2003), have been physiologically characterized from a variety of plants. The first *Arabidopsis CAX* gene, *CAX1* was identified by its ability to suppress the Ca^{2+} sensitivity of a yeast mutant deleted in vacuolar Ca^{2+} transport (Hirschi *et al.*, 1996). CAX1 contains an additional 36 amino acid at the N-terminus that reduces the transport activity in both yeast and plant expression assays (Pittman & Hirschi, 2001; Mei *et al.*, 2007). When the N-terminal truncated version (sCAX1) is ectopically expressed in potato, carrot and lettuce, Ca^{2+} content in their edible tissues increases (Park *et al.*, 2005b; Park *et al.*, 2009). However, in some cases, these changes also produce deleterious phenotypes that impact yield (Hirschi, 1999; Park *et al.*, 2005a). Tempering expression of *sCAX1* results in healthier plants but they often accumulate less Ca^{2+} (Park *et al.*, 2005a).

Tobacco lines expressing *sCAX1* increase Ca^{2+} content in their tissues, but also display severe Ca^{2+} deficiency-like symptoms, such as leaf tip burning and sensitivity to ion imbalances (Hirschi, 1999). In addition, while the fruits of s*CAX1*-expressing tomato plants accumulate higher Ca^{2+} levels than vector control plants, the *sCAX1*-expressing tomatoes show increased incidence of distinct necrotic lesions in the distal portion of fruits, termed blossom-end rot (BER), which is presumed to be caused by aberrant Ca^{2+} homeostasis in fruit cells (Park *et al.*, 2005a). These symptoms are an obstacle for the development of Ca^{2+}-biofortified crops.

Our working hypothesis is that the increased expression of *sCAX1* in conjunction with Ca^{2+} binding proteins on another endomembrane may reduce these deleterious phenotypes by modulating cytosolic Ca^{2+} levels. Calreticulin (CRT), a Ca^{2+}-binding protein mainly resident in the ER, has been known as an effective Ca^{2+} buffer protein that may allow the transient storage of Ca^{2+} and play a role in stress responses (Jia *et al.*, 2009). Over-expression of a maize *CRT*

cDNA in tobacco suspension cells results in a two-fold increase in Ca^{2+} accumulation in the ER-enriched fraction *in vitro* (Persson *et al.*, 2001) and could improve growth of tobacco cell suspensions in high-Ca^{2+} medium (Akesson *et al.*, 2005).

Here, we express a maize *CRT* in *sCAX1*-expressing tobacco and tomato plants to test our hypothesis if the expression of *CRT* gene can mitigate Ca^{2+}-related cellular dysfunction resulted from expressing of *sCAX1* in tobacco and tomato plants while maintaining enhanced Ca^{2+} content. Our findings suggest that co-expressing transporters and binding-proteins may be a means of boosting plant nutrient content without adversely affecting yield. To our knowledge, this study represents the first attempts to increase the Ca^{2+} content of plants using co-expression of two genes that play important roles in the regulation of Ca^{2+}.

Materials and Methods

Bacterial strain and plasmid

The pCaMV::sCAX1 [*sCAX1* driven by the cauliflower mosaic virus (CaMV) 35S promoter] expression vector was previously constructed and described (Park *et al.*, 2005b) (Fig. 4.1a). The maize CRT (NCBI accession number: AF190454) open reading frame was cloned into the *Sac*I site of pE1775 binary vector (Lee *et al.*, 2007) (Fig. 4.1a), and the pE1775::CRT and pCaMV::sCAX1 were introduced into *Agrobacterium tumefaciens* strain LBA 4404 (Hoekema *et al.*, 1983) using the freeze-thaw method (Holsters *et al.*, 1978). The pE1775 expression vector contains a superpromoter, which consists of a trimer of the octopine synthase transcriptional activating element affixed to the *mannopine synthase2'* (*mas2'*) transcriptional activating element plus minimal promoter, and has been proved to be a strong promoter when being expressed in tobacco and maize (Lee *et al.*, 2007). 35SCaMV promoter was intentionally avoided to drive *CRT* gene because previous studies suggest that two transgenes driven by the same promoter might cause silencing of one or both genes (Park *et al.*, 1996).

Plant material, transformation, and growth conditions

Tobacco (*Nicotiana tabacum* L.) cultivar KY14 was used in this study. Tobacco transformation was performed via *Agrobacterium*-mediated leaf disk transformation method as previously described (Horsch *et al.*, 1985). Seeds were surface-sterilized and germinated on MS inorganic salt medium (Murashige & Skoog, 1962) with 30 g/l sucrose, pH 5.7, and solidified

using 8 g/l agar (PhytoTechnology, Shawnee Mission, KS, USA). Transformants were selected on standard MS media containing 100 μg/ml kanamycin for *sCAX1*-, 50 μg/ml hygromycin for *CRT*-, and 100 μg/ml kanamycin plus 50 μg/ml hygromycin for *sCAX1*- and *CRT*-co-expressing tobacco. Tobacco plants were grown in a greenhouse as previously described (Hirschi, 1999). For ion sensitivity analysis, surface-sterilized seeds were germinated in MS media. Ten days after plating, the seedlings were transferred to MS media supplemented with the appropriate ion. For the magnesium stress, 50 mM extra $MgCl_2$ was added to the standard MS media. For the potassium stress, 100 mM KCl was added to the standard MS media. To make media deficient in Ca^{2+}, we deleted the $CaCl_2$ from the standard MS media. The T1 and T2 tobacco plants were grown in the greenhouse under a 16-h photoperiod within a temperature range of 25 ^{o}C to 30 ^{o}C. Leaves from 2-month-old T2 generation tobacco plants were sampled for Ca^{2+} concentration analysis.

Tomato (*Solanum lycopersicum* cv. Rubion) transformation was performed via *Agrobacterium*-mediated transformation method using cotyledon and hypocotyls explants as previously described (Park *et al.*, 2003). *A. tumefaciens* LBA 4404 was used for generating stable transgenic plants. After inoculation with *A. tumefaciens*, the plant cultures were maintained at 25 ^{o}C under a 16-h photoperiod in MS medium. After 6 to 8 weeks, regenerated shoots were transferred to rooting medium for additional six weeks. The rooting medium was the MS agar medium that containing 0.1 mg/l IAA and 2 mg/L Zeatin. The temperature of the greenhouse was maintained within a range of 25 ^{o}C to 28 ^{o}C.

T2 generation of tomato plants were grown on pots containing Micracle Gro (700) growing media in the greenhouse. The temperature of the greenhouse was maintained with a range of 25 ^{o}C to 28 ^{o}C under a 16-h photoperiod. The tomato plants were regularly watered and fertilized on a weekly with 20: 20: 20 fertilizer (Scotts, Marysville, Ohio). We manually pollinated the flowers and marked the date of pollination. The number of healthy and BER fruits was counted and the BER ratio was examined. The fruits of 40-day after pollination (40 DAP) were harvested for Ca^{2+} content determination.

DNA isolation and Southern blot analysis

Genomic DNA of tobacco and tomato was isolated from 100 mg of fresh leaves using a DNeasy Plant Mini-Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's

instructions. DNA gel analysis was carried out as described previously (Park *et al.*, 2009). Genomic DNA (5-10 μg) was digested with *Xba*I, separated in a 0.9% (w/v) agarose gel by electrophoresis and blotted on to a nylon membrane (Zeta-Probe GT membrane, BioRad Laboratories, Hercules, CA, USA). The probe for the *sCAX1* gene was isolated by digesting pBluscript::sCAX1 (Park *et al.*, 2009). The membranes were pre-hybridized at 65°C in 7% sodium dodecylsulphate (SDS) and 0.25 M Na_2HPO_4 for 3 hours, and then hybridized overnight at 65°C in the same solution containing the probe labeled by NEBlot Phototope Kit (New England Biolabs). Membrane were washed twice for 40 min each with 20 mM Na_2HPO_4 and 5% SDS at 65 ^oC and then washed twice again for 30 min each with 20 mM Na_2HPO_4 and 1% SDS at 65 ^oC. The signal was detected using a Phototope-Star Detection Kit (New England Biolabs).

RNA isolation, RT-PCR, and RNA gel blot analysis

Total RNA of tobacco and tomato was extracted from leaves using an RNeasy Plant Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. RNA for RT-PCR was treated with RNase-free DNase prior to the synthesis of first-strand cDNA by oligo (dT) priming using moloney murine leukemia virus-reverse transcriptase (BD Biosciences Clontech, Palo Alto, CA, USA). One microliter of the reverse transcription reaction solution was used as a template in a 25 μl PCR solution. Total RNA (7 μg) was separated on a 1.2% agarose gel containing 1.5% formaldehyde, and blotted on to a Zeta-Probe GT membrane according to the manufacturer's instructions. Hybridization and washing were performed as described previously in DNA gel blot analyses (Park *et al.*, 2009).

Ca^{2+} and other mineral analysis

The tobacco leaves and tomato fruits were dried at 70 ^oC for 4 d. A total of 0.5 g (dry weight) of fruits was digested for analysis as described (Park *et al.*, 2005a). Calcium content per gram of dry weight were determined by inductively coupled plasma emission spectrophotometer (Spectro, Kleve, Germany).

Results

Generation of sCAX1-, CRT-, and sCAX1- and CRT-co-expressing tobacco and tomato plants

The temporal and spatial regulation of s*CAX1* is crucial for proper modulation of Ca^{2+} level in plants (Park *et al.*, 2005a). The 35S promoter confers strong constitutive expression in plants, and is often used to give high level expression of a given gene (Benfey *et al.*, 1990). In previous studies, various *sCAX1*-expressing lines under the control of the 35S promoter showed symptoms similar to Ca^{2+} deficiency (Hirschi, 1999; Park *et al.*, 2005a), and this promoter may therefore be used effectively to identify the capacity to regulate Ca^{2+}-related cellular dysfunction in *sCAX1*-expressing plants through manipulation of CRT. Initially 18 *sCAX1*- and 20 *CRT*-expressing lines were generated, respectively, and then we co-transformed *CRT* into two independent *sCAX1*-expressing T2 homozygous tobacco lines (sCAX1-1 and sCAX1-2) that showed continuously severe Ca^{2+} deficiency-like symptoms (Fig. 4.2a,b). The stable integration of the *35S::sCAX1* chimera in the genome of tobacco plants that were used for *CRT* co-transformation was confirmed by Southern blot analysis (Fig. 4.1b). The line we termed sCAX1-2 appeared to contain a single copy insertion, while line sCAX1-1 and sCAX1-5 had more than one integration event (Fig. 4.1b). Twenty independent *sCAX1*- and *CRT*-co-expressing tobacco lines (hereafter as *sCAX1+CRT*) were generated by *CRT* co-transformation. Expression of *CAX1* and *CRT* transcripts were measured in T1 transgenic lines by RNA gel blot analysis. The results suggest that *sCAX1* and *CRT* transcripts were expressed only in the sCAX1 and CRT transgenic lines, respectively; while both *sCAX1* and *CRT* transcripts accumulated in the sCAX1+CRT-2, -3, and -13 transgenic lines (Fig. 4.1c).

Previous tomato studies demonstrate that *sCAX1* expression also causes apical burning and the development of distinct necrotic lesions in the distal portion of fruits (BER). Thus, we were interested in determining whether co-expression of *CRT* in *sCAX1*-expressing tomato plants would alleviate the symptoms. Initially 24 *sCAX1*- and 15 *CRT*- expressing lines were generated, respectively, and then we co-transformed *CRT* into a *sCAX1*-expressing - 13 (a single-copy insertion) T2 homozygous tomato line that showed severe Ca^{2+} deficiency-like symptoms including BER (data not shown). Twelve independent *sCAX1+CRT*-expressing tomato lines were generated. Two of each *sCAX1*-2 and 13, *CRT*-9 and 21, and *sCAX1+CRT*-4 and 5 expressing

transgenic lines were randomly selected and confirmed by Southern blot and PCR analysis (Fig. 4.1d,e).

The stable integration of the *CRT* in the genome was confirmed by Southern blot (Fig. 4.1d). We found a background band in every line, including wild-type, which might be caused by the endogenous *CRT* in the tomato genome. The Southern-blot result suggests the CRT-21, CAX1+CRT-4, and CAX1+CRT-5 lines contained a single-copy of CRT, while CRT-9 line contained 3 copies of CRT. The integration of *sCAX1* in genome was confirmed by PCR using *CAX1* primers (Table 4.1, Fig. 4.1e). The expression of *CRT* and *sCAX1* was confirmed by RT-PCR using *CRT* and *CAX1* primers, respectively (Fig. 4.1f,g, Table 4.1). All the molecular experiments were conducted using the T2 generation plants.

CRT suppresses sCAX1-induced Ca^{2+} deficiency-like symptoms of tobacco and tomato plants

As shown previously (Hirschi, 1999), *sCAX1*-expressing tobacco lines have altered morphology and growth characteristics. All the *sCAX1*-expressing lines displayed necrosis on the tips of the new leaves from a young stage, which is a Ca^{2+} deficiency-like symptom (Fig. 4.2c). In addition to the necrosis, all the *sCAX1*-expressing tobacco plants showed severe stunting (Fig. 3a, bottom). In contrast, after introducing the *CRT* into *sCAX1*-expressing tobacco plants, the symptoms were alleviated (Fig. 4.2f and 4.3a, top).

To establish that the growth phenotypes were due to co-expression of the *CRT*, 40-45 each of *sCAX1+CRT*-expressing T2 generation plants from 5 independent lines (sCAX1+CRT-2, -3, -6, -13, and -27) were analyzed to determine if *CRT* segregated with the robust growth phenotype. As shown in Figures 34.b (right) and 4.3c, all the *CRT*-co-expressing lines were healthy while the loss of *CRT* caused the reappearance of the symptoms associated with *sCAX1*-expression [Fig. 4.3b (left) and 4.3c]. This result suggests that CRT contributes to the recovering of *sCAX1*-expressing tobacco plants with Ca^{2+} deficiency-like symptoms.

To determine how the expression of *sCAX1*, *CRT* and *sCAX1+CRT* alters Ca^{2+} concentration in the cells, we measured the total accumulation of Ca^{2+} in the tobacco leaves in T2 generation transgenic plants. As shown in Fig. 4.3d, *sCAX1*- and *sCAX1+CRT*-expressing tobacco plants accumulated significantly more (up to 25%) Ca^{2+} than wild-type plants; however, *CRT*-expressing tobacco plants did not significantly enhance Ca^{2+} accumulation as compared

with wild-type plants. In addition, expression of *sCAX1*, *CRT* or *sCAX1+CRT* did not affect the accumulation of other minerals (Cu^{2+}, Fe^{2+}, Mg^{2+}, Mn^{2}+, and Zn^{2+}, data not shown).

In order to ascertain whether CRT can suppress *sCAX1*-induced adverse symptoms in tomato plants, we introduced *CRT* into *sCAX1*-expressing tomato plants. As shown in Fig. 4.4a, the necrosis in leaf tips caused by *sCAX1*-expressing was alleviated by the co-expression of *CRT*. Furthermore, when we counted the number of the BER and healthy fruits of wild-type, *sCAX1*-, *CRT*-, and *sCAX1+CRT*-expressing T2 generation transgenic plants, respectively, the results showed that the BER ratio could be reduced by introducing *CRT* to the *sCAX1*-expressing plants. Although the ratio of BER in *sCAX1+CRT*-expressing plants was not statistically different from that of *sCAX1*-expressing plants, because the BER ratio shows a large variation among different plants even in the same line, the BER symptom in *sCAX1+CRT*-expressing plants was indeed less severe than that in *sCAX1*-expressing plants according to our day-to-day observation (Fig. 4.4b and c, and data not shown).

To determine how the co-expression of *CRT* in s*CAX1*-expressing tomato alters Ca^{2+} concentration in the fruit cells, the total accumulation of Ca^{2+} in the tomato fruits of wild-type, *sCAX1*-, *CRT*-, and *sCAX1+CRT*-expressing T2 generation plants was analyzed. All the *sCAX1*- and *sCAX1+CRT*-expressing tomatoes showed significantly higher Ca^{2+} content than wild-type tomatoes (Fig. 4.4d). However, among 15 *CRT*- expressing tomato lines, the majority of these lines did not significantly enhance Ca^{2+} content as compared to wild-type tomatoes while the fruits of line #9 and #21 increased ~ 9% and ~ 40% more Ca^{2+} than wild-type fruits, respectively (Fig. 4.4d).

CRT suppresses sCAX1-induced ion sensitivity in tobacco lines under ion imbalance growth condition

We further tested whether introducing CRT could mitigate the ion sensitivity caused by sCAX1. After growing lines on standard MS media for 14 d, wild-type and transgenic seedlings (sCAX1-1 and -2; CRT-1; sCAX1+CRT-2, -3, -6, -13, and -27) were transferred to media containing increased levels of Mg^{2+} or K^{+}, or reduced Ca^{2+}. All the *sCAX1*-expressing seedlings were sensitive to the ion imbalance that failed to perturb the growth of wild-type and *sCAX1+CRT*-expressing plants. For example, after being transferred in the Ca^{2+}-depleted media, the *sCAX1*-expressing seedlings could not grow and develop leaves (Fig. 4.5a). In contrast, the

sCAX1+CRT-expressing seedlings grew vigorously without any abnormal morphological developments (Fig. 4.5a). In the medium containing 50 mM $MgCl_2$, the *sCAX1*-expressing seedlings also showed hypersensitivity to the stress, such as necrotic lesions in the young leaves and stunted growth (Fig. 4.5b); however, the *sCAX1+CRT*-expressing seedlings did not display any adverse growth (Fig. 4.5b). The sensitivity of *sCAX1*-expressing tobacco to K^+ salt stress was not as severe as the Ca^{2+} or Mg^{2+} growth phenotypes. However, after transferring the seedlings to the media containing 100 mM KCl for 60 days, the necrotic lesions displayed on the *sCAX1*-expressing leaf tips, but not on the leaves of *sCAX1+CRT*-expressing plants (Fig. 4.5c).

Discussion

Conventional breeding strategies for mineral biofortification of crops rely on germplasm with limited genetic variation for many traits (White and Broadley, 2009). In some cases, genetic diversity may be increased by crossing to distant related species and movement of the traits slowly into the agronomically useful cultivars. However, the variation in a trait, in particular Ca^{2+} concentration, may not cover the range desired for agronomic value. Thus, breeders may not have the appropriate level of genetic variation in Ca^{2+} concentration among varieties. Our genetic engineering approach allows over-expression of Ca^{2+} transporter genes and expression to a level not present in germplasm. However, a major impediment for the development of Ca^{2+}-biofortified crops using Ca^{2+} transporters is that the transgenic lines expressing *sCAX1* dramatically increase Ca^{2+} content in their tissues, but also display severe Ca^{2+} deficiency-like symptoms, leading to significant yield losses. Previous studies in *Arabidopsis* suggest that CRT plays a key role in the regulation of Ca^{2+} status of the plant ER and that the ER, in addition to the vacuole, is an important Ca^{2+} store in plant cells (Persson *et al*. 2001). In fact, *Arabidopsis* plants overexpressing a version of CRT contained up to 35% more total Ca^{2+}, and the increased Ca^{2+} sequestered by the CRT appeared to benefit plants when grown in a Ca^{2+} deficient situation (Wyatt *et al*. 2002). Results from these studies also suggest that the CRT-mediated alteration of the ER Ca^{2+} pool could potentially make Ca^{2+} more readily accessible for release into the cytosol and further strengthens the notion that the increased Ca^{2+}-buffering capacity generated by overproduction of CRT helps maintain Ca^{2+} homeostasis. Indeed, co-expression of a maize *CRT* mitigates the Ca^{2+} deficiency-like symptoms including tip burning and BER (Figs. 4.2, 4.3, and 4.4) and the hypersensitivity to ion imbalance (Fig. 4.5) caused by expression of *sCAX1* in

tobacco and tomato plants. Although *CRT* expression alone was not sufficient to dramatically alter the Ca^{2+} content and incidence of BER in this study, our results here suggest that combining expression of transporters and binding proteins may be a strategy to boost Ca^{2+} levels without negatively impacting plant growth and development.

Most mature plant cells have a central vacuole, which often takes up more than 80% of the cell volume (Martinoia *et al.*, 2000). The vacuole is considered to be the largest intracellular storage compartment for Ca^{2+} (Gelli & Blumwald, 1993), and fluxes of Ca^{2+} across the vacuole are similar in magnitude to those occurring across the plasma membrane. The plant ER, like the vacuole, is thought to function as a substantial Ca^{2+} store (Iwano *et al.*, 2009). In animals, total Ca^{2+} levels can approach micromolar concentrations in the mammalian sarcoplasmic reticulum (SR) (Zucchi & RoncaTestoni, 1997). Measurements of Ca^{2+} efflux from plant ER vesicles indicate that there is rapid exchange of Ca^{2+} across the ER (White & Broadley, 2003). Our data suggest that increased expression of Ca^{2+} binding proteins on the ER can ameliorate the effects caused by increasing sequestration of Ca^{2+} into the vacuoles. Recent technological advances should enable future studies to make a detailed analysis of Ca^{2+} dynamics in different cellular compartments to decipher the temporal and spatial characteristics of Ca^{2+} signatures caused by altered *sCAX1* and *CRT* expression (Krebs *et al.*, 2012).

In Arabidopsis mutants where CAX activity is greatly reduced, the lines show 3-fold more apoplastic Ca^{2+} (Conn *et al.*, 2011). On the other hand, when *sCAX1* expression is increased in tomato plants, apoplastic levels of Ca^{2+} are reduced (de Freitas *et al.*, 2011). Depleting the apoplastic Ca^{2+} pool by expression of *sCAX1* may cause the Ca^{2+} deficiency-like symptoms. One of the important functions of apoplastic Ca^{2+} is cross-linking the homogalacturonans for the biosynthesis of cell wall (Cosgrove, 2005). Thus, reducing the apoplastic Ca^{2+} concentration in *sCAX1*-expressing plants may disrupt the cell wall biosynthesis and further results in growth stunting, tip burning and BER, especially in the tissues that the cell division and wall formation are most rapid (Figs. 4.2, 4.3 and 4.4). Furthermore, recent studies show that suppressing expression of *pectin methylesterases (PMEs)* in tomato fruit reduces the amount of Ca^{2+} bound to the cell wall, subsequently increasing Ca^{2+} available for other cellular functions and, thereby, reducing fruit susceptibility to BER(de Freitas *et al.*, 2012). Therefore, future research may focus on elucidating the effects of co-expression of *CRT* and *sCAX1* on the distribution/partitioning of symplastic and apoplastic Ca^{2+}.

Ca^{2+} disorders, likely involving altered CAX activity, may be responsible for losses in crop production (Ho & White, 2005). These putative Ca^{2+} disorders have been thought to develop similarly (White & Broadley, 2003) and to be associated with a Ca^{2+} deficiency within the cells (Saure, 2001). Recent work suggests that altered CAX activity may modify defense related signaling in barley (Zhang *et al.*, 2009). BER in tomato and bitter pit in apples may also be linked to changes in CAX activity (Park *et al.*, 2005a; de Freitas *et al.*, 2010). To explain the primary causes of BER, two hypotheses have been considered, 1) Ca^{2+} deficiency and 2) aberrant Ca^{2+} homeostasis. The majority of studies on BER in recent years have proposed that Ca^{2+} imbalance events at the cellular level, triggered by environmental stresses, may result in aberrant intracellular Ca^{2+} signals, ultimately leading to BER. It is suggested that this phenomenon may be a consequence of aberrant cytosolic Ca^{2+} regulation, and therefore spatial and temporal control of cellular Ca^{2+} concentration is a key factor determining incidence of Ca^{2+}-related physiological disorders (Hirschi, 2004; Ho & White, 2005; Park *et al.*, 2005a; Karley & White, 2009; White & Broadley, 2009; Dayod *et al.*, 2010; de Freitas *et al.*, 2011). Regardless of mechanisms, our work here shows that elevated expression of *CRT* can reduce the severity of growth abnormalities caused by increased CAX activity.

Utilization of the *sCAX1* for Ca^{2+} biofortification have been extensively investigated in various horticultural crop species (carrot, potato, tomato, lettuce) since the expression of *sCAX1* can dramatically improve the Ca^{2+} accumulation in their edible tissues (Hirschi, 1999; Park *et al.*, 2004; Park *et al.*, 2005a; Park *et al.*, 2005b; Park *et al.*, 2008; Park *et al.*, 2009). Interestingly, not all the increased Ca^{2+} in the transporter-modified carrots was bioavailable (Morris *et al.*, 2008). This may be due to a fraction of the extra Ca^{2+} being bound to antinutrients within the carrot. This serves as a cautionary example for scientists who assume that all increases in nutrient content directly equate to increased bioavailability. However, the modified carrots are a better source of Ca^{2+} because total Ca^{2+} absorbed was higher. Although we postulate that the Ca^{2+} content has increased within the vacuoles of the modified carrots, we have not as of yet experimentally addressed the intracellular Ca^{2+} redistribution in these plants. We postulate that co-expressing various transporters and *CRTs* will differentially increase total Ca^{2+} content and the fractional absorption of Ca^{2+} in animals. However, feeding studies must be conducted to address the bioavailability issues in the double transformants, including the CRT+sCAX1 transformed crops.

Our working hypothesis is that the Ca^{2+} content within these double transgenic plants is more evenly distributed throughout the plant cells. However, in order to decode the relationship between expression of transporters and binding proteins and location of Ca^{2+} within the cell, we must determine the spatial resolution of Ca^{2+} within the plant (Punshon *et al.*, 2009; Conn *et al.*, 2011; Punshon *et al.*, 2012). Various techniques exist to visualize the distribution and abundance of elements within plants. These techniques are useful because, in contrast with bulk or volume-averaged measures (such as inductively coupled plasma mass spectroscopy, ICP-MS) where the sample is homogenized, the confinement of elements within specific plant organs, tissues, cells and even organelles can be seen. The potential of synchrotron x-rays in spatially resolved elemental imaging in plants has begun to be realized (Punshon *et al.*, 2009). In fact, this work has recently been done to demonstrate the alterations of Ca^{2+} partitioning in seeds of Arabidopsis lines altered in CAX expression (Punshon *et al.*, 2012), it will certainly be interesting to apply this technology to the edible portions of crops co-expressing both *sCAX1* and *CRT*.

In conclusion, while genetic engineering strategies to increase Ca^{2+} content by expression of a single gene (either *sCAX1* or *CRT*) alone have provided promising results, co-expressing of *CRT* and *sCAX1* enhances the Ca^{2+} content of plants without any apparent detrimental effects potentially caused by sCAX1 expression. Manipulation of the partitioning of nutrients across various endomembranes may be a means to increase plant nutrient content while maintaining crop productivity.

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Figures and Tables

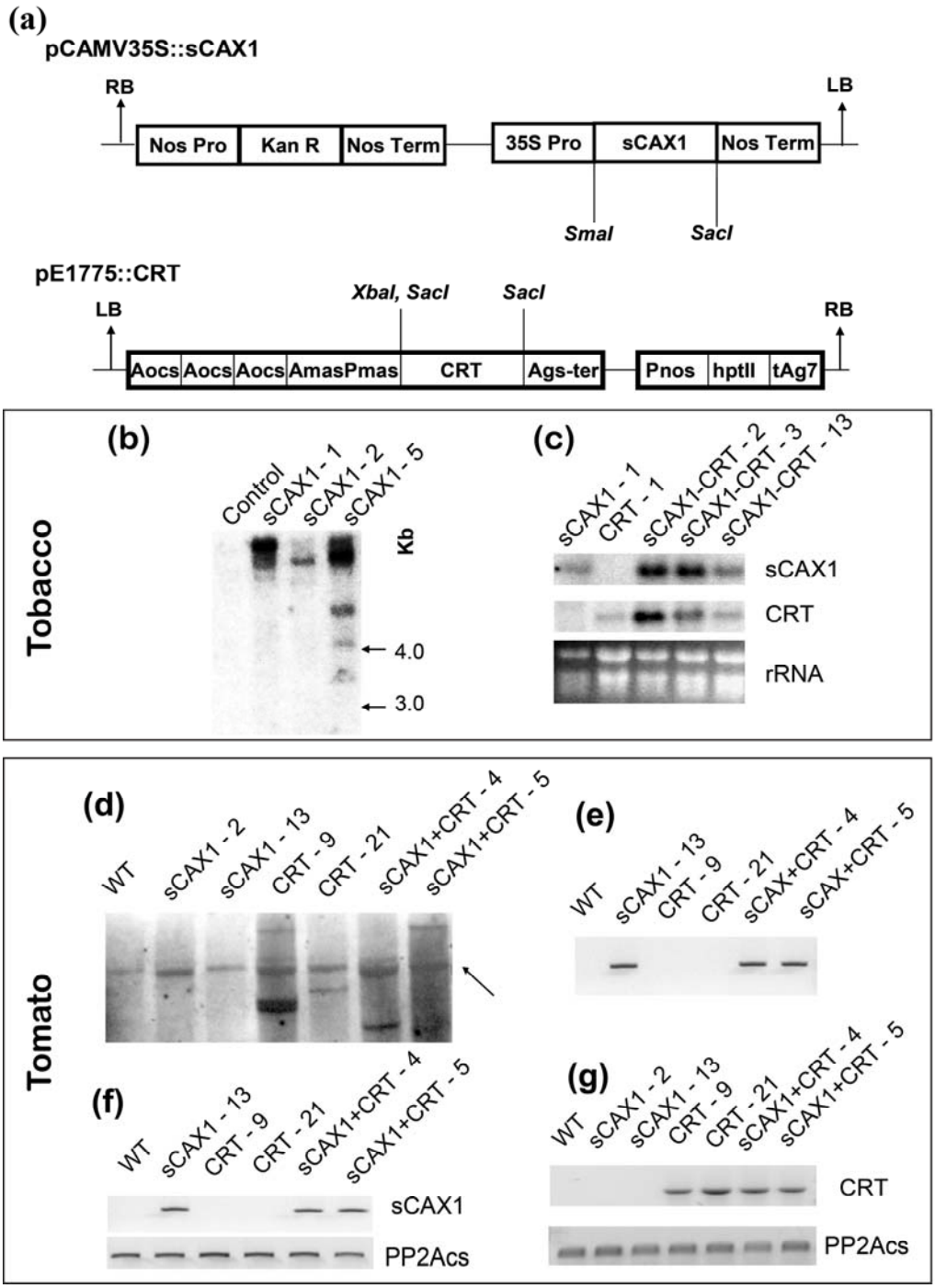

Figure 4.1 Molecular analyses of *sCAX1-*, *CRT*- and *sCAX1+CRT*-expressing tobacco and tomato plants.

(a) T-DNA regions of pCaMV35S::sCAX1 and pE1775::CRT. RB, Right border; LB, left border; Nos-pro, nopaline synthase promoter, Kan R, the gene conferring resistance to kanamycin, neomycin phosphotransferase (NPTII); Nos-ter, nopaline synthase terminator. 35S pro, CaMV 35S promoter; sCAX1, short cut cation exchanger 1 coding region; Aos, octopine synthase transcriptional activating element; AmasPmas, mannopine synthase 2' activating and promoter elements; CRT, maize calreticulin coding region; ags-ter, polyA addition signal from the agropine synthase gene. hptII, gene conferring resistance to hygromycin; Pnos, mopaline synthase promoter; tAg7, poly A addition signal for T-DNA gene 7. (b) Southern-blot analysis of transgenic tobacco plants. Ten micrograms of tobacco genomic DNA were digested with *SacI*, and hybridized with the *sCAX1* probe. (c) Northern-blot analysis of transgenic tobacco plants. Ten micrograms of total RNA from leaves were hybridized with *sCAX1* and *CRT* probe, respectively. Ethidium bromide-strained rRNA (bottom) is shown as a loading control. (d) Southern-blot analysis of transgenic tomato plants with CRT probe. Ten micrograms of tomato genomic DNA were digested with *XbaI*, and hybridized with *CRT* probe. The arrow indicates the endogenous tomato *CRT* gene that was detected by maize *CRT* probe. (e) PCR detection of *sCAX1* in genomic level. (f) RT-PCR detection of the expression of *CRT*. (g) RT-PCR detection of the expression of *sCAX1*. *SlPP2Acs* was used as tomato housekeeping gene.

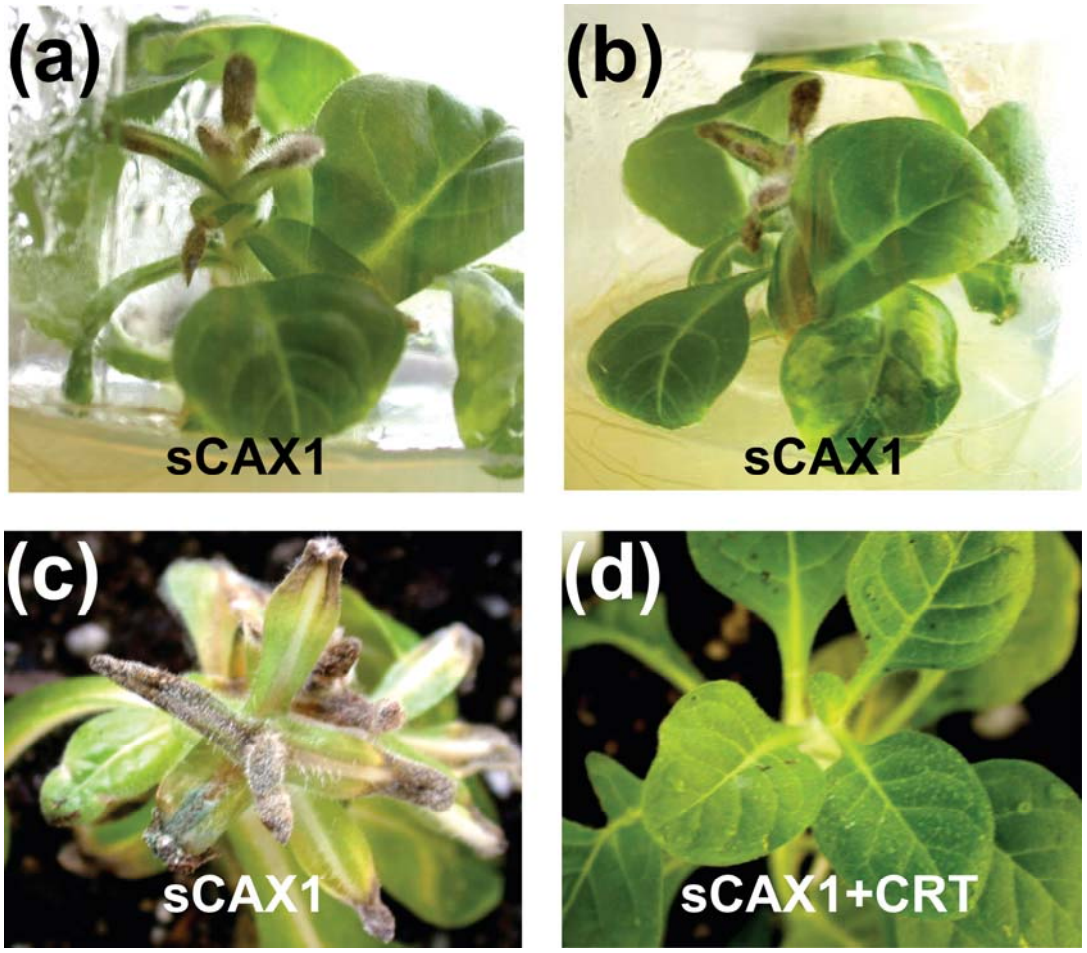

Figure 4.2 Morphology of sCAX1-, and sCAX1+CRT-expressing tobacco plants at young stage.

(a-b) the *sCAX1*-expressing tobacco plants used for *CRT* transformation. (c) the morphology of *sCAX1*-expressing tobacco seedlings. (d) the morphology of *sCAX1+CRT*-expressing tobacco seedlings.

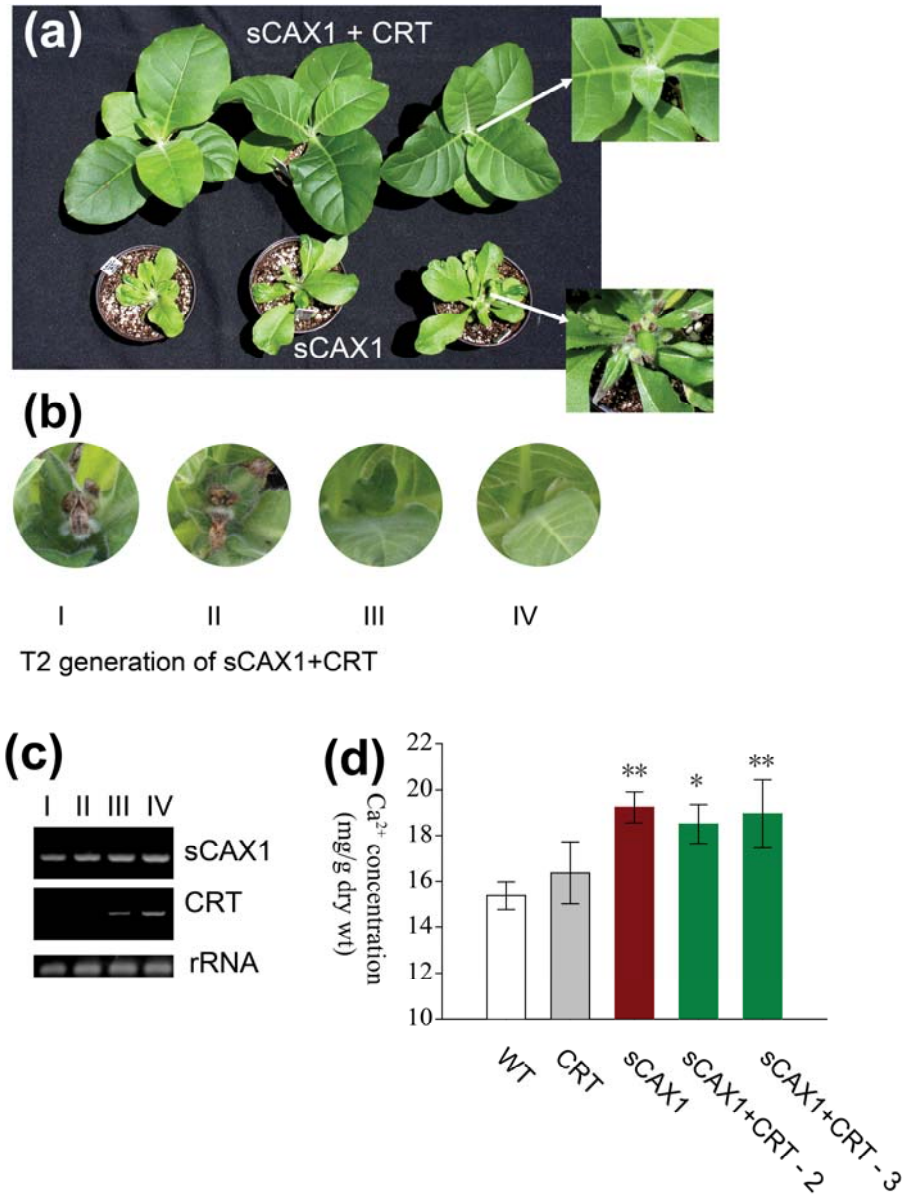

Figure 4.3 Segregation of the Ca^{2+}deficiency-like symptoms.

(a) Morphology of T1 generation of *sCAX1*-, and *sCAX1+CRT*-expressing tobacco plants. (b) Segregation of the morphology in T2 generation of *sCAX1+CRT*-expressing plants. Some of the plants maintained the normal morphology, but some returned to the Ca^{2+}deficiency-like symptoms. (c) detection of the expression of *sCAX1* and *CRT* in T2 generation *sCAX1+CRT*-expressing plants by RT-PCR. (d) Ca^{2+} concentration of T2 generation tobacco leaves of different lines. All results shown here are the means of 3 biological replicates, and the error bars indicate the standard deviations (S.D. n=3) (Student t test, * $p<0.05$, ** $p<0.01$).

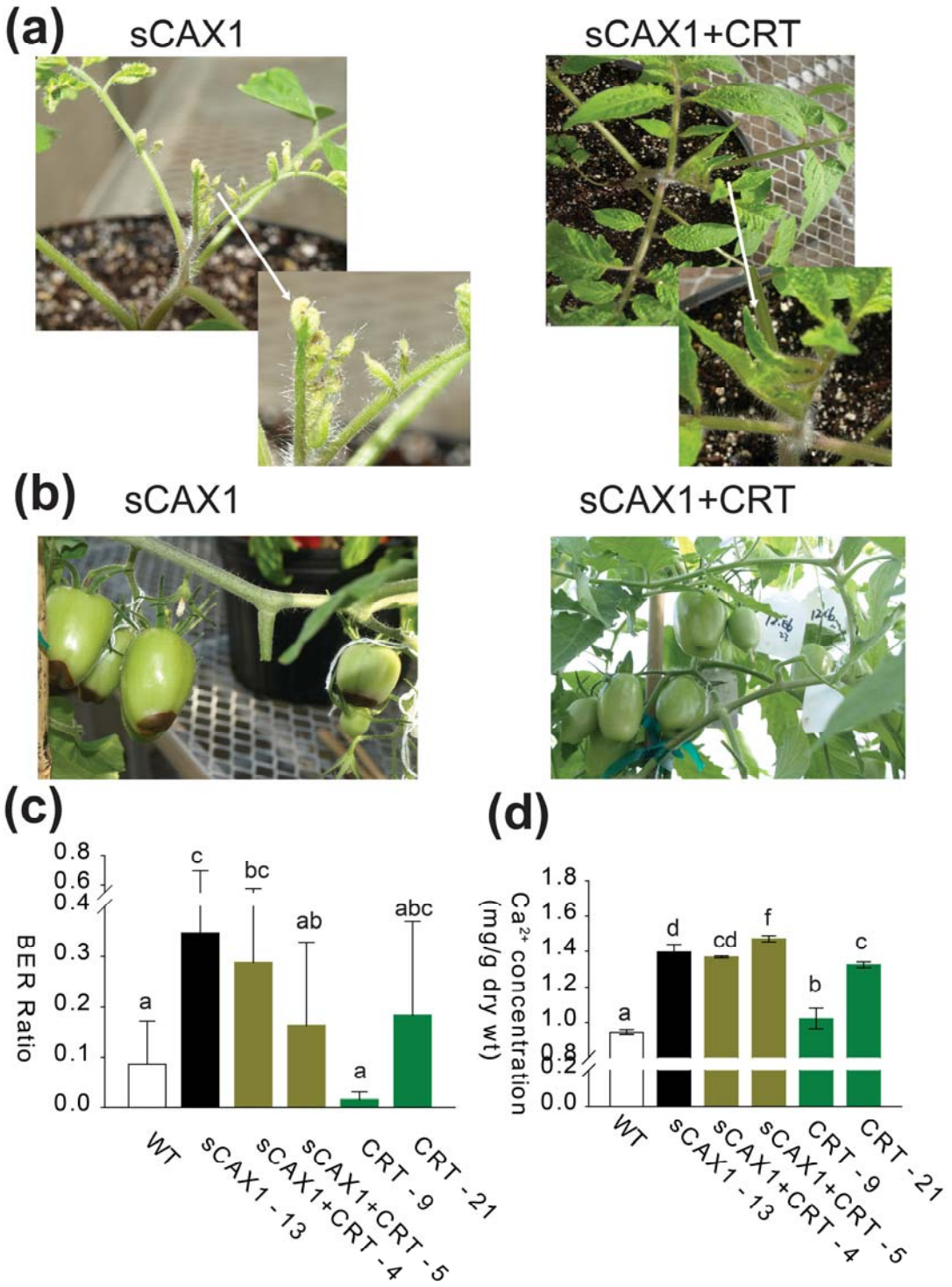

Figure 4.4 Expression of CRT mitigated the Ca^{2+} deficiency-like symptoms of *sCAX1*-expressing tomato plants.

(a) Expression of CRT mitigated the leaf tip burning of *sCAX1*-expressing tomato plants. (b)

Expression of *CRT* reduced the BER incidence of *sCAX1*-expressing tomato plants. Left panel, *sCAX1*-expressing tomato plants; right panel, *sCAX1+CRT*-expressing tomato plants. (c) BER ratio of wild-type, *sCAX1*-, *CRT*-, and *sCAX1+CRT*-expressing tomato plants. (d) Concentrations of Ca^{2+} in fruits of wild type, *sCAX1*-, and *sCAX1+CRT*-expressing tomato plants. All results shown here are the means of 3 biological replicates, and the error bars indicate the standard deviations (S.D. n=3). Means accompanied by the same letter are not significantly different using ANOVA analysis ($p<0.05$).

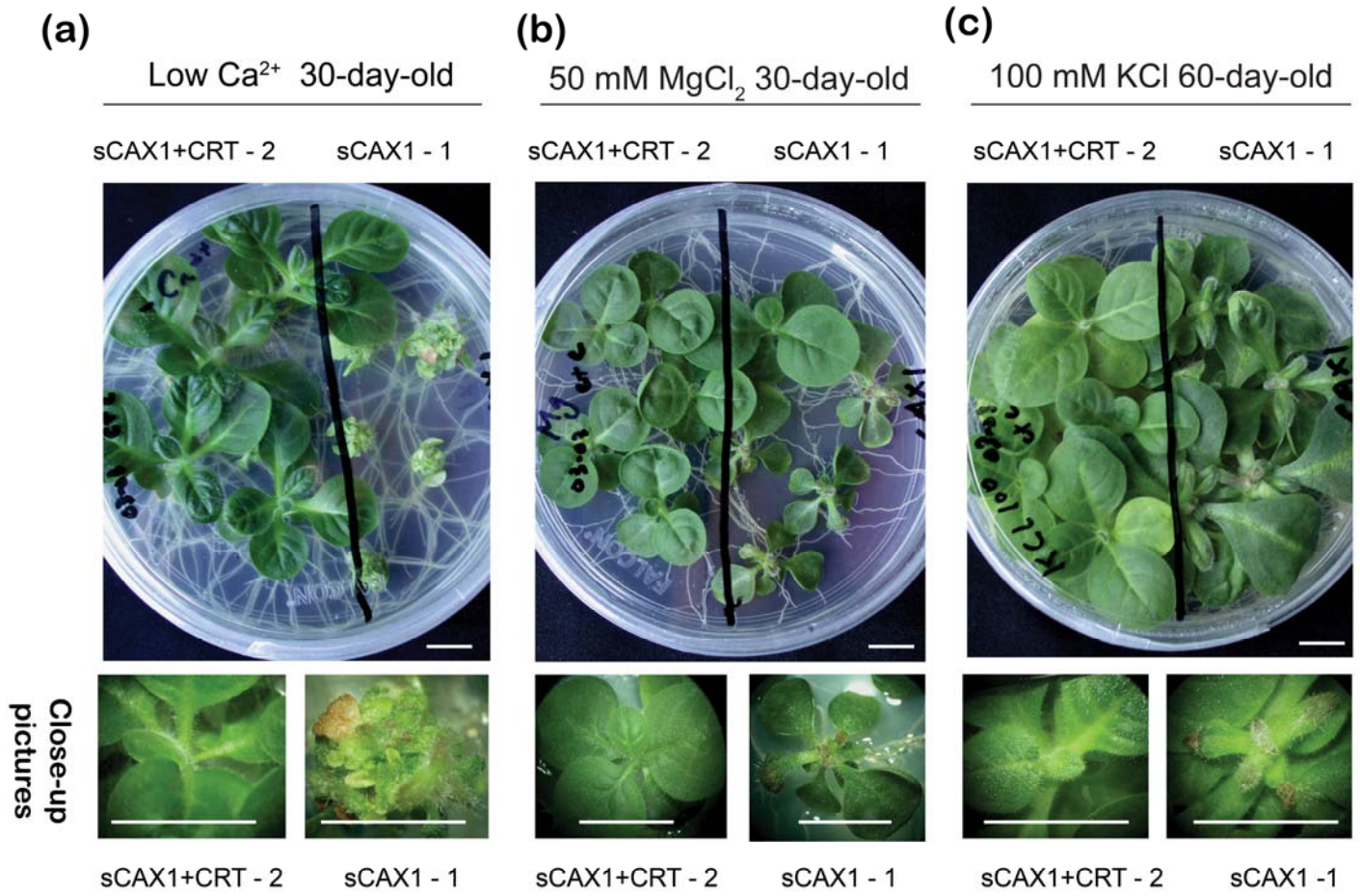

Figure 4.5 CRT suppresses sCAX1-induced ion sensitivity in tobacco plants.

(a) Tobacco seedlings grown in medium with low Ca^{2+} for 30 days. (b) Tobacco seedlings grown in medium with 100 mM $MgCl_2$ for 30 days. (c) Tobacco seedlings grown in medium with 100 mM KCl for 60 days. Upper panel, overview of the plates; lower panel, close up pictures of *sCAX1+CRT*- (lower left) and *sCAX1*-expressing (lower right) seedlings. Scale bar = 1 cm.

Table 4.1 Primers used for PCR and RT-PCR

Gene name	Primer sequence (5’ ⟶ 3’)
CAX1	Forward: AAAGATTTTCTCTCTAATCTCCAAGA Reverse: AATATGATGCTTATGAAACTCACTGA
CRT	Forward: ATGGCGATCCGCAAGGGGTC Reverse: CTAGAGCTCGTCGTGCTTCTC
SlPP2Acs (house keeping gene)	Forward: CGATGTGTGATCTCCTATGGTC Reverse: AAGCTGATGGGCTCTAGAAATC

Chapter 5 - Conclusion

Crop plants are continuously exposed to changing environmental conditions and different signaling pathways converge to help plants counteract numerous stresses. Therefore, manipulating the key genes and proteins may improve the tolerance to multiple abiotic stresses. In the dissertation, we improved abiotic stress tolerance of tomato plants by introduction of a Grx family gene *AtGRXS17*. First, we used yeast heterologous expression systems to characterize AtGRXS17 functions. The results suggest that AtGRXS17 has conserved functions across different organisms. In addition, we generated *AtGRXS17*-expressing tomato plants and determined how this gene influenced heat stress response and tolerance in tomato. The data suggest that AtGRXS17 enhances thermotolerance by mediating the anti-oxidative and HSP/HSF systems. Furthermore, we reported that introducing *AtGRXS17* into tomato plants also enhanced tolerance to drought, cold, and oxidative stress and affected endogenous stress-responsive genes in tomato plants. A possible working model how *AtGRXS17*-expressing tomato enhances tolerance to drought, cold and oxidative stress is summarized (Fig. 3.9, Chapter 3). Expression of *AtGRXS17* inhibits CBF responsiveness during cold stress, but induces *AREB* responsiveness during drought stress. Both cold and drought stress trigger the accumulation of ROS, causing oxidative stress. AtGRXS17 protects plant cells from oxidative stress. This is partly mediated through up-regulating *CAT* expression. Due to the universal existent of GRXs in plant species, manipulation of GRXs across different species may be a useful approach to improve tolerance to abiotic stresses and to understand the plant signaling under abiotic stress conditions in many agriculturally important crop species. Future efforts will focus on manipulation of GRXs in other crop species, such as rice, wheat, or sweet potato, to improve their tolerance to abiotic stresses. In addition, homologous genes of *AtGRXS17* will be cloned in these crop species to facilitate understanding of the abiotic stress signaling.

Conventional breeding strategies for mineral biofortification of crops rely on germplasm with limited genetic variation for many traits. In some cases, genetic diversity may be increased by crossing to distant related species and movement of the traits slowly into the agronomically useful cultivars. However, the variation in a trait, in particular Ca^{2+} concentration, may not cover the range desired for agronomic value. Thus, breeders may not have the appropriate level of genetic variation in Ca^{2+} concentration among varieties. Our genetic engineering approach

allows over-expression of Ca^{2+} transporter genes and expression to a level not present in germplasm. However, a major impediment for the development of Ca^{2+}-biofortified crops using Ca^{2+} transporters is that the transgenic lines expressing *sCAX1* dramatically increase Ca^{2+} content in their tissues, but also display severe Ca^{2+} deficiency-like symptoms, leading to significant yield losses.

We expressed a maize *CRT* in *sCAX1*-expressing tobacco and tomato plants to test our hypothesis if the expression of *CRT* gene can mitigate Ca^{2+}-related cellular dysfunction resulted from expressing of *sCAX1* in tobacco and tomato plants while maintaining enhanced Ca^{2+} content. Our findings suggest that co-expressing transporters and binding-proteins may be a means of boosting plant nutrient content without adversely affecting yield. Results from these studies also suggest that the CRT-mediated alteration of the ER Ca^{2+} pool could potentially make Ca^{2+} more readily accessible for release into the cytosol and further strengthens the notion that the increased Ca^{2+}-buffering capacity generated by overproduction of CRT helps maintain Ca^{2+} homeostasis. Indeed, co-expression of a maize *CRT* mitigates the Ca^{2+} deficiency-like symptoms including apical tip burning and BER and the hypersensitivity to ion imbalance caused by expression of *sCAX1* in tobacco and tomato plants. Although *CRT* expression alone was not sufficient to dramatically alter the Ca^{2+} content and incidence of BER in this study, our results here suggest that combining expression of transporters and binding proteins may be a strategy to boost Ca^{2+} levels without negatively impacting plant growth and development. In conclusion, while genetic engineering strategies to increase Ca^{2+} content by expression of a single gene (either *sCAX1* or *CRT*) alone have provided promising results, co-expressing of *CRT* and *sCAX1* enhances the Ca^{2+} content of plants without any apparent detrimental effects potentially caused by *sCAX1* expression. Manipulation of the partitioning of nutrients across various endomembranes may be a means to increase plant nutrient content while maintaining crop productivity, and future research will focus on visualizing the distribution and abundance of elements within plants using synchrotron x-rays techniques. To our knowledge, this study represents the first attempts to increase the Ca^{2+} content of plants using co-expression of two genes that play important roles in the regulation of Ca^{2+}.