Redox-engineering enhances maize thermotolerance and grain yield in the field

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Summary

Increasing populations and temperatures are expected to escalate food demands beyond production capacities, and the development of maize lines with better performance under heat stress is desirable. Here, we report that constitutive ectopic expression of a heterologous glutaredoxin S17 from Arabidopsis thaliana (AtGRXS17) can provide thermotolerance in maize through enhanced chaperone activity and modulation of heat stress-associated gene expression. The thermotolerant maize lines had increased protection against protein damage and yielded a 6-fold increase in grain production in comparison to the non-transgenic counterparts under heat stress field conditions. The maize lines also displayed thermotolerance in the reproductive stages, resulting in improved pollen germination and the higher fidelity of fertilized ovules under heat stress conditions. Our results present a robust and simple strategy for meeting rising yield demands in maize and, possibly, other crop species in a warming global environment.
Introduction

The years 2014 to 2021 were the warmest on record, in a persistent climate change trend (NOAA, 2022). High temperatures during the reproductive stage can devastate crops due to heat stress and loss of fertility. Affected species include maize (*Zea mays* L.), the largest world crop by grain weight (Tripathi *et al.*, 2016). Crop models predict a 46% to 82% reduction in average yields by the end of the 21st century, and maize may be the most impacted major crop (Schlenker and Roberts, 2009; Zhao *et al.*, 2017). The combination of increases in human population and decreases in crop yields due to heat stress is a direct threat to food security and necessitates development of thermotolerant cultivars, particularly during the reproductive stage, which is the most sensitive and crucial for kernel set and grain yield (Melillo *et al.*, 2014; Tigchelaar *et al.*, 2018; United Nations, 2017).

Due to the spatial separation of the Rubisco complex from oxygen, C4 species have a higher optimal temperature for growth and development than C3 species. Nevertheless, C4 species, including maize, are severely impacted at leaf temperatures above 30°C (Crafts-Brandner and Salvucci, 2002). When exposed to a 4°C increase over normal seasonal temperatures, maize vegetative growth and biomass yields increase, while kernel set and grain yield decrease (Hatfield, 2016). Statistical models indicate that maize grain yield will decrease by 8.3% for every 1°C increase in average temperature (Lobell and Field, 2007). Heat stress decreases yield primarily by reducing fertilization and viability of pollen and ovules, leading to kernel abortion (Lizaso *et al.*, 2018). Pollen production and viability are strongly correlated with ambient vapor pressure deficit (VPD), which is, in turn, a function of temperature (Fonseca and Westgate, 2005). Heat stress-induced yield reductions are also highly correlated to the number of kernels, which can be affected until 15 days post silking (Ordóñez *et al.*, 2015). Indeed, heat-induced kernel abortion can explain up to 95% of yield loss (Cicchino *et al.*, 2010; Rattalino Edreira *et al.*, 2011).

The molecular response of plants to heat stress includes reductions in photosynthesis and increases in reactive oxygen species (ROS), which, in turn, damage cell membranes, proteins, and nucleic acids (Gururani *et al.*, 2015). To combat excessive ROS accumulation, a strict balancing act is maintained within the antioxidant system, and redox enzymes, including glutaredoxins (GRXs), function to moderate ROS levels during normal growth and ROS-inducing stress events (Miller *et al.*, 2008). Glutaredoxins, coupled with reduced glutathione, are small ubiquitous oxidoreductases and present in nearly all living organisms (Wu *et al.*, 2017). The enzymes reduce glutathionylated proteins via the reduced thiol group (Rouhier *et al.*, 2004). The oxidoreductase activity affects the redox state and activity of target proteins in plant cells. Glutaredoxins are also important in heavy metal detoxification, iron-sulfur (Fe-S) cluster binding and transport, floral development signaling, and abiotic stress tolerance (Hu *et al.*, 2015; Inigo *et al.*, 2016; Kruestig *et al.*, 2015; Ströher *et al.*, 2016). A class II GRX family member from *Arabidopsis*, AtGRXS17, has been implicated in multiple stress responses, particularly in abiotic stress adaptation (Wu *et al.*, 2017). *AtGRXS17* is expressed in all tissues of *Arabidopsis* and highly induced under heat stress (Cheng *et al.*, 2011). AtGRXS17 was also identified to be critical for post-embryonic growth and development of *Arabidopsis* when challenged with elevated temperatures (Cheng *et al.*, 2011). A loss-of-function *AtGRXS17* T-DNA insertion line (*atgrxs17*) of *Arabidopsis* and a loss-of-function CRISPR/Cas9-mediated *SlGRXS17* line (*Slgrxs17*) of tomato were both defective in vegetative development and more sensitive to heat stress in comparison to the respective parental lines (Cheng *et al.*, 2011; Kakeshpour *et al.*, 2021). Conversely, constitutive ectopic expression...
of AtGRXS17 in tomato resulted in plants with higher thermotolerance than wild-type (WT) control plants during vegetative growth (Wu et al., 2012). Whether GRXS17 has a conserved thermotolerance function in cereal crops and whether constitutive ectopic expression of AtGRXS17 in maize affects thermotolerance capacity during reproductive stages are unknown. Further, no mechanistic insights of GRXS17 underlying thermotolerance in crops have been delineated, and the potential of this factor has not been tested under field conditions, which is essential to ascertain its agronomic relevance.

Here, we expressed AtGRXS17 in the maize inbred line B104, which is sensitive to heat stress throughout the reproductive stages, to determine if AtGRXS17 affects thermotolerance of the reproductive stages and assess the potential of AtGRXS17 benefits, if any, under field conditions. We explored the functions of AtGRXS17 in response to heat stress using transcriptomics and protein activity and oxidation assays. This study provides prospective thermotolerance mechanisms and the effects of AtGRXS17 in maize on total kernel set and total grain yield when exposed to heat stress during reproductive stages in both greenhouse and field conditions.

**Results**

**Endogenous ZmGRXS17 is induced in response to heat stress**
Maize GRXS17, ZmGRXS17, was cloned from the inbred line B104 using primers based on the B73 reference genome. All three CGFS active site motifs, which are unique to class II GRXs, were present in the maize homolog (Figure S1). The expression profile of ZmGRXS17 was measured in leaf tissue of four inbred lines (B73, B104, A188, and HiIIA) in response to heat stress. In all lines, ZmGRXS17 transcript levels quickly increased in response to heat stress, reaching 3 to 6 fold at 2 h, depending on the line (Figure 1a). ZmGRXS17 expression decreased gradually after 2 h as the period of heat stress was extended to 24 h (Figure 1a).

AtGRXS17-expressing maize plants do not display any discernable changes in growth and fertility
AtGRXS17 driven by the maize ubiquitin-1 (Ubi-1) promoter was designed to generate AtGRXS17-expressing maize (Figure 1b). Four of the 13 independent transgenic lines (Figure 1c) were selected for DNA gel blot analysis after PCR analysis. Southern blot hybridization indicated two, one, multiple, and one T-DNA copies in AtGRXS17-expressing lines S17-4, -5, -6, and -10, respectively (Figure 1d). Lines, S17-5, -6, and -10 events were chosen for further functional analysis. S17-5 displayed the highest expression of the transgene, followed by S17-6, and S17-10 (Figure 1e), while AtGRXS17-expressing maize lines maintained ZmGRXS17 expression steadily in comparison to the WT plants that showed an increase in the expression under heat stress conditions (Figure S2). Morphological or agronomic traits of AtGRXS17-expressing maize were indistinguishable from WT control plants under normal growth conditions in greenhouse and field plots, including plant height during vegetative growth stages (Figures 1f,g,i,j and S3), tasseling, flowering, and kernel set and fill (Figure 1h,k,l).

AtGRXS17 expression in maize increases thermotolerance during reproductive stages
To determine the effects of AtGRXS17 expression on the thermotolerance during the pollination and fertilization stages, a heat stress [37°C/32°C (day/night, 14 h photoperiod)] period of ten days was initiated one day before pollination, and silks were manually self-pollinated daily until pollen shedding was complete (Figure 2b, blue arrow). Though greenhouse temperature was set at 37°C
minimum daytime temperature, maximum daily temperatures exceeded 40°C during the first five days of the treatment due to extremely hot outdoor weather and average daily temperatures exceeded 35°C (Figure 2b). Lines S17-5, -6, and -10, and WT plants displayed no difference in kernel set and development under normal growth temperatures (28°C/22°C, day/night ± 2°C) (Figure 2c,d). Heat stress had less impact on the kernel set of AtGRXS17-expressing maize plants than WT plants (Figure 2e). Kernel set in the heat stress treatment for S17-5, -6, and -10 was at least twice that of WT plants (Figure 2f).

**AtGRXS17-expressing pollen is less sensitive to heat stress**

Maize pollen germination is sensitive to elevated temperatures (Begcy et al., 2019). The effect of heat stress on reduced kernel set is attributed to reduced pollen viability (Lizaso et al., 2018), and the inbred line B104 was chosen to study the effect of AtGRXS17 expression on thermotolerance of reproductive processes as it was found to be sensitive regarding pollen viability (Fonseca and Westgate, 2005). To test whether the expression of AtGRXS17 affects pollen viability, the rate of pollen germination and the development of pollen tubes were analyzed in AtGRXS17-expressing maize lines under heat stress. Pollen from non-stressed plants of all genotypes displayed no differences in morphology or grain diameter. Pollen germinated with more than 80% germination rates and produced pollen tubes more than three times as long as the grain diameter (Figures 3a and S4). Differences between genotypes were apparent when pollen was collected from heat-stressed plants. The tube development of WT control pollen was inhibited after exposure of the plants to 37°C for 48 or 72 h in the greenhouse (Figure 3b,c), and germination rates of WT pollen were reduced when subsequently incubated at high temperatures ranging from 30°C to 40°C after being exposed to 37°C for 48 h in the greenhouse (Figure 3d). Differences became more pronounced after 72 h at 37°C, where WT pollen was almost completely incapable of germination and transgenic pollen maintained a rate near 20% (Figures 3e and S5).

**AtGRXS17-expressing maize has increased kernel set during heat treatment**

A reduction in kernel set when heat treatments were initiated at silking indicates that kernel abortion may be more sensitive to heat stress than pollen germination (Rattalino Edreira and Otegui, 2013). To determine the effects of AtGRXS17 expression on the kernel set and abortion, AtGRXS17-expressing and WT control plants were subjected to heat stress after pollination (Figure 2a, orange arrow). AtGRXS17-expressing lines S17-5 and -10, which were chosen for their consistent thermotolerant phenotypes and single copy T-DNA insertions, displayed significantly higher kernel set than WT plants under heat stress, while no differences were observed in kernel set under normal growth conditions (Figures 4a and S6). Wild-type plants experienced an 81% reduction in kernel set, compared to a 38% and 42% reduction in lines S17-5 and -10, respectively (Figure 4b). Wild-type plants produced an average of nearly 61 kernels per plant compared to 233 and 188 for lines S17-5 and -10, indicating AtGRXS17-expressing plants have a much higher kernel set and less kernel abortion than the WT under heat stress.

**AtGRXS17 alters stress-related gene transcription and protein oxidation**

Expression levels and patterns of several stress responsive genes were examined in maize flag leaf and ovule samples of AtGRXS17-expressing and WT lines. Heat treatments induced expression of heat shock factors (ZmHSF3 and ZmHSF4) (Lin et al., 2011) and heat shock proteins (ZmHSP90, ZmHSP70 and ZmsHSP26) (Frey et al., 2015; Sable et al., 2018) in leaves and ovules at 1 h and returned to near baseline at 24 h at 37°C. Furthermore, the induction of ZmHSFs and ZmHSPs in
leaf, but not in ovule, was substantially higher in AtGRXS17-expressing lines compared with that of the WT plants after a 1 h heat treatment, while no differences associated with expression of AtGRXS17 in leaf and ovule were observed at a 24 h heat treatment (Figures S7 and S8). To adapt to heat stress, plants employ specific enzymatic antioxidants such as catalase (CAT), L-ascorbate peroxidase (APX), and glutathione transferase (GST) to protect cells from oxidative stress. Expression levels and patterns of ZmCAT1, ZmAPX4 and ZmGST1 genes were examined in both leaf and ovule samples of AtGRXS17-expressing and WT lines. Differences in expression levels and patterns of antioxidant genes associated with expression of AtGRXS17 were observed in both leaf and ovule (Figures S9 and S10).

Antioxidant enzyme activities may not be tightly correlated with transcript levels of their respective genes (Hu et al., 2015; Stitt and Gibon, 2014). To test if AtGRXS17 expression in heat stressed maize may affect activity and stability of the antioxidant enzymes, H2O2 accumulation, which is the most general indicator of oxidative stress to plants (Wu et al., 2012), was measured in flag leaf samples at the silking stage from AtGRXS17-expressing and WT plants. H2O2 accumulation was substantially lower in AtGRXS17-expressing lines compared with that of the WT plants after a 24 h heat treatment (Figure 4c). Catalase activity, which is indispensable for oxidative stress tolerance and thermotolerance in plants (Kakeshpour et al., 2021), was measured in both AtGRXS17-expressing and WT lines. Indeed, the activity of CAT was increased in all AtGRXS17-expressing lines when compared to WT plants under heat stress conditions (Figure 4d). Heat tolerance of the AtGRXS17-expressing maize plants to kernel abortion may, therefore, result from enhancement of the antioxidant enzyme activity and reducing ROS accumulation.

Protein carbonyl content is an indicator of severe oxidative protein damage (Stadtman and Levine, 2003). To determine whether AtGRXS17 expression reduces protein oxidation in maize, protein carbonyl content in response to heat stress was measured in flag leaf samples at the silking stage from AtGRXS17-expressing and WT plants. The carbonyl content in WT and AtGRXS17-expressing lines were not different under normal growth conditions. Under a 24 h heat stress treatment, the protein carbonyl content significantly increased in the WT but not in AtGRXS17-expressing lines, indicating that AtGRXS17-expressing plants have higher protein oxidation protection as compared to the WT plants (Figure 4e).

**Heat stress-associated chaperones are up-regulated in the AtGRXS17-expressing maize**

To further identify molecular processes contributing to thermotolerance, RNA-Seq approach was performed using the flag leaf, which is the primary contributor to seed set and grain yield during reproductive stages in cereal crops (Guo et al., 2020), from heat-treated WT and AtGRXS17-expressing line S17-5 (s5) at 1 h and 24 h, as well as from control plants (0 h). Principal component analysis (PCA), using expression data of genes with at least 10 average reads, showed that WT and s5 samples were clustered at 0 h (Figure 5a). Samples with heat treatments for 1 h were shifted to another cluster in both WT and s5 groups. The s5 group at 24 h heat treatment was clustered with 1 h heat treated groups of both s5 and WT, while WT group at 24 h heat treatment formed a separate cluster. The PCA result indicates that s5 had distinct heat-responsive gene regulation as compared to WT at 24 h. Differential expression (DE) comparisons identified 3,893 and 6,982 DE genes between 0 h and 1 h, and between 1 h and 24 h of the WT group, respectively, as well as 5,473 and (0 h vs. 1 h) and 3,893 (1 h vs. 24 h) DE genes of the s5 group at the 5% false discovery rate (FDR). Many genes in the WT group had no expression changes from 0 h to 1 h, while changing to either up- or down-regulated from 1 h to 24 h (Figure 5b, red arrow). In contrast, s5 group featured more gene expression changes from 0 h to 1 h and maintaining expression
patterns from 1 h to 24 h (Figure 5b, green arrow). The time-course expression patterns implied that s5 had a more rapid response in gene regulation upon heat treatments and maintained gene expression steadily in comparison to the WT plants.

We then examined heat responses of s5 and WT at 1 h and 24 h heat treatments separately. Fourteen early (1 h) differentially heat-responsive genes (DHRGs) and 6,777 late (24 h) DHRGs were identified. The results indicate that AtGRXS17 expression does not dramatically rewire transcriptomes under no heat treatment or at the early stage of heat treatment. The gene ontology functioning in protein refolding (GO:0042026) was enriched in the fourteen early DHRGs, consisting of two genes Zm00001d034919 and Zm00001d045544 that both putatively encode chaperonin-60 alpha. Both genes had stronger up-regulation at a 1 h heat treatment in s5 as compared to WT, and their expressions were reduced at 24 h from 1 h heat treatment in both genotypes (Figure 5c). In addition, another putative chaperone gene Zm00001d047302, which encodes a molecular chaperone that is specific for heat stress, showed a similar expression as these fourteen early DHRGs. The AtGRXS17 transgene had a large impact on gene responses upon heat in 24 h. GO enrichment analysis indicated that the regulation of biosynthesis of ribosome RNA, photosynthesis, sexual reproduction, cell wall modification, and translation upon heat were also significantly influenced by the presence of AtGRXS17 (Figure 5d).

**AtGRXS17-expressing maize plants have increased kernel set in the field**

To determine whether AtGRXS17 expression confers thermotolerance under field conditions when the stress is imposed at tasseling through the grain-filling stage, T2 generation plants of lines S17-5, -6, and -10 were grown with and without heat tents and compared to WT plants under the same conditions (Figure 2a, red arrow). On average, heat tents had a maximum daily temperature 3.58°C higher and average daily temperature 1.95°C higher than open air (ambient) conditions (Figure 6a). Each AtGRXS17-expressing line performed better than WT plants and yielded around 6-fold more grain than WT plants, as measured by total grain weight per plant (Figure 6b,c). Ambient conditions, which occasionally exceeded 35°C during pollination causing kernel set and yield decreases, also produced significant differences in total grain yield between AtGRXS17-expressing lines and WT (Figure 6d). Kernel set per plant, both in heat tents and ambient conditions, was higher in all transgenic events than the WT (Figure 6e,f). Kernel number per plant was highly correlated ($R^2=0.98$) with total grain yield across all treatments and genotypes (Figure 6g,h). No differences were observed in ears per plant or nutrient composition of kernels between genotypes or treatments (Table S1).

**Discussion**

This study is one of few examples where genetic engineering has drastically improved maize yield under heat stress field conditions. The global temperature increases have a significant impact on crop production, including maize which faces a yield decline under a prolonged temperature regime over $30^\circ$C (Schauberger et al., 2017). Despite this imminent threat, few approaches have succeeded in genetic engineering thermotolerant maize that performs well in both high stress and non- or low-stress conditions (Casaretto et al., 2016; Mickelbart et al., 2015). Our results demonstrate a robust yield benefit not only at high heat (within the heat tents) but also from ambient conditions (outside the tents), suggesting that these lines will be useful for current warming weather conditions as well as in the future as temperatures continue to rise.
Excessive ROS production, triggered by heat stress, in maize causes oxidative damage to a variety of regulatory proteins, resulting in poor growth and pollen germination, ultimately leading to critical grain yield losses (Janni et al., 2020; Waqas et al., 2021). In response to heat stress, plants have orchestrated molecular chaperone systems as well as an elaborate antioxidant network that judiciously regulates ROS production and scavenging (Foyer and Noctor, 2005; Rouhier et al., 2008). Our results indicated that GRXS17 is a conserved and critical factor in the orchestrated response to heat stress. The gene for the maize homolog is up-regulated in response to heat stress within 2 h, with ZmGRXS17 expression increasing nearly three-fold. Endogenous ZmGRXS17 expression was lower in AtGRXS17-expressing lines than in WT plants, indicating that GRXS17 expression responds to ROS levels, which are driven lower by constitutive expression of the AtGRXS17 gene (Figure S2). This also suggests that constitutive expression of AtGRXS17 further strengthens GRXS17 response to stress and redox imbalance.

Recent studies on GRXS17 demonstrated that GRXS17 itself has both foldase and redox-dependent holdase activities, protecting misfolding and aggregation of proteins caused by heat stress through its chaperone activity (Martins et al., 2020). Moreover, GRXS17 interacted with a different set of proteins upon heat stress, possibly protecting them from heat injuries as a redox-dependent chaperone (Martins et al., 2020). Remarkably, our RNA-seq data revealed that several imperative molecular chaperones for protein quality control were strongly up-regulated in the AtGRXS17-expressing maize during heat stress. In addition, our GO enrichment analysis indicated that the regulation of biosynthesis of ribosome RNA, photosynthesis, sexual reproduction, cell wall modification, and translation which have all been implicated in heat stress responses were significantly influenced by the presence of AtGRXS17 and maintained their gene expression changes steadily in comparison to the WT plants over 24 h of heat treatment. In addition to our protein carbonyl content results, which support that AtGRXS17 expression in maize increases protection against severe protein damage, these findings indicate that a constitutive GRXS17 expression is required for thermotolerance of maize during heat stress through its chaperone activity and modulation of heat stress-associated genes, including chaperones. Taken together, our results suggest that constitutive expression of AtGRXS17 up-regulates genes involved in heat stress response via redox-dependent chaperone activity.

As a large family of molecular chaperones, the induction of HSPs and their HSF transcriptional regulators is also a hallmark response to heat stress (Li and Howell, 2021; Wahid et al., 2007). Constitutive expression of AtGRXS17 in tomato up-regulates the endogenous SIHSF and SIHSP genes in leaf under heat stress. Such changes in gene expression were found to enhance thermotolerance at the vegetative stage by counteracting the oxidative damage caused by excessive ROS accumulation (Wu et al., 2012). Consistently, heat treatments also induced higher increases in expression of ZmHSF and ZmHSP genes in flag leaf in AtGRXS17-expressing maize lines compared with that of the WT plants after a 1 h heat at 37°C; however, none of the differences were associated with expression of AtGRXS17 in ovule. In maize, 25 distinct ZmHSFs have been identified, and tissue localization of the expression of each gene were reported (Lin et al., 2011). ZmHSF4 was reported to be expressed in husks and seeds, and ZmHSF3 was only listed as expressed in seeds. However, both of these factors were found to increase several thousand fold in leaf tissue during heat stress. This drastic increase may be due to the low expression in leaf tissue under normal growth conditions, which may also explain why they were not listed as detected in leaf tissue. Regardless, our results indicated that ZmHSF3 and ZmHSF4 in AtGRXS17-expressing lines were highly expressed in leaves, but not in ovules, as compared to the WT plants under heat stress conditions, suggesting that ZmHSFs, including ZmHSPs, in ovules might be
regulated at the post-translational level, rather than at the transcriptional level, for a thermotolerant response in \textit{AtGRXS17}-expressing lines.

Heat stress negatively affects pollination and fertilization as well as kernel development (De Storme and Geelen, 2014; Devasirvatham \textit{et al.}, 2013; Muhlemann \textit{et al.}, 2018; Rieu \textit{et al.}, 2017). Consistently, we observed that pollen germination and pollen tube growth were significantly reduced and kernel abortion after pollination were markedly increased in WT after heat stress. These features were generally maintained in \textit{AtGRXS17}-expressing pollen grains and kernels when challenged with heat stress. We have shown that constitutive expression of \textit{AtGRXS17} improves thermotolerance at both stages, together resulting in higher yield in high temperature conditions. It remains to be determined whether the pollen and kernel tolerance phenotypes are primarily due to local effects of the \textit{AtGRXS17} transgene or due to a systemic effect, e.g. through changes in photosynthesis or resource allocation from the flag leaf to the reproductive organs. Future experiments with tissue-specific \textit{AtGRXS17} overexpression will provide insight into this.

A genetic engineering approach has been proposed to mitigate grain yield losses in high-nighttime-temperature conditions during grain-filling period by expression of a modified 6-phosphogluconate dehydrogenase in maize seeds (Ribeiro \textit{et al.}, 2020). In addition, numerous studies on engineering thermotolerance across different species have largely focused on HSPs and HSFs. However, few of these approaches have been successful, mainly because increased stress tolerance invariably compromises growth under non-stress conditions (Casaretto \textit{et al.}, 2016; Mickelbart \textit{et al.}, 2015). Furthermore, thermotolerance of these engineered crops has not translated effectively from the greenhouse to the field, and traits that are effective in the laboratory and greenhouse often fail in the field because field conditions vary widely (Nuccio \textit{et al.}, 2015; Shen \textit{et al.}, 2015). Many quantitative trait locus mapping and genome-wide association studies have found significant genomic regions contributing to increased thermotolerance in crop plants (Frey \textit{et al.}, 2016; McNellie \textit{et al.}, 2018). Regardless, gains have been minor, involve many genes or genomic regions, are likely non-transferable to other species, and translating those minor gains into improved cultivars requires years of work and often does not improve cultivars with different genetic pedigrees. While various approaches have also been proposed to improve thermotolerance in maize by manipulating stress-associated traits, to our knowledge, few field tests of genetically engineered thermotolerant maize have been investigated (Casaretto \textit{et al.}, 2016). Here, we demonstrate robust thermotolerance during reproductive developmental and grain-filling stages in maize through constitutive ectopic expression of \textit{AtGRXS17}, resulting in large yield increases without any adverse growth effects in both greenhouse and field conditions. Our findings provide a new avenue to engineer thermotolerant maize and other major crops.
Materials and methods

Cloning \textit{AtGRXS17} for ectopic expression in \textit{Zea mays} and plant transformation

\textit{AtGRXS17} driven by the maize \textit{ubiquitin-1} (\textit{Ubi-1}) promotor was amplified by PCR from previously constructed pSK::\textit{Ubi-AtGRXS17} vector (Hu et al., 2017). CACC was added to the 5’ end of the cassette using forward primer, 5’-CACCTGCAGTGAGCGGTG-3’, for compatibility with the gateway cloning system. Reverse primer, 5’-AATTCCCGATCTAGTAACATAGATGACACCG-3’, complementary to the nopaline synthase (\textit{nos-T}) terminator was used. The blunt end PCR product was directionally cloned into pENTR™/D-TOPO® vector for entry into the gateway system. Plasmids were transformed into competent E. coli cells via the freeze/thaw method, screened by PCR to confirm successful integration of the expression cassette, and a representative clone was sequenced. The vector pTF101.1gw1-\textit{Ubi::AtGRXS17} was created by transferring the sequenced cassette to pTF101.gw1 plasmid (Paz et al., 2004) via gateway cloning. Integration was confirmed by PCR after transferring to E. coli. Vectors were transformed into \textit{Agrobacterium tumefaciens} EHA101 and confirmed by PCR and restriction digestion analysis with EcoRI and EcoRI+ BamH1. The pTF101.1gw1-\textit{Ubi::AtGRXS17} expression vectors were transformed into the maize B104 inbred lines using \textit{Agrobacterium}-mediated transformation (Frame et al., 2002), self-pollinated, and the progeny lines (T1) were genotyped for the presence of T-DNA using standard PCR and Southern blot analysis.

Plant material

The inbred maize line B104 was used to study the effect of \textit{AtGRXS17} expression on thermotolerance of reproductive processes as it was found to be sensitive regarding pollen viability (Fonseca and Westgate, 2005). Furthermore, the line has a high degree of genetic similarity with line B73, which serves as the common reference genome and is parent of many breeding populations (Hallauer et al., 1997; Schnable et al., 2009).

DNA isolation, Southern blot analysis and T2 generation screening

Maize gDNA was isolated from leaf tissue of T1 transgenic and wild-type (WT) plants using 2% cetyl trimethylammonium bromide (CTAB) and phenol:chloroform:isoamyl alcohol (25:24:1). Fifty μg gDNA was digested to completion overnight with HindIII and separated on a 0.7% agarose gel by overnight electrophoresis. The gDNA was blotted onto a positively charged Zeta-Probe GT nylon membrane using the alkali transfer method. All remaining Southern blot steps were carried out according to manufacturer’s instructions for the AlkPhos Direct Labeling and Detection System (CDP-Star GE Healthcare Life Sciences). The probe for the \textit{BAR} gene was generated by PCR from the ptf101.gw1 vector used for transformation using 5’-ATGAGCCCAAGAAGCAGCCC-3’ and 5’-TCAGATCTCGGTAGCGGCACGG-3’. The membrane was prehybridized, hybridized overnight at 60°C, and washed at 65°C. Detection was carried out using CDP-Star with different exposure times to X-ray autoradiography film. All events tested were confirmed to have at least one stable integration of the transgene. Wild-type B104 was used as negative control, and the linearized \textit{pTF101.1gw1-\textit{Ubi::AtGRXS17}} was used as a positive control.

To screen T2 transgenic plants for greenhouse experiments and field trials, gDNA was extracted from leaf tissue of the progeny lines (T2) of each independent, self-pollinated T1 transgenic plant using a standard high-throughput CTAB and chloroform:isoamyl alcohol
method in which TCEP (tris(2-carboxyethyl)phosphine) was used in place of 2-mercaptoethanol. DNA concentrations were assayed using a Quant-iT PicoGreen dsDNA assay kit (ThermoFisher, Waltham, MA, USA) on a FLUOstar Omega fluorescence plate reader (BMG LABTECH, Cary, NC, USA) and normalized to 20 ng/µl. A PCR marker, PCRBAR, was designed on a 500 bp sequence of the herbicide-resistant bar gene used as a selectable marker in the transformation process. An 18 bp tail was added to the 5’ end of forward primer for use with a universal fluorescently labeled primer in a capillary type DNA sequencer. Primer sequences for PCRBAR fragments: PCRBAR-F18 5’-ACGACGGTTGAAACGAGACCAGGCGCCAGAAGCG-3’ and PCRBAR-R 5’-GCTGAAGTCCAGCTGCCAGAAAC-3’. PCR reactions contained 60 ng DNA, 50 nM of tailed forward primer, 100 nM of reverse primer, 50 nM VIC-labeled forward universal primer, 200 µM of each dNTP, 1.3 µl 10X ammonium sulfate PCR buffer, 2.5 mM MgCl₂, and 1 unit of Taq polymerase (NEB, Ipswich, MA, USA) in a 13 µl volume. PCR was performed using a standard 60°C profile in a DNA Engine Peltier Thermal Cycler (Bio-Rad, Hercules, CA, USA). PCR products were detected using an ABI Prism 3730 DNA Sequencer (Applied Biosystems, Foster City, CA, USA) and 518 bp fragments and amplicon polymorphisms were scored using GeneMarker (SoftGenetics LLC, State College, PA, USA).

RNA isolation and qRT-PCR analysis of AtGRXS17 in Zea mays
Total RNA was isolated from leaf tissue of T2 transgenic and WT maize plants using the Qiagen RNeasy Plant Mini kit (Valencia, CA, USA). One µg total RNA was used to synthesize first strand cDNA using Revert Aid First Strand cDNA Synthesis kit. Two µL of cDNA was used as template for PCR. Primers were complementary to the transgene, AtGRXS17. qRT-PCR was carried out according to previous reports using 5’-CACGAGAGCGGTGAACTAAA-3’ and 5’-CCAGCTTCATCCTGACTTTCT-3’ to produce an 80 bp amplicon using CDK as internal control.

Illumina RNA-Seq
AtGRXS17-expressing line S17-5 (s5) and wild-type plants were grown in 1-gallon pots filled with equal volumes of Metro-Mix 900 soilless growing media and were watered as needed with liquid feed. During vegetative growth and tasseling, V1-VT, plants were grown at optimal temperatures (28°C/22°C, day/night ± 2°C). Plants were randomly assigned to the heat stress by moving the plants to greenhouse unit set at 37°C 24 h after pollination. Flag leaf tissues from three biological replicates were collected at 0, 1 and 24 h of heat treatments. Total RNA was isolated from flag leaf tissues of AtGRXS17-expressing lines s5 and WT plants using the Direct-zol RNA Miniprep Plus Kits (Zymo Research, Irvine, CA, USA). RNA quality control, library preparation, and sequencing were performed on an Illumina Novaseq 6000 platform at Novogene (Novogene USA, SACRAMENTO, CA, USA).

Raw RNA-Seq reads were trimmed with Trimmomatic (version 0.38) (Bolger et al., 2014) to remove the adaptor sequence and low-quality bases. Trimmed paired reads both of which were 40 bp or longer were aligned to the B73 reference genome (B73v4) (Jiao et al., 2017; Schnable et al., 2009) using STAR (2.7.3a) (Dobin et al., 2013). Reads that were uniquely mapped and had at least 94% identity and 95% coverage were kept for counting reads per gene. Differential expression between the two groups was performed by using DESeq2 (version 1.26.0) (Love et al., 2014) and, to account for multiple statistical tests, the false discovery rate was controlled at 5% (Benjamini and Hochberg, 1995).
Fall 2015 greenhouse experiment 1
The progeny lines of each independent T1 transgenic plant were genotyped for null, hemizygous or homozygous AtGRXS17-expressing T2 plants using TaqMan analysis (Ingham et al., 2001). T2 transgenic plants of the three AtGRXS17-expressing lines (S17-5, -6, and -10) and WT plants were grown in 3 gallon pots filled with equal volumes of Metro-Mix 900 soilless growth media and were watered as needed with constant liquid feed on a pot-by-pot basis. During vegetative growth, V1-VT, plants were grown at optimal temperatures (28°C/22°C) and under supplemental lighting systems. Before pollinations were carried out, the thermostat was set to a target temperature of 37°C to initiate heat stress. Plants were manually self-pollinated for three days starting on the first day of silking. Twenty-four hours after the last pollination, the thermostat was reset to optimal conditions.

Summer 2017 field trial
T2 transgenic plants of the three AtGRXS17-expressing lines (S17-5, -6, and -10) and WT plants were grown in a split plot design with four replications at the North Agronomy Farm in Manhattan, KS. One row (20 plants per row) per genotype was grown for each replication. Seeds were planted on May 15, 2017 at a spacing of 25 cm and a row width of 60 cm. Approximately two months after sowing (at VT), unique field-based heat tents with a thermostat controlled passive vent system were placed over plots designated for heat treatment (Bergkamp et al., 2018; Sunoj et al., 2017). The thermostat was set to 37°C, opening the vent at this temperature and staying closed below, to increase the temperature compared to ambient conditions. The heat tents remained in place through physiological maturity until harvest.

Spring 2018 greenhouse experiment 2
Greenhouse experiment 2 consisted of two single copy insertion T2 transgenic lines (S17-5 and S17-10) and WT plants grown in one-gallon pots filled with equal volumes of Metro-Mix 900 soilless growth media and were watered as needed with liquid feed on a pot by pot basis. During vegetative growth and tasseling, V1-VT, plants were grown at optimal temperatures of 28°C/22°C. Plants were randomly assigned to several treatments. Six plants per genotype were designated as control and were pollinated. Six plants per genotype were designated as heat and were moved to an adjacent greenhouse exactly 24 h after pollination under the same environmental conditions, except, the thermostat was set to 37°C. In addition to these treatments, twenty-seven plants per genotype had cob tissues collected at different timepoints for gene expression analysis.

Pollen viability and vigor analysis
Pollen stressed for 0, 2, and 3 days at 37°C was collected from plants of each genotype (T2-generation AtGRXS17-5, -6 and -10, and WT) and incubated on a 12% sucrose, 0.03% calcium chloride, 0.01% boric acid media solidified with 0.7% W/V bacto agar at 25°C, 30°C, 35°C, and 40°C for 2 h and then moved to 4°C to arrest development. In the in vitro study, we scored germination as a pollen grain with a pollen tube as long as the grain radius. Vigor was analyzed by measuring the length of the pollen tube divided by the diameter of the pollen grain to account for differences in distance from the objective.

qRT-PCR of leaves and ovules
Flag leaf and ovule tissues collected from greenhouse experiment 2 were used for qRT-PCR analysis. RNA isolation, cDNA synthesis, and qRT-PCR were carried out as described above for different genes listed in the Table S3. HSFs, HSPs, sugar metabolism, receptor kinases, and antioxidant enzyme genes were analyzed.

**Measurement of hydrogen peroxide (H$_2$O$_2$)**
Hydrogen peroxide (H$_2$O$_2$) concentration was measured by following the manufacturer’s instruction using Amplex™ Red Hydrogen Peroxide/Peroxidase Assay Kit (Invitrogen, Carlsbad, CA, USA). H$_2$O$_2$ concentration was determined by measuring the absorbance at 560 nm using a Synergy™ H1 microplate reader (BioTek, Winooski, VT, USA).

**Measurement of the CAT activities**
Catalase (CAT) enzyme activity was measured by following the manufacturer’s instruction using Amplex® Red Catalase Assay Kit (Molecular Probes, Eugene, OR, USA). CAT concentration was determined by measuring the absorbance at 560 nm using a Synergy™ H1 microplate reader.

**Protein carbonyl content assay**
For the protein isolation, 100 mg of flag leaf samples (R1 stage) from control and heat-stressed WT, S17-5 and S17-10 were used according to Abraham-Juárez (Abraham-Juárez, 2019). The level of carbonylated proteins was measured spectrophotometrically using the dinitrophenyl hydrazine (DNPH) method (Levine et al., 1994). Briefly, total soluble proteins (0.5 mg) were incubated with the 1% (w/v) streptomycin sulfate and 0.3% (v/v) Triton X-100 for 20 min and centrifuged at 2,000 g for 20 min. The supernatant was mixed with 10 mM DNPH in 2N HCl. The mixture was incubated at room temperature for 1 h with constant vortexing every 10 min. The protein was precipitated by adding 20% (w/v) trichloroacetic acid, and the precipitated pellets were washed three times using ethanol: ethyl acetate (1:1). The final pellets were dissolved using 6 M guanidine hydrochloride in 20 mM potassium phosphate at pH 2.3, and the absorption was measured at 370 nm. The carbonyl content was calculated based on the molar extinction co-efficient for DNPH (Reznick and Packer, 1994).

**Grain quality parameters**
Nutritional composition for Ca$^{2+}$, Mg$^{2+}$, K$^+$, Cu$^{2+}$, Fe$^{3+}$, Mn$^{2+}$, Zn$^{2+}$, and SO$_4^{2-}$, was determined by nitric-perchloric acid digestion and analyzed by inductively coupled plasma spectrometry. Total carbon was measured by LECO CN 2000 combustion and reported on a weight percentage.

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Conflict of interest
The authors declare that they have no conflict of interests.

Author contributions

Supporting information
Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1 GRXS17 is highly conserved between Arabidopsis and maize at the protein level.
Figure S2 The relative expression levels of maize endogenous ZmGRXS17 in leaf tissue.
Figure S3 Morphological traits of AtGRXS17-expressing maize plants in the field.
Figure S4 Germination rates of pollen at different incubation temperatures collected from all non-stressed plants.
Figure S5 Pollen tube length at different incubation temperatures.
Figure S6 Kernel set under both control and heat stress conditions after pollination.
Figure S7 Effects of AtGRXS17 expression on the expression levels and patterns of heat responsive genes in leaves during heat stress (37°C) at the silking stage.
Figure S8 Effects of AtGRXS17 expression on the expression levels and patterns of heat responsive and ovule developmental genes in ovules during heat stress (37°C).
Figure S9 Effects of AtGRXS17 expression on the expression levels and patterns of antioxidant enzyme genes in leaves at the silking stage during heat stress (37°C).
Figure S10 Effects of AtGRXS17 expression on the expression levels and patterns of antioxidant enzyme genes in ovules during heat stress (37°C).
Table S1 Nutrient composition of maize kernels from 2017 field trial
Table S2 Number of pollen grains scored
Table S3 Primers used for qRT-PCR
References


Figure legends

Figure 1 Characterization of AtGRXS17-expressing maize plants under normal growth conditions [28°C/22°C (day/night, 14 h photoperiod)]. (a) Maize ZmGRXS17 transcript levels in leaf tissue of inbred lines, B73, B104, A188, and HillA, after heat treatments for 24 h at 37°C. (b) Map of T-DNA region of the binary vector pTF101.1gw1-Ubi::AtGRXS17 used for transformation. RB, right border; LB, left border; Tvsp, 3’ terminator from soybean vegetative storage protein gene; BAR, phosphinothricin acetyl transferase; TEV, Tobacco Etch Virus translational enhancer; p2x35s, tandem repeat of the cauliflower mosaic virus (CaMV) 35S promoter; pZmUbi, maize ubiquitin-1 promoter; AtGRXS17, Arabidopsis monothiol glutaredoxin S17; tnos, nopaline synthase terminator. (c) PCR analysis of independent transgenic maize lines expressing AtGRXS17 (lines S17-1 to S17-20). Lanes: WT, wild-type; +, positive control (plasmid). The arrow indicates PCR detection of AtGRXS17 in genomic level. (d) DNA gel blot analysis with BAR probe confirmed the stable integration of AtGRXS17 into genome of randomly selected four Ubi::AtGRXS17 transgenic maize plants. (e) The relative expression level of AtGRXS17 was measured by qRT-PCR in 4-week-old WT and three AtGRXS17 transgenic maize plants grown at 28°C. The maize CDK was used to normalize expression levels. Values are means ± SD (n=3). (f-l) Typical morphology at different developmental stages. The growth and development of the AtGRXS17-expressing maize plants are phenotypically indistinguishable from WT plants under normal growth conditions: (f) Early vegetative growth, (g) late vegetative growth, (h) tasseling, (i and j) height (S17, pooled all AtGRXS17 transgenic lines), (k) kernel set (scale bar = 10 cm), and (l) number of kernels between AtGRXS17-expressing (S17, pooled all AtGRXS17 transgenic lines) and WT plants. Note that there were no differences in ears per plant (one to two ears per plant) between AtGRXS17-expressing and WT plants. Data represent means ± SD and were analyzed using Student’s t-test (n = 3).

Figure 2 AtGRXS17-expressing maize plants display higher kernel set than WT in heat treated greenhouse trials. (a) Heat treatment period in greenhouse experiments and field trial: Blue arrow, pollination and fertilization stages (greenhouse experiment 1); Orange arrow, fertilization, kernel set, and grain-filling stages (greenhouse experiment 2); Red arrow, pollination, fertilization, kernel set, and grain-filling stages (field trial). VE, emergence; V2, second leaf; V4, fourth leaf; V8, eighth leaf; V(n), nth leaf; VT, tassel; R1, silking; R6, maturity. (b) Average and maximum daily temperatures within heat treated greenhouses. During the heat stress period, temperatures often exceeded 35°C, indicated by the red bar, and maximum daily temperatures neared 40°C at midday, while average daily temperatures stayed near the climate control target of 37°C. Arrow indicates conclusion of the heat stress period. Under optimal conditions, greenhouse temperatures never exceeded 35°C, and average daily temperatures hovered near the target of 28°C. (c) Greenhouse grown maize plants display high vigor under normal growth conditions and kernel set of WT and AtGRXS17-expressing plants are indistinguishable (scale bar = 10 cm). (d) Number of kernels between WT and AtGRXS17-expressing plants does not differ when growth under optimal conditions. (e) Representative cobs from greenhouse grown plants exposed to heat stress. Scale bar = 10 cm. (f) AtGRXS17-expressing lines have higher kernel set when exposed to heat stress during pollination and fertilization stages. Data are means ± SE of four plants per genotype and were analyzed with Student’s t-test.
Figure 3 *AtGRXS17*-expressing maize pollen is less sensitive to heat stress than WT pollen. (a) Representative image of pollen grains and germination tubes from non-stressed plant of all genotypes. Pollen was collected from plants before heat treatment was initialized. Red arrows indicate a pollen germination tube and the corresponding pollen grain. (b) Representative image of pollen grains and germination tubes from plants stressed for two days at 37°C. (c) Representative image of pollen grains and germination tubes from plants stressed for three days at 37°C. (d and e) Germination rates of pollen at different incubation temperatures collected from plants stressed for two days (d) and three days (e) at 37°C, respectively. In total, almost 8,000 pollen grains were scored (Details are presented in Table S2). Data are means ± SE (n = 8) and were analyzed using two-way ANOVA and Student’s t-test. Scale bars = 100 μm in (a) to (c).

Figure 4 Effect of ectopic expression of *AtGRXS17* in kernel set response, H$_2$O$_2$ accumulation, CAT activity, and protein carbonyl content to high temperature treatment. (a) Representative plants with intact cobs exposed to a 37°C temperature treatment 24 hours after pollination and continued through physiological maturity (scale bar = 10 cm). (b) Kernel set of *AtGRXS17*-expressing and WT plants. Data are means ± SE of six per genotype (n = 6) and were analyzed using Student’s t-test. Effect of expression of *AtGRXS17* in maize on H$_2$O$_2$ accumulation (c), CAT activity (d), and protein carbonyl content (e) under normal or heat treatments for 24 h at 37°C. Data are means ± SE and were analyzed using Student’s t-test (n=3).

Figure 5 PCA plot of RNA-Seq samples. (a) Two top components, PC1 and PC2, explaining 23.6% and 17.5% percentages of variation in RNA-Seq data, respectively, are used to display sample relations in term of gene expression. (b) Time-course expression changes. The top barplot shows the number of genes with time-course expression changes as the corresponding pattern displayed on the bottom box. Each box represents a time-course expression pattern. Numbers 0, 1, and 24 stand for time points at 0 h, 1 h, and 24 h. No changes in expression between time points are represented by horizontal lines, and up- and down-regulation are represented by upward and downward slopes, respectively. (c) Expression heatmap of early WT vs. s5 DHRGs. The genes in the heatmap show differential heat responses between WT and s5 in 1 h (early) heat treatment. Average expression per group per gene was determined and was scaled from 0 to 1. Six groups include WT_C (WT control), WT_1 (WT 1 h heat), WT_24 (WT 24 h heat), s5_C (s5 control), s5_1 (s5 1 h heat), and s5_24 (s5 24 h heat). For each gene, the highest expression was set as 1 and expression of other samples was scaled by dividing their expression to the highest expression value. (d) GOs enriched in genes showing differential heat responses between WT and s5 in a 24 h (late) heat treatment with p-values less than 0.001 were plotted. The total length of each bar represents the number of genes of a GO term in our examined gene set, and the blue bar represents the number of genes of the GO term which were differential responsive.

Figure 6 *AtGRXS17*-expressing maize plants confer thermotolerance and subsequently increase total kernel set and grain yield under heat stress in the field. (a) Temperatures measured in Summer (July 15th through August 30th) during the 2017 field trial. Red line indicates temperatures measured within heat tents and the black line indicates ambient temperatures. Maximum daily temperatures within heat tents were slightly higher (1.5°C on average) in the ten-day period leading up to silking, indicated by arrow. Maximum daily temperatures within heat tents were 3.3°C, 3.9°C, 3.8°C, and 3.0°C higher, on average, than ambient in the first, second, third, and fourth week post silking, respectively. Temperatures often exceeded 35°C, indicated by the red bar. (b)
Representative cobs harvested from the 2017 field trial. Representative cobs harvested from plants grown inside heat tents (left panel) and from plants grown at ambient temperatures (right panel) during pollination through grain-filling stages. Scale bar = 10 cm. (c and d) Total grain yield harvested from plants grown inside heat tents (c) and at ambient temperatures (d). (e-g) Representative kernel in 2017 field trial. Kernel set per plant in heat tents (e, bottom panel in g) and ambient conditions (f, top panel in g) in all transgenic events. (h) Correlation between kernel number per plant and total grain yield. Data are means ± SE of four rows (n = 20 plants per row) per genotype (n = 4 genotypes) and were analyzed using two-way ANOVA and Student’s t-test.
Heat treatment period

- P and F
- F, KS, and GF
- P, F, KS, and GF

Pollination (P), fertilization (F), kernel set (KS), and grain filling (GF).

(a) Developmental stages (VE, V1, V2, V4, V8, VT, R1, R6)

(b) Greenhouse Temperature

(c) Under optimal temperatures

(d) Kernels/Plant

(e) Heat treatments during pollination and fertilization

(f) Statistical analysis