Genetic engineering of maize and rice to enhance tolerance against abiotic and biotic stresses

by

Tej Man Tamang

B.Sc., Purbanchal University, 2013
M.S., Fort Hays State University, 2016

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Horticulture and Natural resources
College of Agriculture

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2021

Abstract

Genetic engineering has allowed biologists to make genetic changes for crop improvement. Being sessile, plants are exposed to the different biotic and abiotic stresses throughout their life stages. Numerous genes have been studied to improve pest resistance and abiotic stress tolerance in plants. Here, we expressed *Arabidopsis* monothiol glutaredoxin gene in maize and studied the effect of the gene in improving drought and heat tolerance. Similarly, we expressed *Pi9* gene in elite cultivar of rice to develop the resistance against blast disease through cisgenic approach.

Heat and drought are two of the major abiotic stresses that usually appear simultaneously. Various climate models suggest that these stresses will increase in intensity and frequency. The reproductive phases of the development are very sensitive to both drought and high-temperature stress. Reactive oxygen species (ROS) accumulate in response to the stresses and cause oxidative damage to many biological molecules, including lipids, proteins, and DNA. In our study, we performed ectopic expression of *Arabidopsis* monothiol glutaredoxin, *AtGRXS17*, to improve tolerance against drought stress and combined heat and drought (HTD) stress at the reproductive stage of maize. Glutaredoxins (GRXs) are oxidoreductases that maintain redox homeostasis. Ectopic expression of the *AtGRXS17* in maize resulted in increased tolerance against drought stress and HTD stress. Under both types of stresses, *AtGRXS17*-expressing maize lines showed higher yields than WT plants. Our results also indicated that *AtGRXS17*-expressing pollen grains showed better pollen germination than WT under desiccating environment.

Despite the huge potential of genetic engineering in improving crops, concerns have been raised about the presence of foreign genes in plants and their unpredictable consequences. Because of this, commercialization of transgenic crops is highly regulated. Removing selection marker and unnecessary transgene can potentially address the biosafety concerns of regulatory bodies. In our

research, we demonstrated successful incorporation of a rice blast resistance gene *Pi9* into an elite US rice cultivar via cisgenic method. We used a co-transformation strategy to eliminate the marker gene (*hygromycin B phosphotransferase*) from the cisgenic rice. We also found that the success rate of co-transformation was very low. Nevertheless, the co-transformation approach provides an effective way to develop marker-free cisgenic plants.

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Approved by:

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Sunghun Park

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Acknowledgments

I would like to thank everyone who helped and supported me during my studies at Kansas State University. I also thank everyone who helped me to get into this outstanding program.

First, I want to express my sincere gratitude and appreciation to Dr. Sunghun Park who is an amazing mentor. As an apprentice, I learned a lot at a personal and professional level from him. He is caring, kind, loving, and full of compassion. He constantly boosted my confidence and inspired me to work harder. He told me that hard work and being smart is the key to success. He offered his advice on experiments, teaching, and professional development. He provided valuable suggestions whenever needed. He provided his insights to grow a scientist inside me. He was always there when I needed him. He is a true inspiration and I hope to continue this relationship throughout my career.

I would also like to thank my committee members for their constant support and guidance during my doctoral training. I would like to thank Dr. Steve Keeley who taught me to teach more effectively. I learned more than ever when I assisted him in teaching HORT 201. I would like to thank him for his constant faith and support. I would like to thank Dr. Kimberly Williams for being amazing support. She was always there whenever I needed something in the greenhouse. Without Dr. Williams's help, some of the greenhouse work would not have been possible. I would like to express my gratitude to Dr. Harold Trick for helping and supporting me during conferences. He actively helped me to network with the people in our field. I have unforgettable memories with him from the conference days. His suggestions during my preliminary exams have helped me to grow as a learner. Finally, I would like to thank Dr. Ari Jumpponen for his advice and suggestions for my dissertation and defense.

This lab has been like a home to me. I have spent a huge chunk of my life's time in this lab. The lab members are like family to me. I would like to thank Kim Park for her support. Whenever possible she provided suggestions that helped me to be a better researcher. I learned a lot about tissue culture and transformation from her. She has magic in her hand and is one of the best plant tissue culture experts I have met. My tissue culture works would not have been possible without her help and support. I would also like to thank Dr. Tayebeh Kakeshpur for being a great colleague. I spent amazing 4 years with her where I learned a lot from her. Dr. Stuart Sprague helped me during my initial days to settle and get used to the research and lab environment. I would like to thank Gergerly Motolai, Zachary Fleming, Spencer Navrude, Trevor Steiner, and many undergraduate students who helped me in the greenhouse and lab. I would like to thank Dr. Qingyu Wu and Dr. Ying Hu who constantly provided advice and collaboration for my work.

I would like to thank Dr. Sanzhen Liu, Dr. Barbara Valent, Melinda Dalby, Dr. Alina Akhunova, and many other researchers around the country who helped me with a different part of the research and doctoral career. Dr. Liu was a great help in providing technical advice whenever needed. He is a close collaborator and a wonderful teacher. The greenhouse crew of our department deserves the best accolade. They were there all the time whenever I needed assistance. Lea Westerveldt and Jacob Hueste kept the greenhouse problems away.

I would also like to thank my friends and family who made my stay in Manhattan very comfortable. I want to thank my parents for sending love from half globe away. I want to thank all my friends from the Nepalese Student Association and the international community. My special cheers to the friends from G18 (our special group). Dr. Rashmi Thapa has been the person who I look up to. She is my spiritual guide and a person who brings all the positive vibes.

Most importantly, my career would not have moved forward without the support of my beautiful and caring wife. She kindles the light of hope whenever I am frustrated. She is my happiness and motivation. She is the best thing that has ever happened in my life. Thank you all, this work would not have been possible without you.

Dedication

I would like to dedicate this work to my family. My parents who made sacrifices in their life and worked hard for my success and happiness. My wife, Anuja without whom this work would not have accomplished.

Chapter 1 - Ectopic expression of a heterologous glutaredoxin enhances drought tolerance and grain yield in field grown maize

Abstract

Drought stress is a major constraint in global maize production, causing from 30 to 90% of the yield loss depending upon growth stage and the degree and duration of the stress. Here, we report that ectopic expression of *Arabidopsis* glutaredoxin *S17* (*AtGRXS17*) in field grown maize conferred tolerance to drought stress during the reproductive stage, which is the most critical for seed set and grain yield. *AtGRXS17*-expressing maize events displayed higher kernel sets in the field, resulting in 2-fold and 1.5-fold increase in yield in comparison to the non-transgenic counterparts when challenged with drought stress at the tasseling stage and silking/pollination stage, respectively. In the leaf tissues, *AtGRXS17*-expressing lines showed higher relative water content, higher chlorophyll content, and less hydrogen peroxide accumulation than wild-type (WT) control plants under drought conditions. *AtGRXS17*-expressing lines also exhibited at least 2-fold higher pollen germination rate than WT under drought stress. Compared to the transgenic maize, WT controls accumulated higher amount of proline, indicating that WT plants were more severely stressed over the same period of drought. Our results present a robust and simple strategy for meeting rising yield demands in maize under water limiting conditions.

Introduction

The world population is expected to grow to 9.7 billion by 2050, and global food production needs to be increased by twice to keep up with the demands of increasing population (Tilman *et al.*, 2011; United Nations, 2019). Maize is one of the most important crops in the world and widely used for food, feed, and biofuel. More than 1,100 million metric tons of maize were produced worldwide, and over 30% was produced in the U.S., making the country the largest producer and exporter of this crop (USDA 2020).

Drought is one of the environmental stresses that affects the quality and quantity of crop yield (Lesk *et al.*, 2016; Mishra and Cherkauer, 2010). Drought severely affects plant growth and development and, consequently, leads to death of the plant by affecting various physiological and biochemical processes such as photosynthesis, respiration, and nutrient uptake and translocation (Jaleel *et al.*, 2009). It is estimated that drought events will be more frequent, intense and longer in agriculturally important regions and pose a serious threat to food security (Lesk *et al.*, 2016; Xu *et al.*, 2016; Zipper *et al.*, 2016). Since 1990, drought has affected almost 2 billion people and has caused global economic losses of $6 to $8 billion annually (Zipper *et al.*, 2016). Drought impacts maize production and growth from vegetative stages to the reproductive stages (Zheng *et al.*, 2010). The 2012 US drought had a substantial impact in the global agricultural market (Al-Kaisi *et al.*, 2013; Rippey, 2015). During this drought period, corn production declined by more than 25% (Rippey, 2015). Similarly, the 1988 drought in the Midwest region caused 30% decrease in corn production and cost $3 billion in relief payments (Rosenzweig *et al.*, 2001).

Transgenic approaches have been made to improve drought tolerance in plants. These include engineering of genes that encode proteins related to osmotic adjustment, plant hormone synthesis, ion transporters, chaperones, transcription factors, and reactive oxygen species (ROS) (Deikman

et al., 2012; Umezawa *et al.*, 2006; Yang *et al.*, 2010). In maize, overexpression of *ZmZAR1* improved the plant growth and traits related the drought-stress tolerance (Guo *et al.*, 2014). Ectopic expression of the *Arabidopsis NF-YB2* in maize exhibited higher chlorophyll index, photosynthesis rate, stomatal conductance and 50% more yield in field (Nelson *et al.*, 2007). Similarly, RNAi lines created by knocking down the ACC synthase in maize showed reduced anthesis-silking interval, thereby, increasing the kernel set (Habben *et al.*, 2014). Drought tolerance in maize has also been reported to be improved by ectopically expressing genes such as *NPK1* from tobacco*, TsCBF1* and *TsVP* from *Thellungiella halophila, betA* from *E.coli, HVA1* from Barley, and *TPP* from rice (Li *et al.*, 2008; Nuccio *et al.*, 2015; Quan *et al.*, 2004; Shou, 2004; Xuan Nguyen, 2013; Zhang *et al.*, 2010).

ROS function as the signaling molecules under the control of the antioxidant system (Thannickal and Fanburg, 2000). However, under abiotic and biotic stresses, including drought, the ROS production is rapidly increased. Overproduction of the ROS disables the antioxidant defense system and causes oxidative damage to the lipids, proteins and DNA (Sofo *et al.*, 2015). Glutaredoxins (GRXs) are small ubiquitous oxidoreductase that belongs to thioredoxin family. This group of proteins maintain the cellular ROS homeostasis by catalyzing the reduction of disulfide bonds of target proteins through dithiol or monothiol mechanism in the presence of glutathione (Mashamaite *et al.*, 2015). Glutaredoxins are predicted to be found in various parts of the cells such as cytosol, chloroplast, and mitochondria and play a role in the oxidative stress response (Rouhier, 2010). They are also involved in Fe-S cluster assembly which are required for several processes, including photosynthesis, respiration, nitrogen and sulphur assimilation, ribosome synthesis and DNA repair (Rouhier, 2010). Based on the active site motifs, plant GRXs are grouped in the four classes. Class I, class II, class III and class IV have CxxC/S, CGFS, CCxx

and CxDC/s or CPxC active site motifs, respectively (Wu *et al.*, 2017). *Arabidopsis AtGRXS17* is a member of the class II GRXs that was shown to be involved in maintaining the ROS accumulation, auxin signaling and temperature-dependent postembryonic growth in the plants (Cheng *et al.*, 2011). Ectopic expression of *AtGRXS17* in plants has been reported to improve the plant tolerance against several abiotic stresses, such as heat, chilling and drought, at the vegetative stages (Hu *et al.*, 2015; Wu *et al.*, 2012; Wu, Hu, *et al.*, 2017). The expression of *AtGRXS17* is also crucial for maintaining the ROS homeostasis for protecting plants from oxidative damage (Wu *et al.*, 2012; Yu *et al.*, 2017). However, the effect of *AtGRXS17* in response to drought stress during the reproductive stage, particularly in the field, has not been studied yet. Here, we investigate the effects of *AtGRXS17* in field grown maize on the drought tolerance during the reproductive growth stages, and demonstrate that ectopic expression of this single gene in maize can increase the grain yield under drought stress conditions.

Materials and Methods

Cloning of *AtGRXS17*

Arabidopsis glutaredoxin, *AtGRXS17*, was driven by the maize ubiquitin promotor and terminated by nopaline synthase terminator. A detailed study of *AtGRXS17*-expressing lines were performed previously (Sprague, 2018). Three lines, *AtGRXS17-5*, *AtGRXS17-6* and *AtGRXS17-10* were used to study the effect of drought stress. Based on previous studies, *AtGRXS17-5* and *AtGRXS17-10* had one stable integration of T-DNA and *AtGRXS17-6* had multiple integration of T-DNA into the genome. The phenotype of transgenic plants and WT maize, B104 was indistinguishable throughout the life cycle of the maize.

qRT-PCR analysis of the maize endogenous monothiol glutaredoxin 17, *ZmGRXS17*

Maize inbred lines B73, B104, A188 and HiIIA were grown in PRO-MIX®BRK MYCORRHIZAE™ (Premier Horticulture Ltd.) at 27°C/22°C (day/night) in the greenhouse. At V5 growth stage, the seedlings were assigned to the drought treatments with three biological replicates. Plants were imposed drought stress by holding the water. Leaf samples were collected from each plant on day 0, 2, 4, 6 and 8, and immediately frozen in liquid nitrogen and stored in the -80°C freezer for further analysis.

Total RNA was isolated from leaf tissues of inbred maize lines using the Direct-zol RNA Miniprep Plus Kits (Zymo Research). One µg total RNA was used to synthesize first strand cDNA using Revert Aid First Strand cDNA synthesis kit (Thermo Scientific™). Each 20uL qRT-PCR reaction consisted of 9.4 µL diluted cDNA solution (2ng/µL), 0.3 µL of each primer (20µM), and 10 µL iQ SYBR Green Supermix (Bio-Rad). Primers 5'-CCGTCATCGCCTCACGAAGAG-3' and 5'-AGAGCCTGCCTTACGGAATTGG-3', which are based on the cyclin-dependent kinase gene CDK, were used as an expression control.

Field trial

WT B104 and transgenic plants were grown in a randomized block design with 10 replications at the Rockyford Research station in Manhattan, KS. Seeds were germinated in 4-inch pots. Three weeks old seedlings were transferred into the 5-gallon pots containing PRO-MIX®BRK MYCORRHIZAE™ and pasteurized field soil in 1:1 ratio, and subsequently, transferred to the field. Approximately at V10 stage, poly film cover was placed on the rainout shelter to prevent the effect of rainwater during drought treatment. The first drought treatment that started at VT stage was continued for 6 days. The second drought treatment was started at R1 stage and continued for 5 days.

Greenhouse experiment

WT and *AtGRXS17*-expressing lines were grown in the greenhouse at 27°C/22°C (day/night) in 1-gallon pots filled with equal volume of Pro-Mix®BRK Mycorrhizae™ until VT stage. At VT stage, drought stress was imposed by withholding water for 5 days. After 5 days, watering was restarted to recover, and pollination was performed manually under well-watered condition.

Pollen germination analysis

Pollen grains from *AtGRXS17*-expresing lines and WT were collected at T1 stage. Pollen grains were incubated on a liquid media comprising of 0.0005% boric acid, 10mM calcium chloride, 0.05 mM monopotassium phosphate, 10% sucrose and 6% polyethylene glycol 4000 for 30 minutes and observed under the light microscope. The tassel was kept in the room temperature inside a Petri dish to mimic drought environment. The pollen germination was observed at 0 hours, 4 hours and 8 hours. In the *in vitro* study, germination was scored when pollen tube length is at least equal to the diameter of the pollen grain.

Proline content measurement

B104 and three *AtGRXS147*-expressing lines were grown until the T1 stage. The plants were grown under the control and drought (8 days) conditions. The flag leaf tissues were collected and proline content was determined using a colorimetric assay as described previously (Hu *et al.*, 2017). Briefly, 100 mg of ground leaf samples were suspended into 500 µl of 3% sulfosalicylic acid and centrifuged for 5 minutes at 13,000 rpm for 5 minutes. 100 µl of supernatant was added to 500 µl of reaction mixture that contains 100 µl of 3% sulfosalicylic acid, 200 µl of glacial acetic acid and 200 µl of acidic ninhydrin solution. The mixtures were incubated at 96°C for 1 hour and reaction was terminated in ice. 1 ml of toluene was added to the reaction mixture and the samples were vortexed for 20 seconds. The reaction was left in the room temperature for 5 minutes to allow the separation of the organic and water phases. The upper organic phase was used to determine proline content by measuring the absorbance at 520 nm using toluene as reference. The proline concentration was determined using a standard concentration curve and calculated on FWbasis ($mg \cdot g^{-1}$).

Chlorophyll content measurement

WT along with three transgenic lines were grown in the greenhouse under optimum conditions. Drought stress was imposed on the maize plants at VT stage for 8 days. Chlorophyll content (chlorophyll a and b) along with the carotenoid contents were measured from control and drought-stressed plants as previously explained (Lichtenthaler 1987). Briefly, chlorophyll contents along with carotenoids were extracted from 0.2 grams of leaf samples using 100% acetone. The extract was centrifuged for 5 minutes at 500 x g. Chlorophyll and carotenoid contents were determined by measuring the absorbance at 662 nm, 645 nm and 470nm. The following equations were used to determine the chlorophyll and carotenoid contents.

Chlorophyll content: $C_a = 12.25\ A_{662} - 2.79\ A_{645}$

$C_b = 21.50\ A_{645} - 5.10\ A_{662}$

Total carotenoids $(x+c) = (1000\ A_{470} - 1.82\ C_a - 85.02 C_b)/198$

The weight ratio of Chls a and b to the total carotenoid, (a+b)/(x+c), was determined.

Relative water content (RWC)

WT along with two *AtGRXS17*-expressing lines with one insertion were grown at 27°C/22°C (day/night) with 14-hour photoperiod in the growth chamber. After 4 weeks, the seedlings were subjected to the drought treatment by withholding water for 5 days. The measurement of RWC was performed as described previously (Barrs and Weatherley, 1962). Briefly, the leaves (2cmX3cm) from the control and drought-stressed seedlings from were excised, and fresh weight (FW) was measured. Then the leaves were immersed into the distilled water for 4 hours and turgid weight (TW) was measured. Finally, dry weight (DW) was measured after drying the leaves at 80°C overnight. The RWC was calculated as follows: RWC= (FW-DW)/(TW-DW).

Stomatal conductance and chlorophyll fluorescence measurement

WT and *AtGRXS17*-expressing lines were grown until the T1 stage. The plants were subjected to the drought. Stomatal conductance was measured using SC-1 leaf porometer (Decagon Devices) on day 1, day 5 and day 8 of the drought treatment. Stomatal conductance was measured in the abaxial surface of the leaves. Similarly, phytochemical efficiency of the leaves was determined by chlorophyll fluorescence ratios (Fv/Fm) using portable chlorophyll fluorometer (OS30p+, Opti-Sciences). The chlorophyll fluorescence measurement was taken from dark-adapted leaves on day 0, day 2, day 4, day 6 and day 8 of drought treatment.

H_2O_2 assay

Hydrogen peroxide was measured from WT and *AtGRXS17*-expressing maize plants grown in controlled condition and drought stressed condition using Amplex® Red Hydrogen Peroxide/

Peroxidase Assay kit (Invitrogen). Briefly, 100 mg of ground leaf samples were suspended in 1X reaction buffer and mixed well. 50 µl of the working solution of 100 µM of Amplex® Red reagent and 0.2U/ml Horseradish Peroxidase was mixed to 50 µl of the sample solution in a well of a microplate and the reaction was incubated in the room temperature in dark for 30 minutes. The H_2O_2 concentration was determined by measuring the absorbance at 560 nm using no-H_2O_2 as control. H_2O_2 concentration was determined using a standard concentration curve and calculated on FWbasis ($\mu g.mg^{-1}$).

qPCR of leaf tissues

Leaf tissues from three biological replicates of WT and *AtGRXS17*-expressing lines grown in the well-watered condition and drought stressed condition were collected. Total RNA was isolated from leaf tissues of inbred maize lines using the Direct-zol RNA Miniprep Plus Kits (Zymo Research). One µg total RNA was used to synthesize first strand cDNA using Revert Aid First Strand cDNA synthesis kit (Thermo Scientific™). qRT-PCR was carried for different genes listed on the Table 1.1.

Results

Upregulation of *ZmGRXS17* expression under drought stress

Maize endogenous monothiol glutaredoxin S17, *ZmGRXS17*, expression was measured in the leaf tissues of B73, B104, A188 and HiIIA in response to the drought stress. The expression pattern of *ZmGRXS17* under the drought stress was similar across the inbred lines (**supplementary fig. 1.1a**). *ZmGRXS17* transcripts increased in response to the drought on day 2 and decreased on day 4 of drought stress in all the inbred lines. However, the *ZmGRXS17* expression increased gradually after 4 days as the period of drought was extended. In B104, the transcript expression was 5.9 times higher than the initial levels after 2 days of drought stress (**supplementary fig. 1.1a**). The relative expression of *ZmGRXS17* was 2.2 times on day 4, 3.6 times on day 6 and 4 times higher on day 8 of the drought stress compared to the initial level of the expression.

AtGRXS17-expressing maize plants increase yield under drought stress field conditions

Previously, a binary vector containing *AtGRXS17* driven by the maize *ubiquitin-1* (*Ubi-1*) promoter was transformed to generate *AtGRXS17*-expressing B104 maize lines and subsequently homozygous *AtGRXS17*-expressing T2 generation seeds were obtained (Sprague, 2018). Three *AtGRXS17*-expressing maize lines (*S17-5*, *S17-6*, and *S17-10*) along with wild-type (WT) B104 plants were grown in the field. Drought stress was imposed at VT (tassel) and R1 (silking) stages by withholding watering, and the yield was compared. During the period of the drought stress imposition, the average maximum daily temperature and average daily temperature were 30.4°C and 23.5°C, respectively (**Fig. 1.1a**).

When drought stress was imposed at VT and R1 stages, all the *AtGRXS17*-expressing lines had higher yield than WT, while there was no significant difference in the phenotype and yield between *AtGRXS17*-expressing lines and WT under well-watered conditions (**Fig. 1.1b-d)**. Drought stress

had less impact on the kernel number set in each ear of *AtGRXS17*-expressing lines compared to the WT (**Fig. 1.1d**). Kernel numbers under drought treatments at the VT stage were 75.5, 176.4, 204.2 and 168.1 for WT, *S17-5*, *S17-6*, and *S17-10*, respectively. Similarly, Kernel numbers under the drought treatments at the R1 stage were 169.7, 287.5, 260.3 and 259.4 for WT, *S17*-5, *S17*-6 and *S17*-10, respectively. In WT and transgenic maize, the weight of seeds per thousand was not different in control and drought during the R1 stage. However, when drought stress was imposed at the VT stage, *AtGRXS17*-10 had significantly higher seed weight compared to the WT and other *AtGRXS17*-expressing lines (**supplementary fig. 1.1b)**. On average, *AtGRXS17*-expressing lines showed at least 127% and 48% more yield than WT when drought stress was imposed at VT and R1 stage in the field, respectively (**Fig. 1.1c)**. Overall, *AtGRXS17*-expressing lines had the higher yield per plant than the WT in field. Similar results were observed in the greenhouse experiments (**supplementary fig. 1.2a-c**). Our data also indicated that the drought stress was more severe if imposed at the VT stage when compared with the R1 stage in the field (**supplementary fig. 1.2c**).

***AtGRXS17*-expressing maize pollen grains are more drought tolerant than WT pollen grains**

In order to find the effect of ectopic expression of *AtGRXS17* in maize pollen grains under drought, the fresh tassels were collected and kept in the petri dish to mimic drought environment. The pollen grains were collected at 0, 4 and 8 hours and incubated in the pollen germination media. A total of 15,176 pollen grains were scored for the germination. Germination was scored when the pollen tube was formed and the tube length was at least equal to the diameter of the pollen grain (**Fig. 1.2a).** Fresh pollen grains collected from the tassels at 0 hour had an average of 64% germination rate. At 0 hour, germination rate for WT, *AtGRXS17-5*, *AtGRXS17-6* and *AtGRXS17-10* was 63.10%, 70%, 64.30% and 58.5% respectively. After keeping the tassels in the petri dish for 4 hours, the pollen germination rate decreased. WT pollen germination was 23.4% and *AtGRXS17*-

expressing maize pollen grains had an average germination rate of 42.9%. After 8 hours, the germination rate reduced more; however, the pollen germination rate was more than double in *AtGRXS17*-expressing lines compared to WT (**Fig. 1.2b-c**).

Effect of ectopic expression of *AtGRXS17* on proline accumulation, chlorophyll content, relative water content, and stomatal conductance under drought conditions

To measure the physiology and biochemical activities in *AtGRXS17*-expressing maize, the transgenics and WT plants were grown in the greenhouse and were subjected to the drought stress. The proline content was not different between WT and *AtGRXS17*-expressing maize plants under control conditions. After 8 days of drought treatment, the proline content increased in both WT and *AtGRXS17*-expressing maize plants. However, the proline content of WT plants was higher than that of transgenic plants (**Fig. 1.3a**). The result indicates that the WT plants were more stressed under drought conditions as compared to the transgenic maize plants.

Chlorophyll (a and b) and carotenoid contents of the transgenics and WT plants were not different under the well-watered conditions, while the *AtGRXS17*-expressing plants had higher concentration of chlorophyll and carotenoid than the WT under drought conditions (**Fig. 1.3b**). Similarly, the ratio of chlorophyll to carotenoid content was also significantly higher in *AtGRXS17*-expressing maize plants under drought conditions (**Fig. 1.3c**).

Relative water content (RWC) declined in both *AtGRXS17*-expressing and WT maize plants after 5 days of drought treatment. However, transgenic maize plants significantly retained more water than the WT. The RWC in WT was dropped to 46.6% but RWC in transgenic maize plants dropped to 66% on an average (**Fig. 1.3d**).

Stomatal conductance of both *AtGRXS17*-expressing and WT plants gradually decreased as the drought stress was imposed. The stomatal conductance remained similar for both the transgenic

and WT lines on day 1 and day 5 of drought treatments but significantly decreased in WT on day 8 (**Fig. 1.4a**). Chlorophyll fluorescence, as measured by Fv/Fm ratio, gradually decreased under drought treatment. However, no difference in the chlorophyll fluorescence was observed on ear and second leaves in both WT and *AtGRXS17*-expressing lines (**Fig. 1.4b**).

***AtGRXS17*-expressing maize plants produce less H_2O_2 compared to the WT**

H_2O_2 content increased after imposing drought stress. H_2O_2 content was similar in both WT and *AtGRXS17*-expressing maize plants when grown in well-watered conditions, while H_2O_2 content was significantly increased in WT when compared with *AtGRXS17*-expressing maize plants under drought conditions (**Fig. 1.4c**).

Drought stress results in altered gene expression in maize plants

The effect of drought stress on the expression of several drought-stress related genes was measured in both WT and *AtGRXS17*-expressing maize plants. Drought treatments induced the changes in expression of genes (**Fig. 1.5a-h)**. *S17-5* displayed the highest expression of the transgene *AtGRXS17*, followed by *S17-6*, and *S17-10* (**Fig. 1.5a)**. However, we did not observe the yield difference among the transgenic lines. *ZmGRXS17*, an endogenous GRXS17 of maize, had significant increase in the expression in WT than the *AtGRXS17*-expressing maize lines under drought conditions (**Fig 1.5b)**. Similarly, antioxidation enzymes showed varied effect of drought stress on their expression (**Fig. 1.5c, 1.5d**). Under drought, catalase (*ZmCAT1)* expression was highly increased in WT, while L-ascorbaste peroxidase (*ZmAPX*) expression was lower in WT than *AtGRXS17*-expressing maize lines. *ZmABI5*, ABSCISIC ACID-INSENSITIVE 5, expression was higher in the WT than *AtGRXS17*-expressing maize lines after drought treatment (**Fig. 1.5e)**. Brassinosteroid synthesis 1 (*ZmBRS1*) expression decreased under drought conditions. WT showed lower expression of *ZmBRS1* than *AtGRXS17*-expressing maize lines (**Fig. 1.5f)**. Heat

shock proteins (HSPs) experienced higher expression under drought stress, with the expression of both *ZmHSP*s significantly higher in the WT than *AtGRXS17*-expressing maize lines after drought treatment (**Fig. 1.5g, 1.5h)**.

Discussion

Maize is a water-sensitive crop. Water-deficit conditions during the pollination and early grain-filling stages causes serious yield loss (Çakir, 2004). Similarly, drought imposition at the VT stage was found to have greater negative impact in corn grain yield than the vegetative stage (Atteya 2003). We also found that drought stress imposition at T1 stage had negative yield impact. Like other abiotic stresses, drought enhances the ROS production which can affect the cellular processes by causing oxidative damage. *GRXS17* plays an important role in maintaining the H_2O_2 accumulation during abiotic stress. The loss of function mutants (*atgrxs17*) of *Arabidopsis* had high accumulation of ROS in growing tissues compared to the WT under heat stress (Cheng *et al.*, 2011). Similarly, *OsGRXS17* RNAi lines of rice showed higher H_2O_2 accumulation in the presence or absence of abscisic acid, indicating that *OsGRXS17* is important to maintain the ROS homeostasis during drought (Hu *et al.*, 2017). In agreement with these studies, ectopic expression of *AtGRXS17* in tomato reduced the H_2O_2 accumulation under heat and chilling stress (Hu *et al.*, 2015; Wu *et al.*, 2012). We also found lowered H_2O_2 accumulation in *AtGRXS17*-expressing maize under drought stress indicating that *AtGRXS17* plays a role in reducing the ROS accumulation during drought stress.

Pollen viability can be affected by several factors, including moisture. Maize pollen is considered 'desiccation intolerant' and loses water and viability rapidly (Aylor, 2003; Luna V. *et al.*, 2001). It was reported that pollen moisture content is reduced by half within an hour at 32.5°C and 50% relative humidity, and B104 pollen viability is lost when pollen moisture content is around 32% (Fonseca and Westgate, 2005). We also found that pollen germination rapidly decreased when pollen grains were kept for a longer period in a desiccating condition. However, *AtGRXS17*-expressing plants maintained higher RWC under drought stress and had higher pollen viability

compared to WT after 2 and 4 hours of desiccation period. WT pollen viability after 8 hours remained below 15% while *AtGRXS17*-expressing maize pollen viability was 28%, suggesting that the high kernel set in the *AtGRXS17*-expressing plants is due to higher pollen viability at limited water conditions.

Drought stress and low water content generally lead to stomatal closure, resulting in decreased transpiration and photosynthesis (Ouyang *et al.*, 2017). This, in turn, increases ROS accumulation that can cause heavy damage to cells if remained unchecked (Cruz de Carvalho, 2008; Kar, 2011). A high RWC is a drought resistance mechanism rather than drought-escape mechanism (Atteya 2003; Merah, 2001). In our study, we found that *AtGRXS17*-expressing maize lines have higher stomatal conductance and RWC than WT under drought stress. We also observed higher chlorophyll and carotenoid contents in *AtGRXS17*-expressing maize lines than WT under drought stress. Drought tolerant maize have been shown to possess higher chlorophyll content under drought stress. Drought tolerant corn, genotypes BC678 and BC404, had higher chlorophyll content and higher yield due to less damaged chloroplasts (Khayatnezhad *et al.*, 2011). Similarly, drought tolerant cultivars of wheat had higher RWC and chlorophyll content (Keyvan 2010). We found that the ratio of chlorophyll to carotenoid was significantly low in the WT under drought stress. Lower ratio of chlorophyll to carotenoids is the indicator of plant stress and damage to the photosynthesic apparatus (Lichtenthaler and Buschmann, 2001). Taken together, these data suggest that *AtGRXS17*-expressing maize plants were less stressed than WT and, consequently, more drought tolerant. Furthermore, we found that after 8 days of drought stress, WT plants accumulated approximately 32% more proline and approximately 36% more H_2O_2 than *AtGRXS17*-expressing maize lines. Proline is a compatible osmolyte that accumulates when plants are stressed. Proline accumulation is dependent upon H_2O_2 accumulation. H_2O_2 is found to

promote the ABA-induced proline accumulation in *Arabidopsis* (Verslues *et al.*, 2007). Elevated proline accumulation could be an indicator of the degree of drought stress in maize plants. Proline accumulation could be due to reduced water status in the maize, rather than the drought resistance mechanism (Ibarra-Caballero *et al.*, 1988). We postulate that *AtGRXS17* expression assisted maize plants in maintaining water status during drought which, in turn, caused the transgenic plants to accumulate less H_2O_2 and, subsequently, proline.

Abscisic acid (ABA) is a stress hormone that acts as a key regulator for abiotic stress resistance in plants along with several aspects of plant growth and development (Vishwakarma *et al.*, 2017). Endogenous ABA is reported to increase under drought stress in maize (Beardsell and Cohen, 1975; Wang *et al.*, 2008) and participates in a network of stress-signaling mechanisms (Brocard *et al.*, 2002). Exogenous application of ABA can improve drought tolerance in various plant species (Waterland *et al.*, 2010; Wei *et al.*, 2015). Exogenous application of ABA is also known to enhance the activities of the antioxidant enzymes, such as catalase, peroxidase, ascorbate peroxidase, superoxide dismutase and glutathione reductase (Wang *et al.*, 2011). ABA INSENSITIVE 5 (*ABI5*), a leucine zipper transcription factor, is regulated by ABA (Finkelstein and Lynch, 2000; Lopez-Molina *et al.*, 2001) and functions in various abiotic stress adaptation (Skubacz *et al.*, 2016). Several homologs of *ABI5* have been studied in relation to the abiotic stresses. *HvABI5*, a barley *ABI5*, activated the stress-responsive genes under drought stress ensuring the plant adaptation to the limited water conditions (Collin *et al.*, 2020). Similarly, wheat *ABI5*, *WABI5*, improved the freezing and osmotic tolerance in the transgenic tobacco (Kobayashi *et al.*, 2008). However, ectopic expression of *ZmABI5* in tobacco showed that transgenic lines were more sensitive to drought, salt, high and low temperature, suggesting that *ZmABI5* plays a negative regulatory role in stress response (Yan *et al.*, 2012). Transgenic *Arabidopsis* plants expressing *OsABI5* were also

sensitive to the salinity and drought stress, indicating negative regulatory role in stress response (Zou *et al.*, 2008). In our study, *ZmABI5* expression was upregulated by 1.5-folds compared to the WT under drought stress, supporting that WT lines were more sensitive to the drought stress than *AtGRXS17*-expressing maize plants as previously reported.

Catalase (CAT) activity has been shown to increase rapidly under drought conditions in several plant species (Luna, 2004; Mafakheri *et al.* 2011; Ren *et al.*, 2016). On the other hand, the CAT activity can also decrease or remain unchanged (Smirnoff, 1993). CATs have been identified as interacting target proteins of GRXs (Lee *et al.*, 2010; Rouhier *et al.*, 2005). We observed interesting expression pattern of *ZmCAT1* in our study under drought stress in maize. *ZmCAT1* expression increased in maize plants after drought stress, however, the *CAT1* expression was greater in WT. This result contradicts the studies involving heat, chilling and drought stress where CAT activity increased in *AtGRXS17*-expressing tomatoes (Hu *et al.*, 2015; Wu *et al.*, 2012; Wu, Hu, *et al.*, 2017). Consistent with this study, *OsGRXS17* knock-down RNAi rice plants showed increased H_2O_2 accumulation and reduced CAT activity under drought conditions (Hu *et al.*, 2017). An explanation is that higher *CAT1* expression in WT is due to higher H_2O_2 accumulation during drought stress. Conversely, we found *ZmAPX* expression decreased by more than half under drought stress in both transgenic maize and WT. APX activity is shown to decrease in several plant species such as sorghum, rice and maize under drought stress (Anjum *et al.*, 2017; Zhang *et al.*, 2013; Zhang and Kirkham, 1996). Our results showed at least two-fold increase in expression of *ZmAPX* in transgenic maize compared to WT under drought. The increase in expression of *ZmAPX* might compensate to detoxify the ROS in transgenic maize. A balance of ROS scavenging enzymes is crucial for ROS level suppression. When CAT activity decreases, activity of

scavenging enzymes such as APX should increase as a compensatory mechanism (Apel and Hirt, 2004).

Heat shock proteins (HSPs) are also one of the targets of GRXs (Rouhier *et al.*, 2005). Heat shock proteins, also termed molecular chaperones, stabilize proteins at intermediate stages of their formation and assist in folding, association, translocation, and degradation of the proteins across the membranes (Priya *et al.*, 2019). Heat shock proteins are induced by several abiotic stresses, including drought stress (Augustine, 2016). Drought sensitive plants have been shown to be negatively regulated by HSPs. In *Arabidopsis*, overexpression of *AtHsp90* conferred tolerance to calcium but plants were more sensitive to salt and drought stresses (Song *et al.*, 2009). Similarly, ectopic expression of the *AsHSP26.8a* in *Arabidopsis* resulted in the hypersensitivity to ABA and salinity stress and reduced tolerance to the heat stress (Sun *et al.*, 2020). We observed that expression of *ZmHsp90* and *ZmHsp26* was moderately higher in WT as compared to *AtGRXS17*-expressing maize plants under drought stress. This suggests that WT could require higher expression of the *HSPs* due to greater sensitivity to the drought stress than transgenic maize plants. Though HSPs might be the interacting target protein of GRXs, it remains unclear how AtGRXS17 interacts with HSPs.

In summary, we found that *AtGRXS17*-expressing maize plants were less stressed under conditions of drought stress. This is reinforced by data showing that transgenic lines had higher RWC than the WT under drought stress. We hypothesized that WT plants close stomata sooner than the *AtGRXS17*-expressing lines under drought stress due to reduced plant water status. This contributed to higher accumulation of ROS in WT as compared to *AtGRXS17*-expressing maize plants. The hypothesis is supported by our stomatal conductance and hydrogen peroxide content. The result that *AtGRXS17*-expressing maize plants are more drought tolerant than WT is further

supported by the proline accumulation and several drought stress-related gene expression data. The evidence, along with the higher pollen viability in *AtGRXS17*-expressing maize plants under drought stress, makes clear that ectopic expression of *AtGRXS17* in maize confers tolerance to drought stress during the reproductive stage and improves the overall grain yield.

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Figures

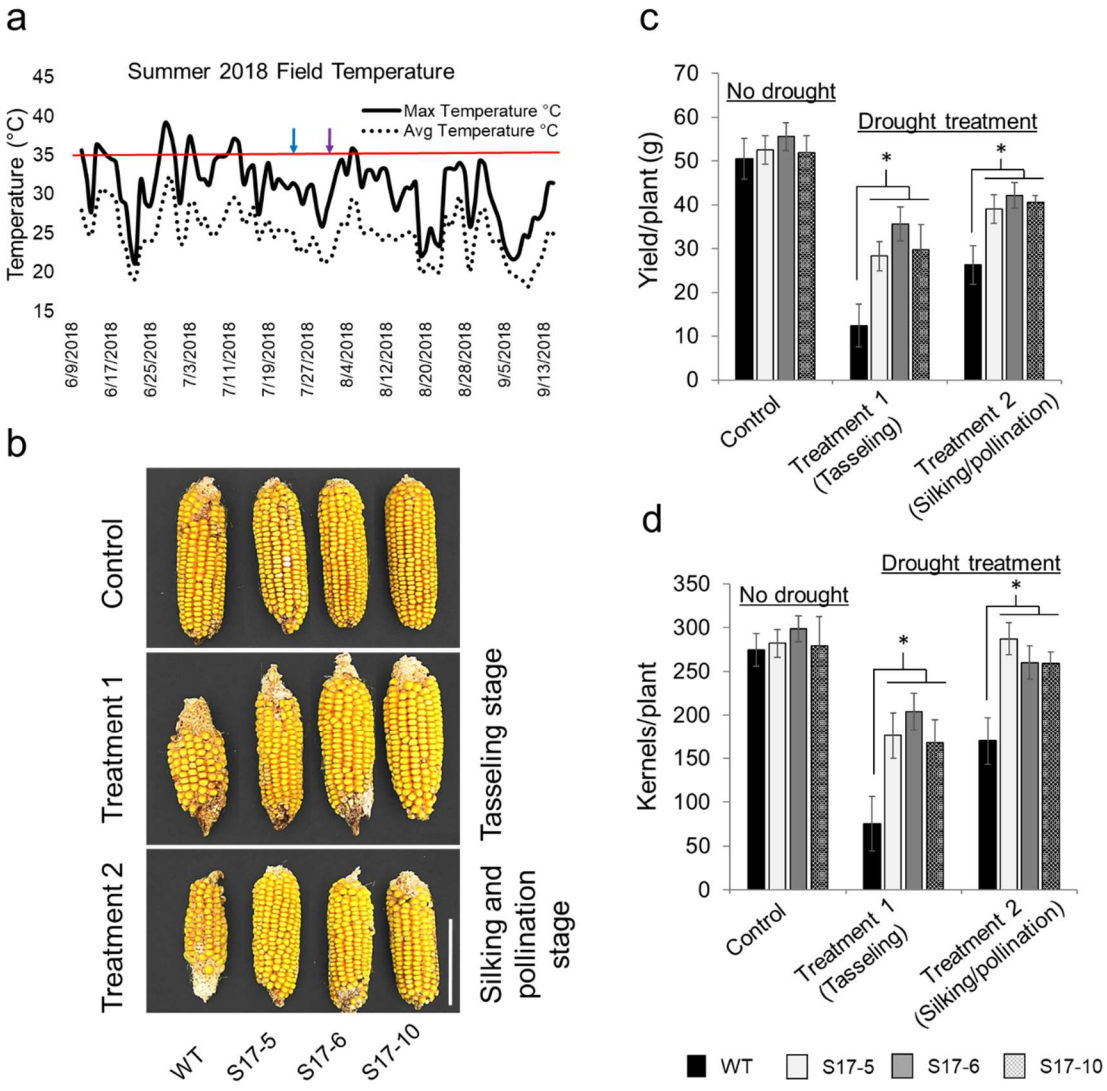


Figure 1.1 Effect of ectopic expression of *AtGRXS17* in maize yield under drought in the field. **(a)** Average and maximum daily temperature during the drought stress. The blue arrow represents the drought stress initiation at the VT stage and purple arrow represents the drought initiation at the R1 stage. During the period of the drought stress, the maximum temperature remained below 35°C as represented by a red bar. **(b)** Cobs from WT and *AtGRXS17*-expressing lines grown in the field under controlled condition and drought condition at VT (Tasseling stage) and R1 (Silking stage). **(c)** Average yield per maize plant harvested from field grown maize at well-watered

condition (n=12) and drought at the VT (n=12) and R1 (n=12) stages. **(d)** Number of kernels per plant grown in the field under well-watered condition and drought at the VT and R1 stages. Data shown are Mean ± SE and were analyzed using one-way ANOVA. Asterisks indicate level of significance (*P<0.05).

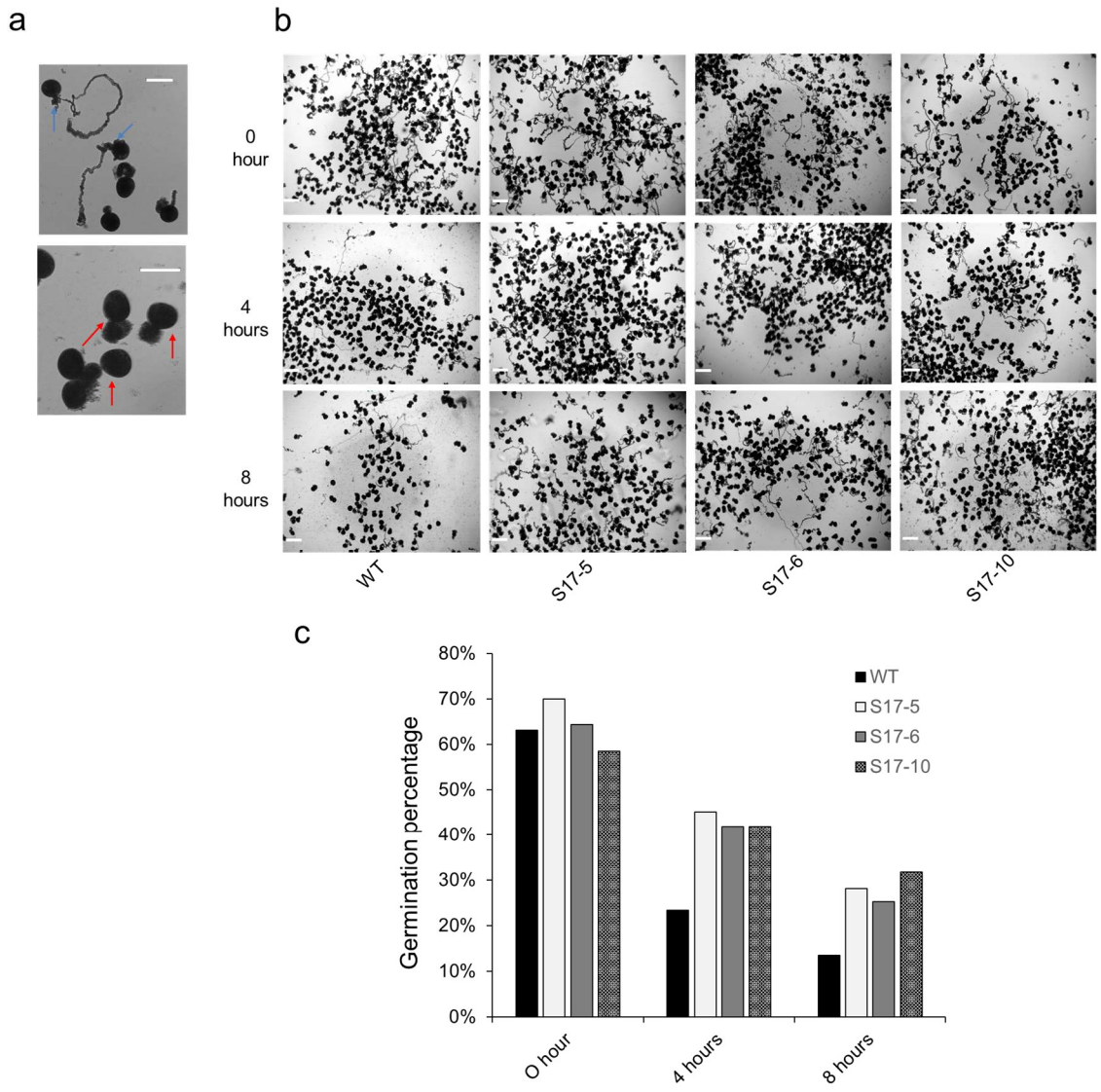


Figure 1.2 Pollen germination of the *AtGRXS17*-expressing and WT maize under drought stress.

(a) Pollen germination (Upper panel; blue arrow) and pollen grains that failed to germinate (Lower panel; red arrow) in the pollen germination media. **(b)** Time course germination rate of pollen grains kept at room temperature. **(c)** Pollen germination rate at 0 hour, 4 hours and 8 hours timepoint of the WT and *AtGRXS17*-expressing maize plants. Scale bars = 100µm.

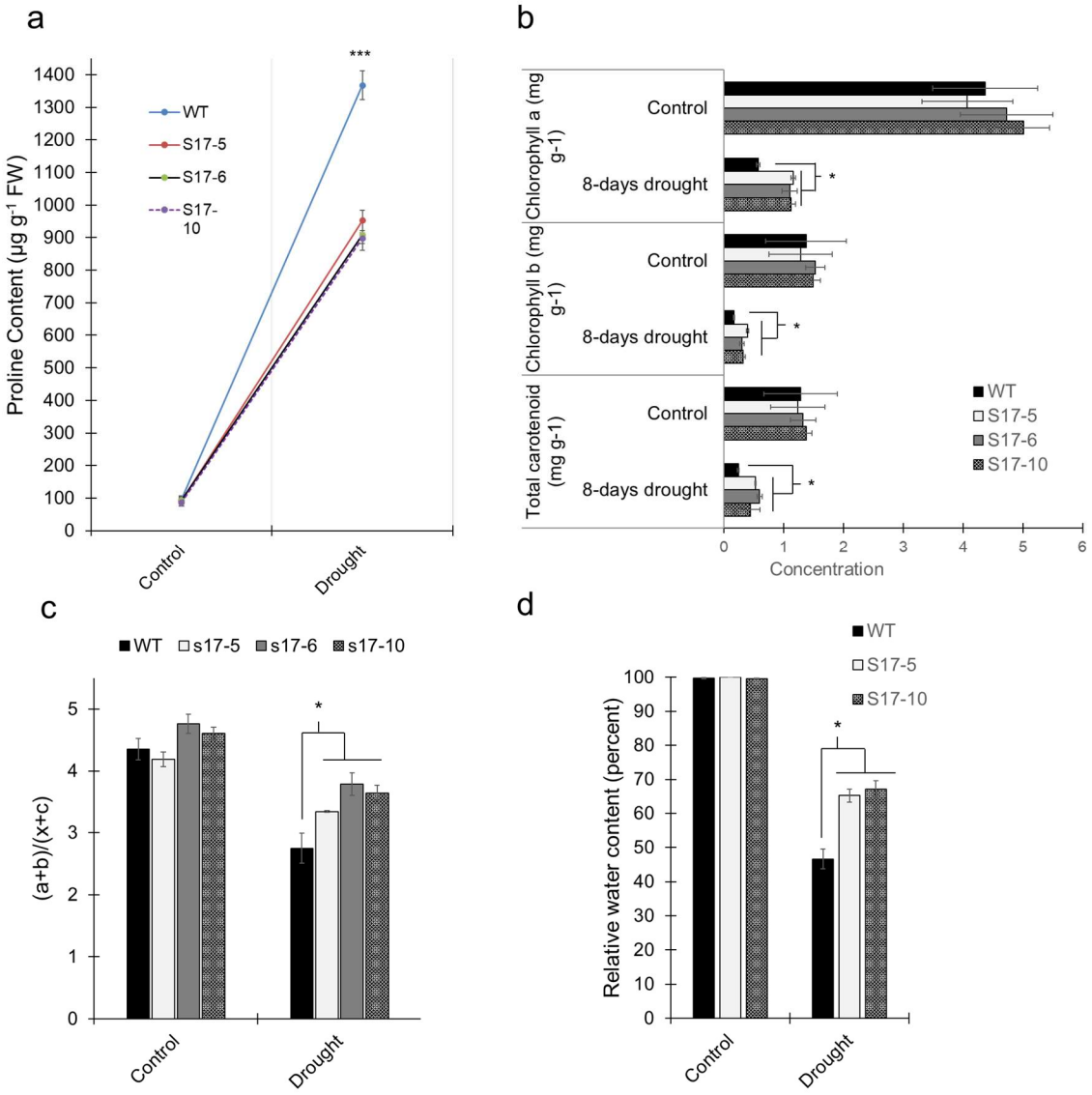


Figure 1.3 *AtGRXS17*-expressing maize were less stressed than WT under drought stress.
(a) Proline accumulation of WT and *AtGRXS17*-expressing maize plants under the control (well-watered) and drought stress after 8 days. **(b)** Total chlorophyll and carotenoid content in the WT and *AtGRXS17*-expressing maize plants under the control (well-watered) and drought stress after 8 days. **(c)** Graph represents the stress as measured by the ratio of total chlorophyll to total carotenoids. **(d)** Relative water content of 4-week-old WT and two *AtGRXS17*-expressing maize seedlings under the control (well-watered) and drought stress after 5 days. Data shown are Mean

± SE (n=6) and were analyzed using one-way ANOVA. Asterisks indicate level of significance (*P<0.05, ***P<0.001).

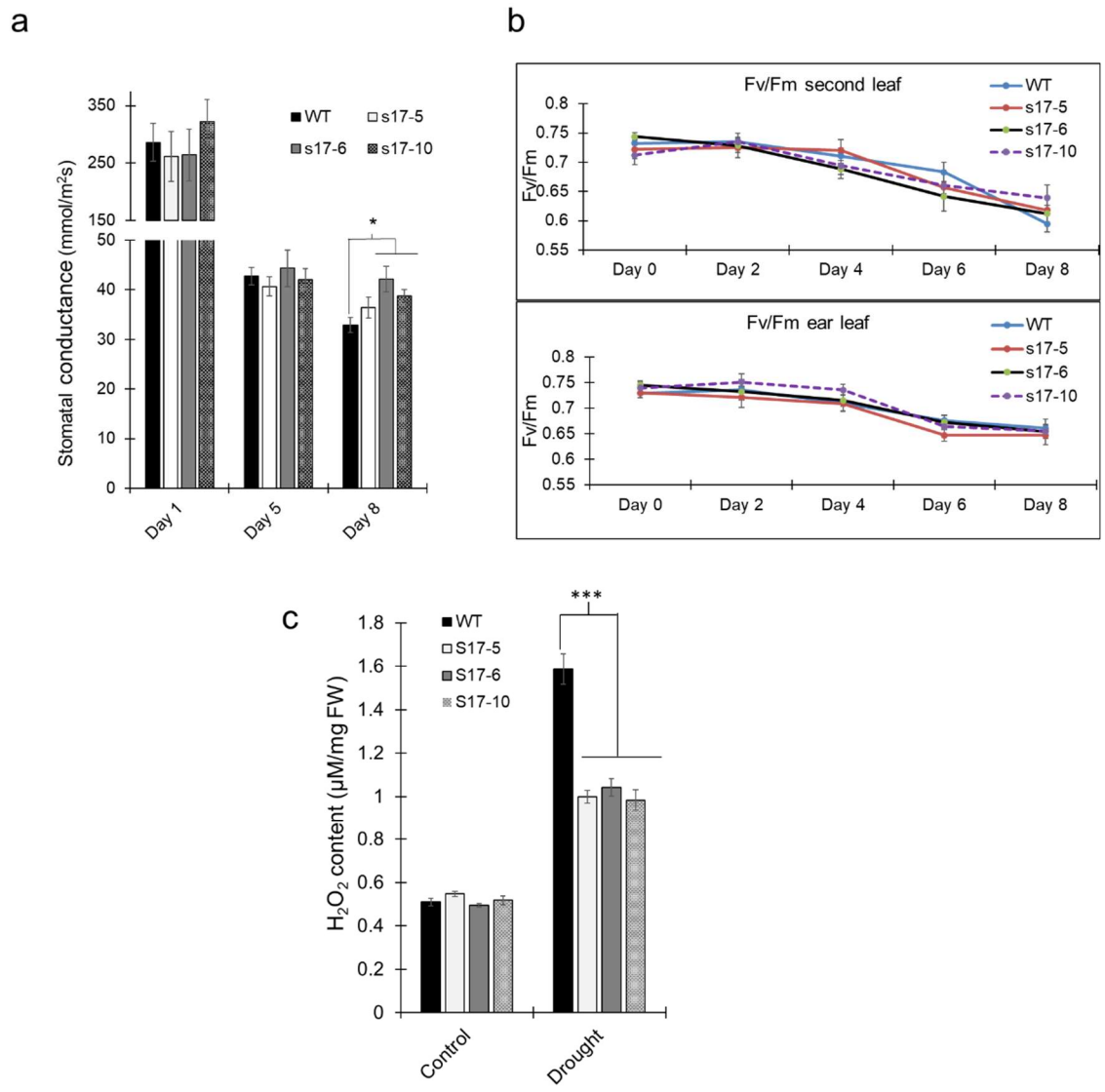


Figure 1.4 Effect of ectopic expression of *AtGRXS17* in stomatal conductance and H_2O_2 content. **(a)**Stomatal conductance of WT and *AtGRXS17*-expressing maize plants at VT stage as the drought progresses. **(b)** Chlorophyll fluorescence measurement of the ear leaf and second leaf from top in WT and *AtGRXS17*-expressing maize plants at VT stage as the drought progresses. **(c)** H_2O_2 content of the WT and *AtGRXS17*-expressing maize plants under the control (well-watered) and drought stress after 8 days. Data shown are Mean ± SE (n=6) and were analyzed using one-way ANOVA. Asterisks indicate level of significance (*P<0.05, ***P<0.001).

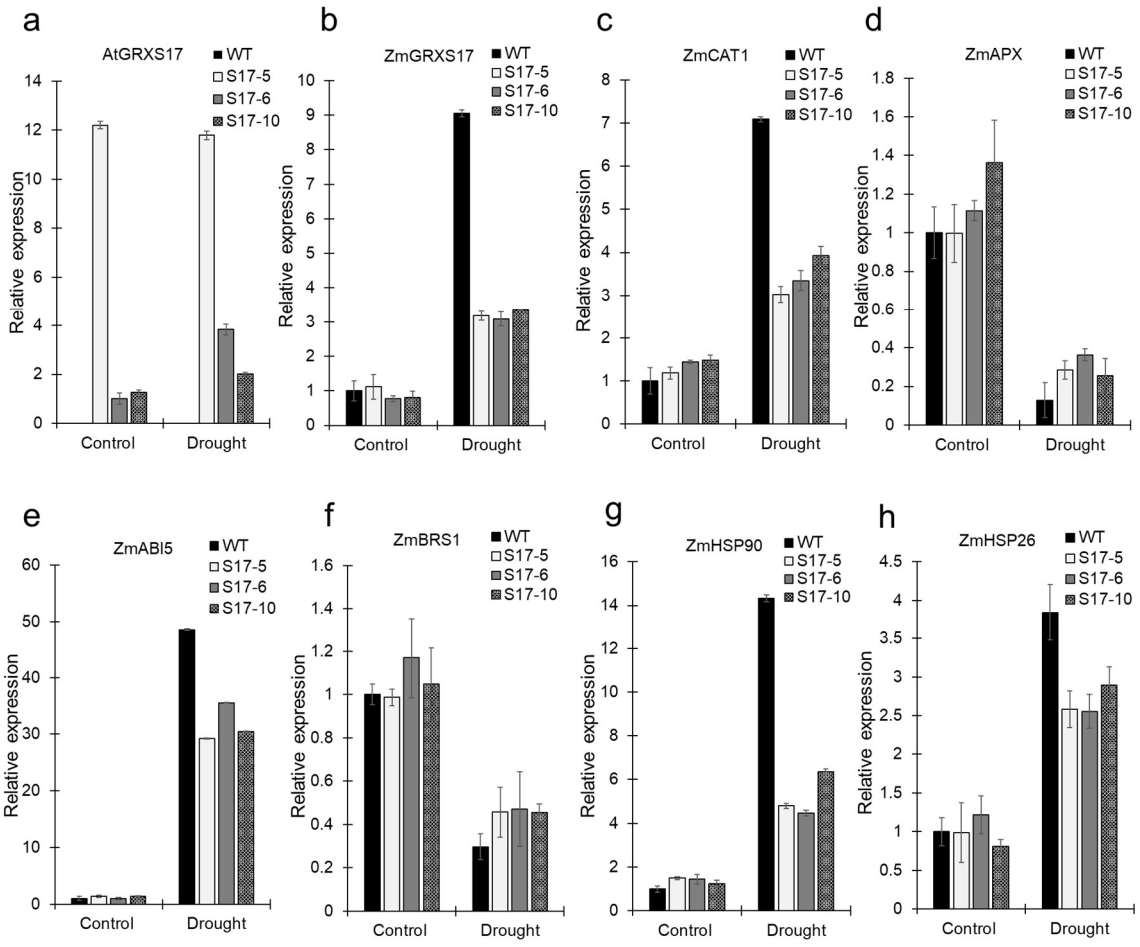


Figure 1.5 A relative comparison of transcript levels between *AtGRXS17*-expressing and WT maize plants in response to the drought stress.

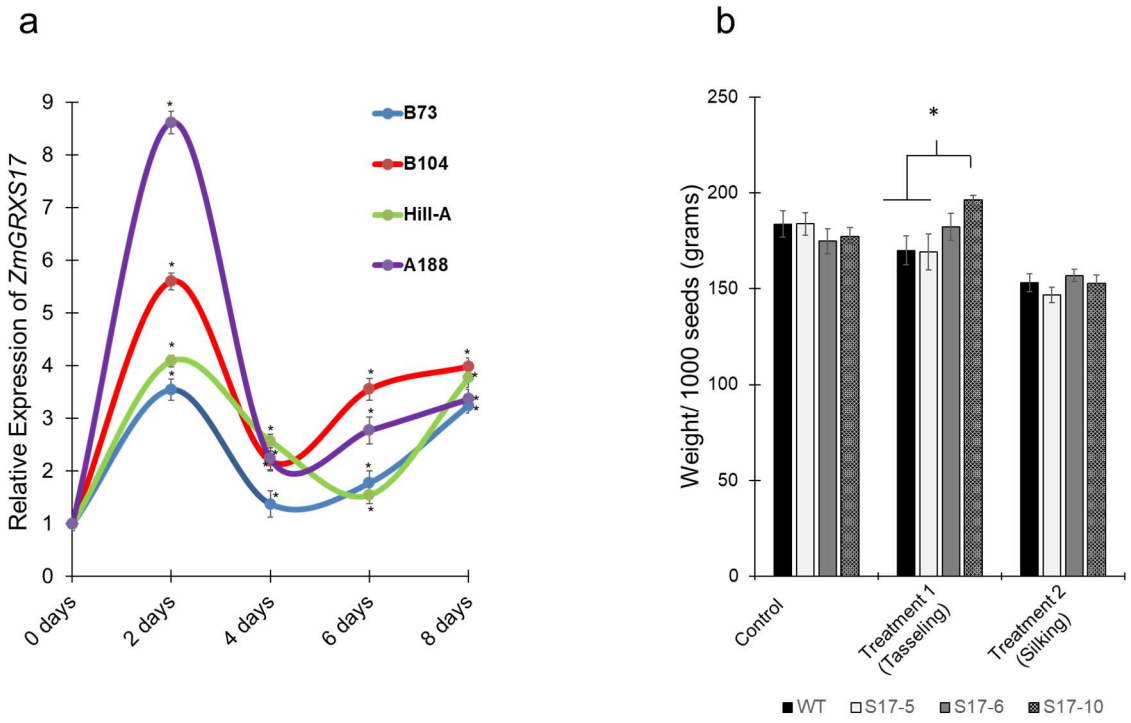
(a) *AtGRXS17* **(b)** *ZmGRXS17* **(c)** Catalase (*ZmCAT1)* **(d)** L-ascorbate peroxidase (*ZmAPX*) **(e)** ABSCISIC ACID-INSENSITIVE 5 (*ZmABI5)* **(f)** Brassinosteroid synthesis 1 (*ZmBRS1*) **(g)** Heat shock protein, 90 KDa (*ZmHSP90)* **(h)** Heat Shock protein, 26 KDa (*ZmHSP26)*.

Table

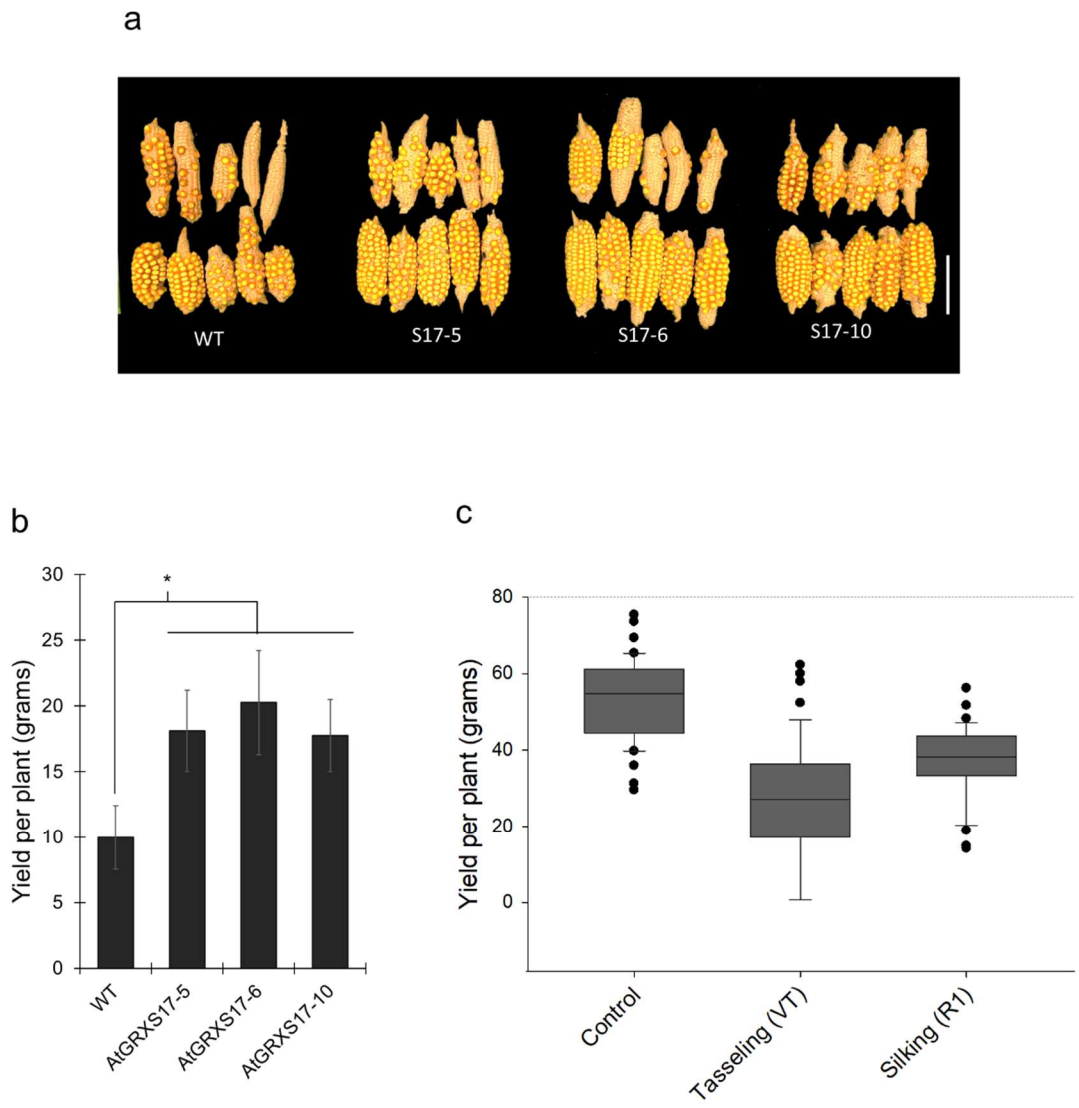
Gene	Gene ID	Primers
Arabidopsis Glutaredoxin 17 (AtGRXS17)	AT4G04950	Forward: AGTCGTGCTTCACTTCTGGG Reverse: CCAACCCTAAGCTTGCAGGA
Maize Glutaredoxin 17 (ZmGRXS17)	GRMZM2G131769	Forward: CCTGAGTCTGCAACTGAGAAG Reverse: CATCACTGGGCTGGAGTTAAT
Catalase 1 (ZmCAT1)	GRMZM2G088212	Forward: ACAGGCTGTCGTGAGAAGTG Reverse: TACGGTGTTCATGGGTCACG
L-ascorbate peroxidase (ZmAPX)	GRMZM2G156227	Forward: TAAAACAGGCCTTCCAGGGG Reverse: TGCCTTTCTGGAGATCGCTG
ABSCISIC ACID-INSENSITIVE 5 (ZmABI5)	GRMZM2G479760	Forward: CGCTAAGCCACTCTACTCCG Reverse: GAGAGAGTACACCGACCCCT
Brassinosteroid synthesis 1 (Zmbrs1)	GRMZM2G065635	Forward: AGGAAGATGGAGGACAGGCT Reverse: CGTTTATAACCCGGAGTTCTTGC
Heat shock protein, 90 KDa (ZmHSP90)	GRMZM5G833699	Forward: TTTCGGGTCTGCTCTGAGTG Reverse: CTTGGGCATCTGTTGAGCTTC
Heat Shock protein, 26 KDa (ZmHSP26)	GRMZM2G149647	Forward: TGTCCGGAAGCTTCAACGTC Reverse: TTTCCTGGTTGGAGACCGTG

Table 1.1 Primers used for qRT-PCR

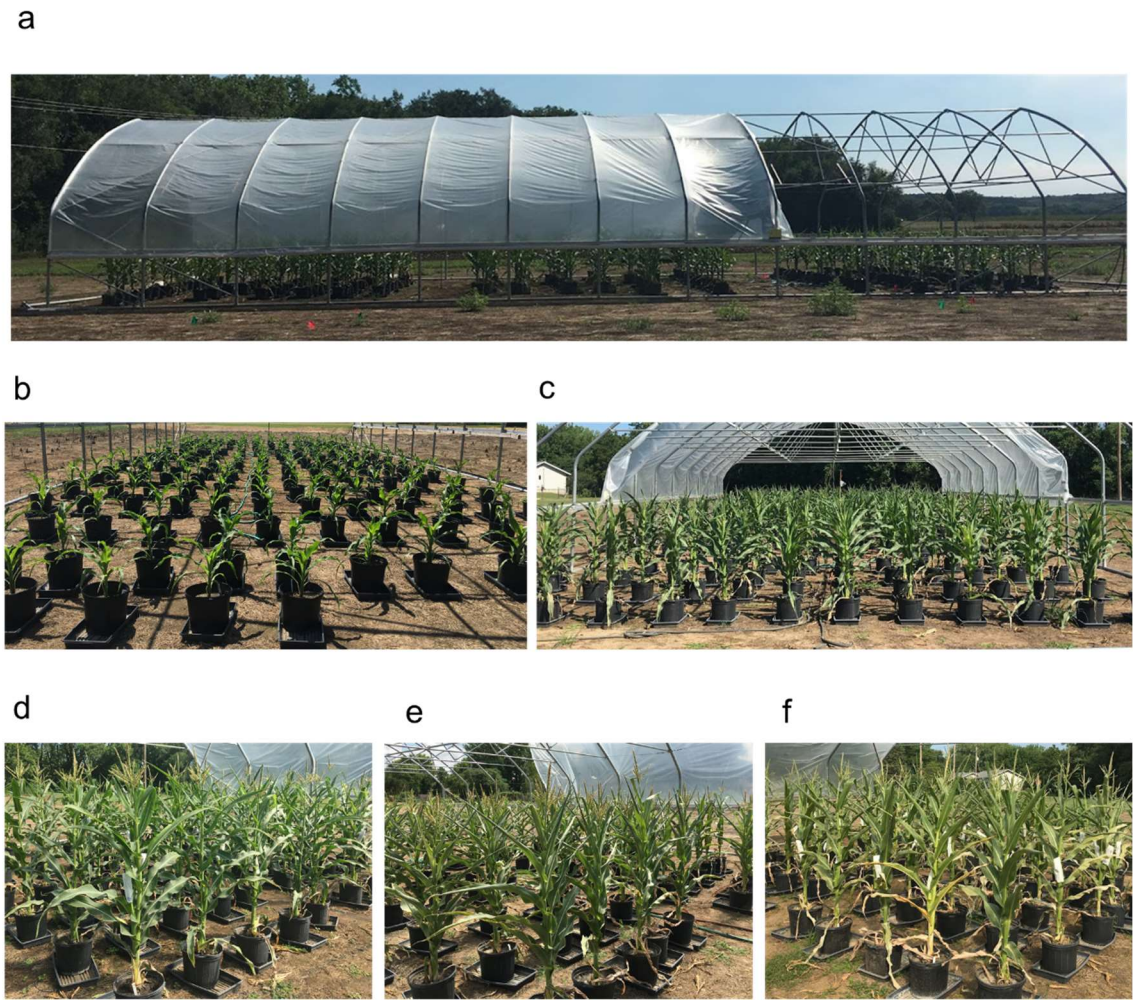
Supplementary Figures



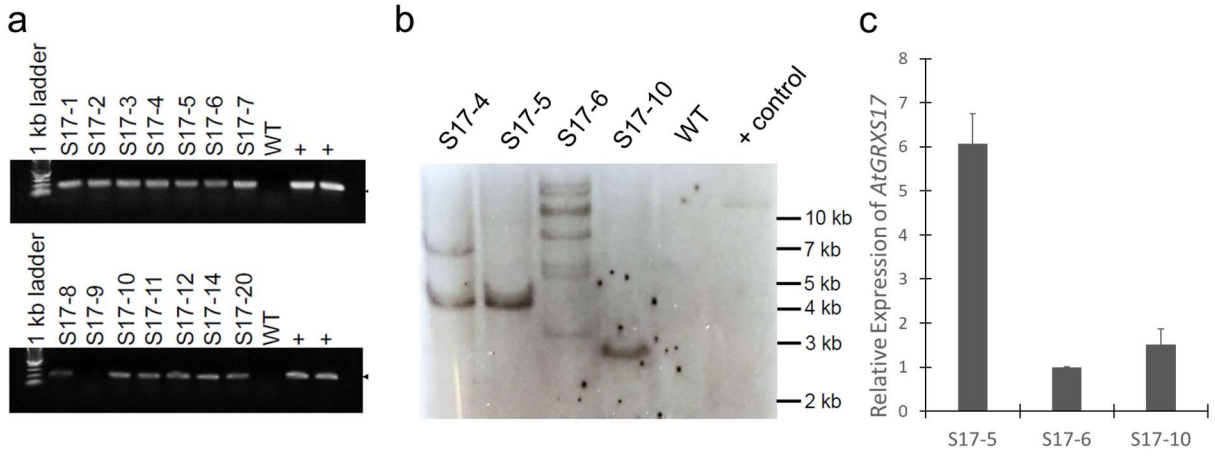
Supplementary figure 1.1 Expression levels of *ZmGRXS17* in inbred lines. **(a)** Time course normalized relative expression of *ZmGRXS17* in response to drought over 8-day period in greenhouse (26°C/22°C, day/night) of B73, B104, HiII-A and A188. Leaf samples were collected at 2 days interval. Data shown are Mean ± SE (n=3) and were analyzed using Student's *t*-test. Asterisks denote the difference between the corresponding time point and day 0 control (*P<0.05). **(b)** Weight of 1,000 seeds per plant from maize plants grown in well-watered condition and drought at the VT (n= 12) and R1 (n=12) stages in the field. Data shown are Mean ± SE and were analyzed using one-way ANOVA. Asterisks indicate level of significance (*P<0.05).



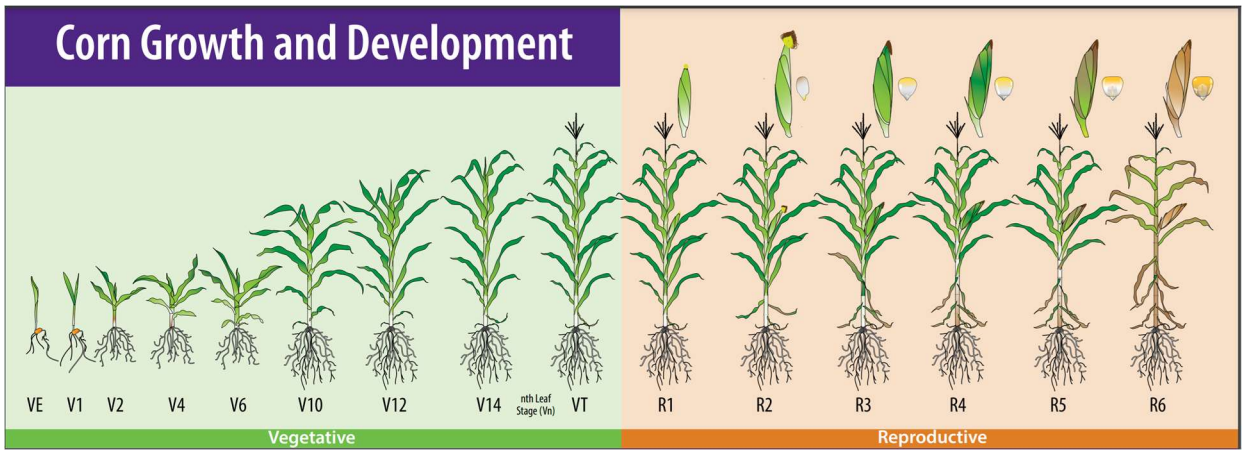
Supplementary figure 1.2 Effect of *AtGRXS17* expression in maize yield under drought stress. **(a)** Representative cobs from WT and *AtGRXS17*-expressing lines grown in the greenhouse under drought condition at VT stage. **(b)** Average yield per maize plant grown under the drought stress in the greenhouse (n= 15). **(c)** Comparison of yield per plant under drought condition at VT and R1 stages. The drought had severe effect in the yield of the maize. The median yield per plant in grams for control, treatment 1(VT) and treatment 2 (R1) were 54.7 g, 27.2 g and 38.2 g respectively. Data shown are Mean ± SE and were analyzed using one-way ANOVA. Asterisks indicate level of significance (*P<0.05).



Supplementary figure 1.3 WT and *AtGRXS17*-expressing maize plants grown in the field. **(a)** Rainout shelter was constructed before the drought stress imposition**. (b)** Maize plants at the seedling stage. **(c)** VT stage. WT plants were phenotypically indistinguishable from the transgenic plants. **(d)** Maize plants before drought stress. **(e)** Maize plants during drought stress showed phenotypes such as wilted leaves and rolling of the leaves. **(f)** Maize plants were recovered by rewatering after drought stress. Many leaves did not revert to normal form.



Supplementary figure 1.4 PCR and expression analysis of the transgene *AtGRXS17* (Sprague 2018). **(a)** PCR analysis of *AtGRXS17*. **(b)** Southern blot hybridization to detect the T-DNA integration. **(c)** Relative expression of *AtGRXS17* in three transgenic maize lines.



Supplementary figure 1.5 Maize growth and development stages. VT represents tasseling stage and R1 represents silking stage. Vn is the n^{th} leaf stage. Source: K-State Research and Extension.

Chapter 2 - Ectopic expression of a monothiol glutaredoxin, *AtGRXS17*, improves maize yield under combined heat and drought stress

Abstract

Arabidopsis monothiol glutaredoxin, *AtGRXS17*, has been demonstrated to improve abiotic stress tolerance at the vegetative stages. However, the effect of *AtGRXS17* expression on stress tolerance at the reproductive stage and the overall yield are unknown. In this study, we imposed a combined heat and drought (HTD) stress in *AtGRS17*-expressing and Wild-type (WT) maize lines and compared the effect of HTD stress at Silking (R1) stage. We found that *AtGRXS17*-expressing maize lines displayed improved kernel set and yield than WT when imposed with HTD stress at the R1 stage. The H_2O_2 content increased in WT when compared with *AtGRXS17*-expressing maize lines under HTD stress, indicating that the WT plants are more stressed than transgenic maize lines. Stomatal conductance analysis showed that stomatal closure occurred early in the WT maize plants under HTD stress. Additionally, the expression of heat shock factors and heat shock proteins was higher in *AtGRXS17*-expressing maize lines under HTD stress. These results indicate that the ectopic expression of *AtGRXS17* in maize protects the plants from combined heat and drought stresses and improves the overall maize yield.

Introduction

Climate variability and extreme weather adversely affect food security. Climate models indicate that the Earth's temperature will continue to rise and is expected to warm on average by 2.6 to 4.8 °C by the end of the 21st century [1]. Along with this, drought and extreme temperature events such as heatwaves are projected to be frequent and more intense [2]. These predicted climate changes adversely affect the agriculture production system. While all the abiotic stresses are unfavorable for plant growth and development, heat and drought stresses are the most important limiting factors to crop productivity.

Drought can lead to significant yield loss when imposed at different stages of plant growth [3–6]. Drought reduces the crop yield by affecting photosynthesis, respiration, relative water content, leaf water potential, nutrient relations, and assimilate partitioning [7]. Similarly, high heat stress causes leaf burn, foliar senescence, and abscission, root and shoot growth inhibition, fruit damage, and discoloration [8,9]. At the physiological level, heat stress causes adverse effects in photosynthesis, membrane stability, source and sink activities, and hormonal stability that lead to significant yield loss [9]. Both drought and heat stress cause the yield reduction in several crops. A meta-analysis of the studies done between 1980 and 2015 showed that the drought reduced the wheat and maize yield by 29.6% and 39.3% respectively at a global scale [10]. Drought stress, heat stress, and the combined stress of heat and drought caused the yield reduction of 57%, 31%, and 76% respectively in winter wheat [11]. From 1964 to 2007, the national cereal production was reduced by 10.1% and 9.1% on average by drought and extreme heat respectively [12]. Likewise, drought and heat stress cause yield reduction in other crops such as rice [13,14], chickpea [15], and soybean [16]. When high heat and drought stresses are accompanied together, they can cause a negative influence on plant physiology and reproductivity in much larger intensity than the sum of effects of separate

heat and drought stresses [17,18]. The combined stresses reduce the plant productivity and drought by reducing the function of PSII, weakening nitrogen anabolism, strengthening protein catabolism, and provoking lipid peroxidation [19]. Heat and drought stresses can cause significant damages to the plants. However, the extent of damage depends upon the severity of the stress and plant growth stage with the reproductive phase being very sensitive [20].

Maize is one of the most widely grown crops in the world. Maize is a water-sensitive crop and the yield depends on the proper irrigation [21]. Drought stress and heat stress at the flowering and early grain-filling stages negatively affect the maize grain yield [22]. Drought at the pre-flowering and grain filling stage results in the yield reduction by decreasing leaf number, increasing anthesis silk interval and maturity time, and losing the normal root architecture [23]. Similarly, temperature spike at the pollen development stage results in male sterility by reducing the starch content and enzymatic activity in pollen [24]. Heat also affects the silk development and reduces the kernel set [25].

Like other abiotic stresses, heat and drought stresses lead to the production of the reactive oxygen species [26–28]. Reactive oxygen species (ROS) are generated due to incomplete reduction of oxygen and are involved in intracellular signaling at a low level [29]. During stress, ROS accumulation increase in excess and causes oxidative damage to the proteins, nucleic acids, and lipids [30]. This disruption of ROS homeostasis is maintained by the antioxidative system that is comprised of enzymatic as well as nonenzymatic antioxidants [31]. Glutaredoxins (GRXs) maintain ROS homeostasis by contributing towards ROS detoxification [32]. GRXs are the small oxidoreductases that use the reduced thiol group to reduce the glutathionylated proteins [33]. The reduced form of glutaredoxins is mediated by reduced glutathione which uses electrons donated by NADPH [34]. A class II *Arabidopsis* glutaredoxi S17, AtGRXS17 contains a Trx motif in the

N-terminus and three Grx motives with CGFS active site in C-terminus [35]. Apart from redox activity, AtGRXS17 plays critical role in responding to the iron deficiency, iron-sulfur cluster binding and transport, floral development, and abiotic stress tolerance [27,28,36–38]. AtGRXS17 is also required for post-embryonic growth and maintaining auxin sensitivity under elevated temperature [39]. In the same study, *Agrxs17* mutants were found to have impaired polar auxin transport and abnormal root growth. Consistent with this study, AtGRXS17 is found to play a critical role in maintaining the shoot apical meristem at high temperatures and under long-day photoperiod [40]. Moreover, ectopic expression of *ArGRXS17* in tomato resulted in thermotolerance and drought tolerance during vegetative growth [27,28,41].

Heat Shock Factors (HSF) regulate the expression of heat shock proteins (HSPs) and act as the ROS sensors [42]. During stress, the accumulation of the nonnative proteins leads to the formation of the HSF trimers[43]. The trimer relocates to the nucleus and activates the HSP and several heat-responsive genes expression [44]. HSFs have been studied to improve the abiotic stress tolerance in plants. Overexpression of *HSF* genes such *HSFA2, HSF3, HSFA2, HSFA1* is found to improve the thermotolerance in various plants [45–48]. Overexpression of *HSFs* has also been found to improve tolerance against other abiotic stresses [45,49,50]. Heat shock proteins (HSPs) are molecular chaperones that help in proper protein folding, assembly, organellar localization, secretion, regulation, and non-native protein degradation [51]. Abiotic stresses such as extremes of temperature induce HSP expression. In the presence of the stress, plants reduce the synthesis of normal protein and increase the production of HSPs [52]. Overexpression of heat shock proteins in plants have found to enhance tolerance against several abiotic stresses [53–55].

In this study, we demonstrated that the ectopic expression of *AtGRXS17* improves the maize tolerance against HTD stresses and enhances the overall yield. We examined and quantified the

stomatal conductance, photosynthetic efficiencies, H_2O_2 accumulation, and expression of several stress-responsive genes. These findings suggest that the ectopic expression of a single gene, *AtGRXS17*, can improve heat and drought stress tolerance at the reproductive stage of maize.

Materials and Methods

qRT-PCR of the endogenous monothiol glutaredoxin 17, *ZmGRXS17*

WT and *ATGRXS17-5* were grown at 27°C/22°C (day/night) in the greenhouse until the R1 stage. Leaf tissues from three biological replicates were collected on day 0, day 1, day 2, and day 3 of the HTD stress imposition. Leaf tissues were immediately frozen in liquid nitrogen after collection. Total RNA was isolated from leaf tissues of inbred maize lines using the Direct-zol RNA Miniprep Plus Kits (Zymo Research). One µg total RNA was used to synthesize the first strand cDNA using Revert Aid First Strand cDNA synthesis kit (Thermo Scientific™). Primers 5'-CCGTCATCGCCTCACGAAGAG-3' and 5'-AGAGCCTGCCTTACGGAATTGG-3', which are based on the cyclin-dependent kinase gene CDK, were used as an expression control. Timecourse expression pattern of *ZmGRXS17* was performed in WT and *ATGRXS17-5*. Similarly, expression analysis of *AtGRXS17* was also measured for the stress period.

Greenhouse experiment

Wild-type and At*GRXS17*-expressing corn lines (*S17-5, S17 -6*, and S17-*10*) were grown in 1-gallon pots filled with equal volumes of Pro-Mix®BRK Mycorrhizae™ growth media and were watered as needed with constant liquid feed on a pot by pot basis. During vegetative growth, plants were grown at optimal temperatures of 28ºC/22ºC. At the R1 stage, self-pollination was performed and kept under the well-watered and optimal condition for 24 hours. After 24 hours of self-pollination, the experiments were systematically performed by imposing combined heat (37ºC / 31ºC) and drought stress and continued for 72 hours. After the end of heat and drought imposition, the plants were grown in the optimal condition and rewatered. The experiment was replicated three times. Agronomic traits such as kernel number and total yield per plant were measured.

Stomatal conductance and chlorophyll fluorescence measurement

WT and *AtGRXS17*-expressing lines were grown until the R1 stage. After selfing, the maize plants were subjected to HTD stress. Stomatal conductance measures the degree of stomatal opening and indicates the plant water status (Gimenez and Thompson 2013). Stomatal conductance was measured using SC-1 leaf porometer (Decagon Devices) on the flag leaf. The observation was recorded in six replicate plants in each line under study from day 0 to day 3 of the stress period.

Chlorophyll fluorescence can be used to obtain the photosynthetic performance of the leaf (Baer 2004). The phytochemical efficiency of the photosystem II was determined by chlorophyll fluorescence ratios (Fv/Fm) using a portable chlorophyll fluorometer (OS30p+, Opti-Sciences). The measurement was taken on the flag leaf following a 30-minute dark adaptation. The observation was recorded on six replicate plants in each line understudy from day 0 to day 3 of the stress period.

H_2O_2 assay

WT, *AtGRXS17-5,* and *AtGRXS17-6* were grown until the R1 stage. After selfing, the maize plants were subjected to the HTD stress for 3 days. Hydrogen peroxide was measured from the leaf tissues of WT and *AtGRXS17*-expressing maize plants using Amplex® Red Hydrogen Peroxide/ Peroxidase Assay kit (Invitrogen). Briefly, 100 mg of ground leaf samples were suspended in 1X reaction buffer and mixed well. 50 µl of the working solution of 100 µM of Amplex® Red reagent and 0.2U/ml Horseradish Peroxidase was mixed to 50 µl of the sample solution in a well of a microplate and the reaction was incubated in the room temperature in dark for 30 minutes. The H_2O_2 concentration was determined by measuring the absorbance at 560 nm using no-H_2O_2 as control. H_2O_2 concentration was determined using a standard concentration curve and calculated on FWbasis ($\mu g.mg^{-1}$).

qRT-PCR analysis of abiotic stress-related genes

Leaf samples from WT and two *AtGRXS17*-expressing lines (*S17-5* and *S17-6*) grown in the optimal condition and drought-stressed condition were collected. Total RNA was isolated from leaf tissues of inbred maize lines using the Direct-zol RNA Miniprep Plus Kits (Zymo Research). One µg total RNA was used to synthesize the first-strand cDNA using Revert Aid First Strand cDNA synthesis kit (Thermo Scientific™). qRT-PCR was carried for different genes listed in Table 2.1. Each 10uL qRT-PCR reaction consisted of 4.4 µL diluted cDNA solution (2ng/µL), 0.3 µL of each primer (20µM), and 5 µL iQ SYBR Green Supermix (Bio-Rad). Primers 5'-CCGTCATCGCCTCACGAAGAG-3' and 5'-AGAGCCTGCCTTACGGAATTGG-3', which are based on the cyclin-dependent kinase gene CDK, were used as an expression control.

Results

ZmGRXS17 expression changes in response to the HTD stress

Maize endogenous glutaredoxin *S17*, *ZmGRXS17*, expression response to the HTD treatment was measured in WT and *AtGRXS17-5* transgenic line. In WT, the *ZmGRXS17* was upregulated gradually in response to the HTD treatment. WT *ZmGRXS17* expression increased by 2-folds, 3-folds, and 5.5-folds respectively on day 1, day 2, and day 3 of the stress period (**Fig. 2.1a**). Compared to this, *ZmGRXS17* in the transgenic maize, as measured on *AtGRXS17-5,* increased by 2-folds on day 1 of HTD treatment. The expression returned to near baseline on day 2 of stress treatment followed by the sharp upregulation by 2.2-folds on day 3 of HTD treatment. The transgene, *AtGRXS17*, expression remained near the baseline from day 0 to day 3 of the stress treatment. Among three transgenic lines, *AtGRXS17-5* had the highest transgene expression (**Fig. 2.1b**). *AtGRXS17-5* had a 6-fold higher expression than *AtGRXS17-6*.

AtGRXS17-expressing maize lines have a higher yield compared to the WT

The effect of the HTD stress was severe as compared to the heat and drought stress alone over the same period as observed in the maize phenotype (**Fig. 2.2a**). While symptoms like the rolling of the leaf occurred in plants treated with heat and drought stress alone on day 3, the plants that were imposed with HTD stress had irreparable damage of 5 to 6 lower leaves within 3 days of stress. The pot capacity decreased to nearly 43% after three days of continuous HTD stress (**supplementary fig. 2.1**). A HTD stress had a severe effect on the yield of the maize plants. In the greenhouse experiment, we found HTD stress reduced the kernel numbers in the corn. The kernel number was 189 per ear under the optimum condition. The number reduced to an average of 68 kernels per ear under combined stress (**Fig. 2.2b**). The overall yield as measured by the total weight of kernels per plant was also reduced because of the combined stress. The HTD stress reduced the

overall yield by nearly 74% on average (**Fig. 2.2c**).

AtGRXS17-expressing lines performed better than WT under the combined stress (**Fig. 2.3a**). Under the optimal condition, the kernel number in the transgenic maize and WT remained similar (**Fig 2.3b, upper panel**). Although the kernel number reduced severely in both transgenic maize and WT, the *AtGRXS17*-expressing maize produced a significantly higher number of kernels per plant (**Fig. 2.3b, lower panel**). Kernel numbers per ear in *S17-5, S17-6,* and *S17-10* were 98, 92 and 86 respectively compared to 54 of the WT under HTD stress (**Fig. 2.4a**). Because of kernel numbers, the overall yield calculated based on the total kernel weight per plant was higher in the *AtGRXS17*-expressing maize plants (**Fig. 2.4b**). A mean yield reduction of 79.8% per plant was observed in WT as compared to the mean yield reduction of 59.5% In transgenic maize.

Effect of ectopic expression of *AtGRXS17* on stomatal conductance and chlorophyll fluorescence

Stomatal conductance and chlorophyll fluorescence were measured to observe the status of the stress in maize plants under combined drought and heat stress. Stomatal conductance of both *AtGRXS17*-expressing and WT plants decreased as the combined stress was imposed. The stomatal conductance remained similar for both the transgenic and WT lines on day 0 and day 1 of the stress treatments. However, on day 2 and day 3 of the stress imposition, stomatal conductance decreased in WT compared to the transgenic maize indicating the closing of the early stomatal closure (**Fig. 2.5a**).

Phytochemical efficiency of the PSII, as measured by Fv/Fm ratio, was affected by HTD stress. The efficiency remained constant from day 0 to day 2 of the HTD treatment. However, the efficiency lowered on day 3. On day 3, a 13% of reduction in Fv/Fm was observed with WT showing a comparatively higher reduction compared to the transgenic maize (**Fig. 2.5b**). However,

the reduction of Fv/Fm in WT was not statistically significant as compared to the *AtGRXS17*-expressing maize.

***AtGRXS17*-expressing maize lines accumulated less H_2O_2 under the HTD stress**

H_2O_2 content increased after imposing HTD stress. No difference was found in H_2O_2 content in both WT and *AtGRXS17*-expressing maize plants when grown in optimal condition, however, H_2O_2 content significantly increased in WT when compared with *AtGRXS17*-expressing maize plants under the combined stress (**Fig. 2.6**).

HTD stress affects the expression of stress-responsive genes in maize

The effect of HTD on the expression of the stress-responsive genes was measured using the leaf tissues. The expression analysis was performed in WT and two of the *AtGRXS17*-expressing lines. HTD stress-induced the changes in the expression of HSFs, HSPs, ROS-responsive genes, and *ADP-pyrophosphorylase* (*ZmAGPase*). The genes of heat shock factors and heat shock proteins were strongly upregulated under the HTD stress. *ZmHSF03* and *ZmHSF04* expression were respectively upregulated by an average of 2.3-folds and 1.8-folds in transgenic maize lines than WT under HTD stress (**Fig. 2.7a-b**). *ZmHSP90* expression was not detected in plants grown under the optimum condition. However, Under HTD stress, *ZmHSP90* in *AtGRXS17-5* and *AtGRXS17-6* was upregulated by 10-folds and 4-folds respectively when compared with the WT (**fig**. **2.7c** Expression of *ZmHSP70* and *ZmHSP26* were also upregulated under HTD stress (**Fig. 2.7d-e**). *AtGRXS17*-expressing maize lines showed higher expression of *ZmHSP70* and *ZmHSP26* than WT under HTD stress. Expression of the ROS-related genes such as catalase (*ZmCAT*) and L-ascorbate peroxidase (*ZmAPX)* was measured (**Fig 2.8a-b**). *ZmCAT* was upregulated while *ZmAPX* was downregulated by the HTD stress. Interestingly, *ZmCAT* expression was higher in WT under HTD stress. Conversely, *ZmAPX* expression was higher in transgenic maize under HTD stress.

Expression of the regulatory starch biosynthesis gene, *ADP-glucose pyrophosphorylase* (*ZmAGPase*), was significantly reduced under HTD stress (**Fig. 2.8c**).

Discussion

Plants encounter a combination of multiple abiotic stresses throughout their life stages. Crop production depends on the proper development of gametes, successful pollination, fertilization, and the development of the endosperm. Any abiotic stress during these processes negatively impacts crop production [56]. Drought and heatwaves are climatic hazards for crop production. Studies have shown that a combination of heat and drought stress is more destructive to crop yield and performance than stresses occurring separately [57,58]. Heat and drought stresses are positively associated. High temperature causes drought severity by enhancing evaporation [59,60]; a dry surface is favorable to increase the monthly average temperature [61]. Maize being a summer crop encounters heat and drought stress that decreases its net primary productivity [62]. Heat and drought stresses during the seed filling stage can lead to a reduction in seed size and number [63,64]. In maize, heat and drought stresses at the seed filling stage can severely suppress the endosperm development and starch accumulation [65,66]. Excessive heat and drought stress in the first two weeks following pollination can cause kernel abortion in maize [67]. Thus, the development of maize plants to tolerate both heat and drought stresses has been a major objective of breeders for many decades [68]. Ectopic expression of *AtGRXS17* in different plants has been shown to improve the tolerance against various abiotic stresses. In our study, we found that a combination of heat and drought stress led to a severe reduction in the kernel numbers per plant. We also found that expression of the *AtGRXS17* in maize improves the kernel number and hence, the overall yield when maize gets exposed to a short period of the combination of heat and drought stresses at the reproductive stage.

Stomata are the active interface for gas and water exchange. Both heat and drought stress negatively affect the carbon and water use efficiency in plants. Stomatal conductance was found

to be drastically reduced under heat and drought stresses in various plants [69–71]. In our study, we found that stomatal conductance increased on day 1 of stress and gradually decreased on day 2 and day 3 of stress imposition. An increase in leaf temperature causes an increase in stomatal conductance under well-watered conditions [72]. The opening of the stomatal aperture on day 1 could be because of elevated temperature. Stomatal closure occurs because of the increased level of ROS generation and ABA [73]. In our study, we found that stomatal conductance decreased significantly in WT than *AtGRXS17*-expressing lines on day 2 and day 3 of the stress imposition. This is possibly due to the expression of the *AtGRXS17* that acted as an additional ROS scavenger. Heat and drought stresses affect the efficiency of the photosystem (PS) II [74]. The Fv/Fm ratio is often used as an indicator of stress. The decrease in the Fv/Fm ratio indicates the photodamage to PS II reaction centers [75,76]. In our study, the quantum efficiency of the photosystem, as measured by Fv/Fm ratio indicates that the efficiency remained constant until day 2 of the heat and drought stress. On day 3, the efficiency decreased in WT as compared to the transgenic lines although no statistical difference could be found. Enhanced antioxidant activities protected PS II performance in maize [77]. In the previous studies, *AtGRXS17*-expressing tomatoes demonstrated higher quantum efficiency of PS II compared to the WT under heat and chilling stresses [27,28]. Consistent with previous studies, our result indicates that expression of the *AtGRXS17* alleviates photodamage to the PS II as glutaredoxin is a part of the antioxidant defense system.

Heat shock factors (HSFs) are the major regulators of the heat shock proteins (HSPs) and are upregulated in response to several environmental stresses. 58 *ZmHSFs* have been identified so far [78]. *ZmHSF03* and *ZmHSF04* expression were induced by a 30-minute heat shock in the leaf tissues at the anthesis and post-anthesis stages [78]. In another study, *ZmHSF03* and *ZmHSF04* expression were very low to non-existent under normal conditions, however, their expression was

found to be strongly upregulated under heat stress [79]. In our study, we also found that *ZmHSF03* and *ZmHSF04* were upregulated under the HTD stress indicating that they play a crucial role when plants are under stress. Along with *ZmHSF*, we found that *ZmHSP26, ZmHSP70,* and *ZmHSP90* were also upregulated under the HTD stress. Remarkably, *HSFs* and *HSPs* expression is upregulated by more than two-folds in many instances in *AtGRXS17*-expressing maize lines than WT. Consistent with this study, *AtGRXS17* expressing tomatoes were thermotolerant and had higher expression of *HSFs* and *HSPs* [27]. Several studies have found that the application of H_2O_2 leads to the expression of the heat-responsive genes [80,81]. Activation of HSFs and increase in ROS scavenging activity suggest that crosstalk between HSF, HSP, ROS, and ROS scavenging system exists [44]. Glutaredoxin, being an antioxidant protein, is a part of the ROS scavenging system. Furthermore, several HSPs such as HSP70 and HSP60 were identified as a target of plant glutaredoxin. These HSPs contain at least one conserved cysteine [82]. Similarly, it has been reported that several HSFs contain cysteine which can engage in redox-sensitive disulfide bond [83–85]. It is also suggested that thioredoxin restores the DNA-binding activity of oxidized HSF which in turn regulates the HSP generation under prolonged heat stress [86]. Our result suggests that crosstalk or interaction occurs between the heat-responsive genes and glutaredoxin that induces the activation of HSFs and HSPs to improve the plant's tolerance against HTD stress.

Catalase (CAT) and ascorbate peroxidase (APX) are major enzymatic antioxidant systems in the living system. Although both enzymes metabolize H_2O_2, CAT is located in the peroxisome, and APX is located in other organelles such as chloroplast, cytosol, and mitochondria [87]. In our study, both *ZmCAT* and *ZmAPX* expression changed after a HTD stress. *ZmCAT* expression was highly upregulated in WT and *ZmAPX* activity was highly upregulated in transgenic maize under heat and drought stress. Previously, tomato CAT activity and expression were increased in

response to the heat, chilling, and drought response in *AtGRXS17*-expressing tomatoes [27,28,41]. Conversely, we found that CAT expression decreased in *AtGRXS17*-expressing maize plants under stress condition. Both CAT and APX were identified as the interacting partner of glutaredoxin [82]. One explanation is that ROS generation was very high in WT under the stressed condition and because of this, catalase expression was highly upregulated. CAT is only active when cellular H_2O_2 concentration is high whereas APX detoxifies H_2O_2 at a lower concentration [88]. As stated earlier, we found that *ZmAPX* expression was much higher in transgenic maize. The decrease in *ZmCAT1* expression and increase in *ZmAPX* expression as seen in this study could be explained by the fundamental principle of the genetic network called redundancy [89] where deficiency of *ZmCAT1* expression results in the enhanced expression of *ZmAPX*.

Starch is the major source of carbohydrates for plants and animals. *ADP-glucose pyrophosphorylase (AGPase)* catalyzes the rate-limiting reaction in the biosynthesis of starch [90]. *AGPase* converts ATP and Glc-1-phosphate into ADP-Glc and releases pyrophosphate in the initial step of the starch biosynthetic pathway. High temperature and water deficit condition affects the expression of the *AGPases* and in turn, the yield of the plants. Endosperm *AGPase* activity reduced by 96% when endosperm was heat shocked for 10 minutes at 57°C [91]. Under HTD stresses, the expression of all five isozymes of *AGPases* was significantly reduced in wheat [92]. In another study conducted in wheat, mean AGPase activity decreased under heat and drought stresses. Interestingly, the reduction of AGPase activity was significant in the drought-sensitive wheat [93]. Like this study, we found that the expression of *ZmAGPase* reduced in WT and transgenic maize when a HTD stress was imposed. Particularly, *AtGRXS17-5 and AtGRXS17-10* had 4-folds and 2.4-folds higher expression than WT respectively indicating higher starch biosynthesis under the stress. It is reported that *AGPase* is identified as one of the target proteins

of glutaredoxin [82]. Interestingly, cysteine residues were found in the N terminus motif of the small subunit in heat-stable AGPase [94,95]. In potatoes, cysteine improves the AGPase stability at high temperatures by forming an intermolecular disulfide bridge [96,97]. Importantly, AGPases were activated by the chloroplast thioredoxin probably by interacting with the conserved cysteine residue [98]. Based on these previous findings, we posit that the ectopic expression of *AtGRXS17* might play a role to keep up the activity of AGPase during high heat and drought stress.

In summary, our results suggest that ectopic expression of *AtGRXS17* enhances the HTD stress tolerance in maize plants. Improved yield, improved stomatal conductance, reduced accumulation of H_2O_2, and improved expression of *HSFs* and *HSPs* are all associated with the tolerance against HTD stress. Together with previous results, we believe that manipulation of the glutaredoxin gene could prove to be a great tool to improve abiotic stress tolerance at any growth stage of the plant life cycle.

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Figures

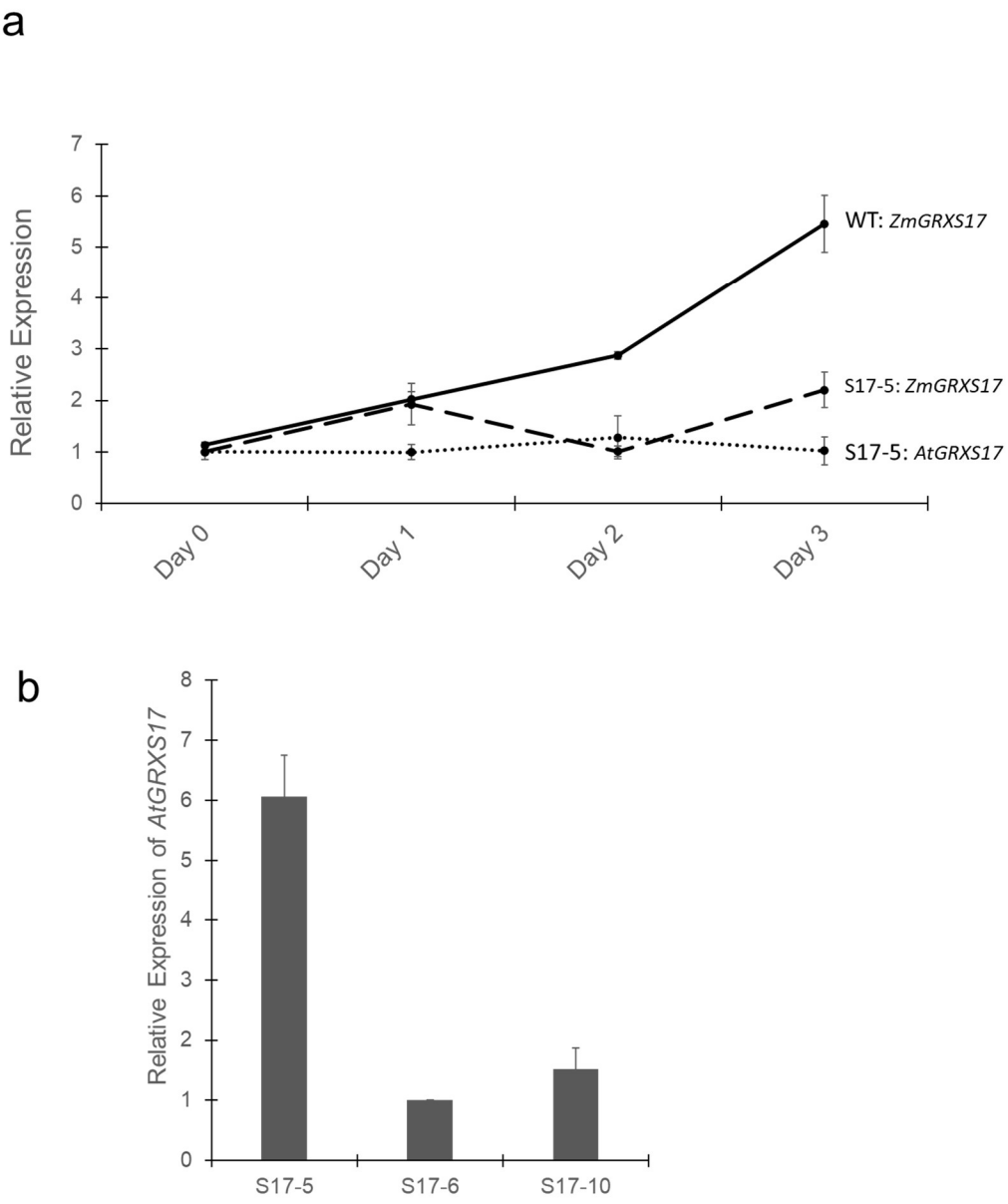


Figure 2.1 Relative expression of *ZmGRXS17* in response to the HTD stress in WT and *AtGRXS17*-5.

a) Time course normalized relative expression of *ZmGRXS17* and *AtGRXS17* in WT and *AtGRXS17*-expressing maize line, *AtGRXS17-5* in response to HTD stress. Leaf tissues were collected on day 0, day 1, day 2, and day 3 of stress treatment. Data shown are Mean± SE (n=3).

b) Relative expression of the transgene, *AtGRXS17*, in three transgenic maize lines. Leaf tissues were used to quantify the expression level. Data shown are Mean± SE (n=3).

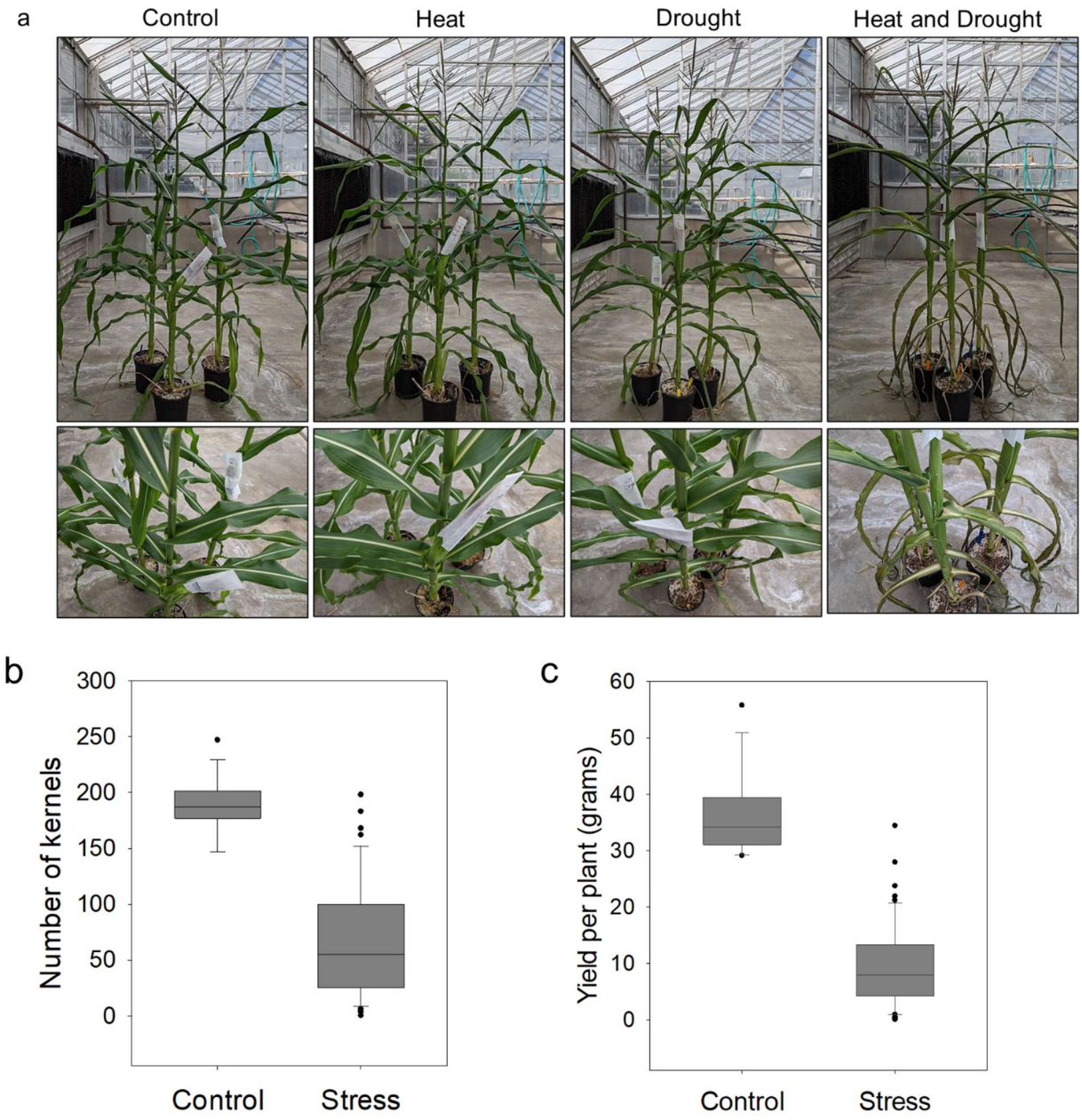


Figure 2.2 HTD stress causes significant damage to the leaves and yield of the plant.

a) Comparative phenotype analysis of the maize plants kept in control, 3-days heat stress, 3-day drought stress, and 3-day HTD stress (left to right). 3 days-HTD stress was severe and lower leaves were irreversibly affected. b) Effect of HTD stress on the number of kernels per plant (n=12) c) Effect of HTD stress on the yield per plant as measured by total weight of kernels per plant (n=12).

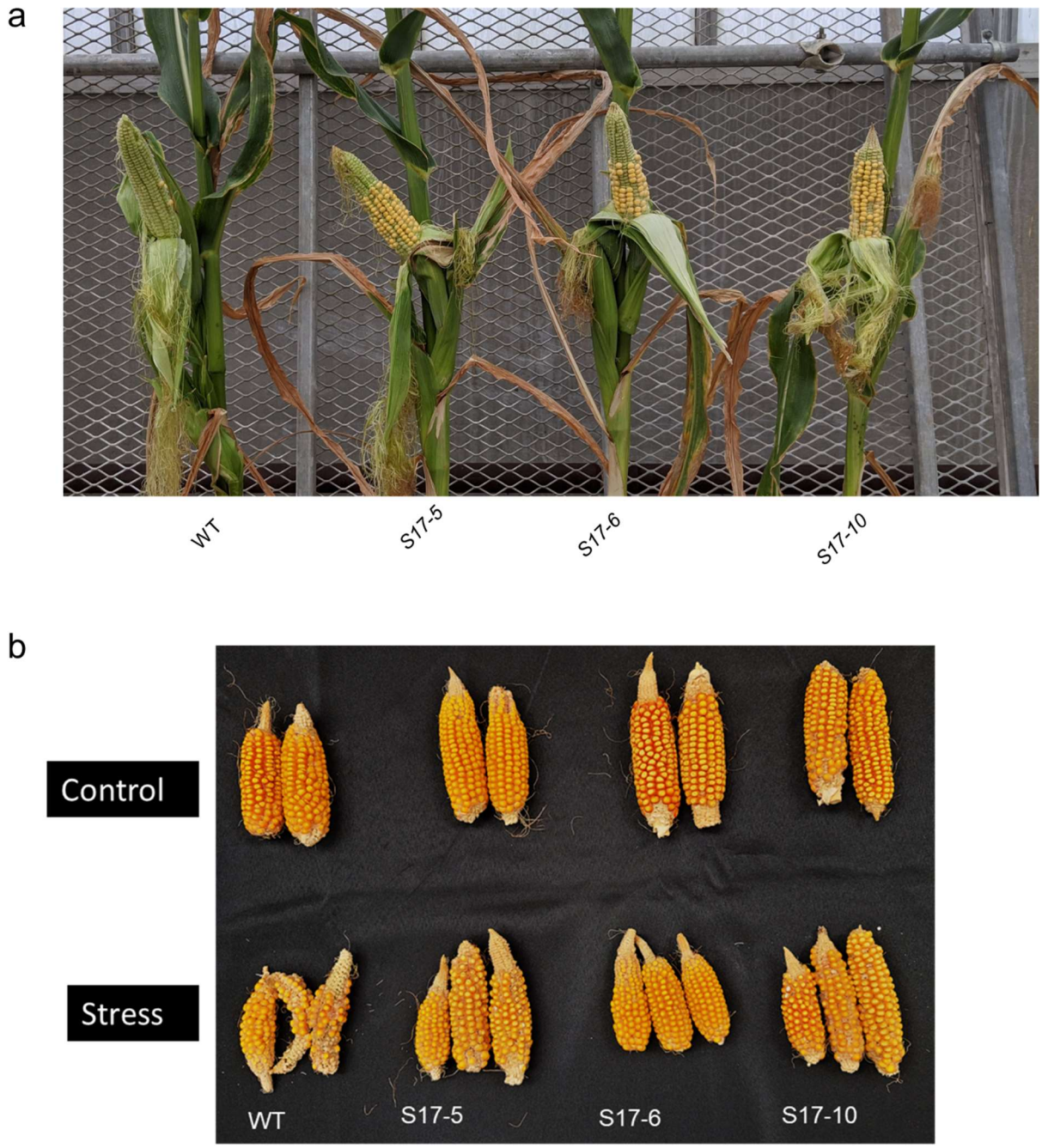


Figure 2.3 Effect of HTD stress in WT and *AtGRXS17*-expressing maize lines.

a) In the greenhouse experiment, HTD stress resulted in severe loss of yield in WT and AtGRXS17-expressing maize lines. **b)** Representative cobs from WT and *AtGRXS17*-expressing maize lines grown under optimum condition (upper panel) and HTD stress (lower panel).

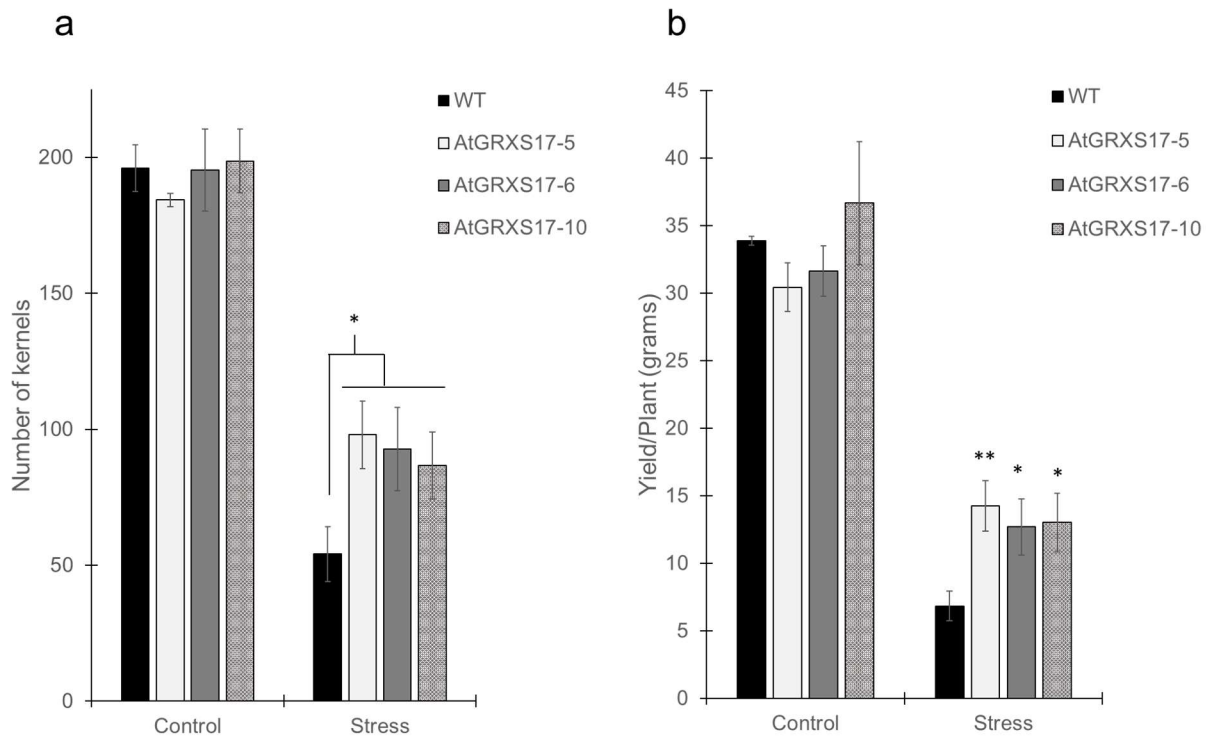


Figure 2.4 *AtGRXS17*-expressing maize lines have a higher yield than WT under HTD stress. **a)** Number of kernels per plant in *AtGRXS17*-expressing maize lines and WT under the optimal condition and HTD stress (n=18). **b)** Average yield per plant in *AtGRXS17*-expressing maize lines and WT under the optimal condition and HTD stress (n=18). Data shown are Mean ± SE and were analyzed using one-way ANOVA. Asterisks indicate a level of significance (*P<0.05; **<P<0.01).

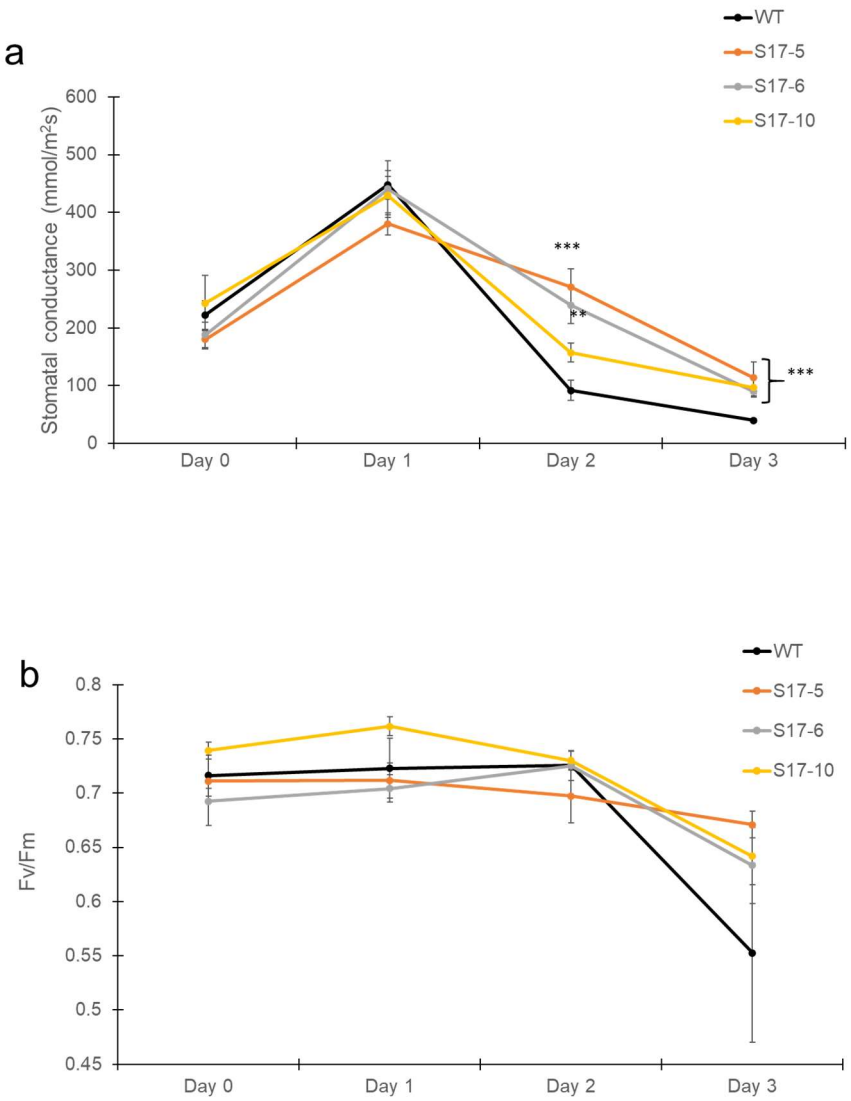


Figure 2.5 Effect of ectopic expression of *AtGRXS17* in stomatal conductance and quantum efficiency of PS II.

a) Stomatal conductance of WT and *AtGRXS17*-expressing maize plants as the HTD stress progresses from day 0 to day 3. **b)** Quantum efficiency of the PS II of WT and *AtGRXS17*-expressing maize plants as the HTD stress progresses from day 0 to day 3. Data shown are Mean ± SE (n=6) and were analyzed using one-way ANOVA. Asterisks indicate a level of significance (**P<0.01, ***P<0.001).

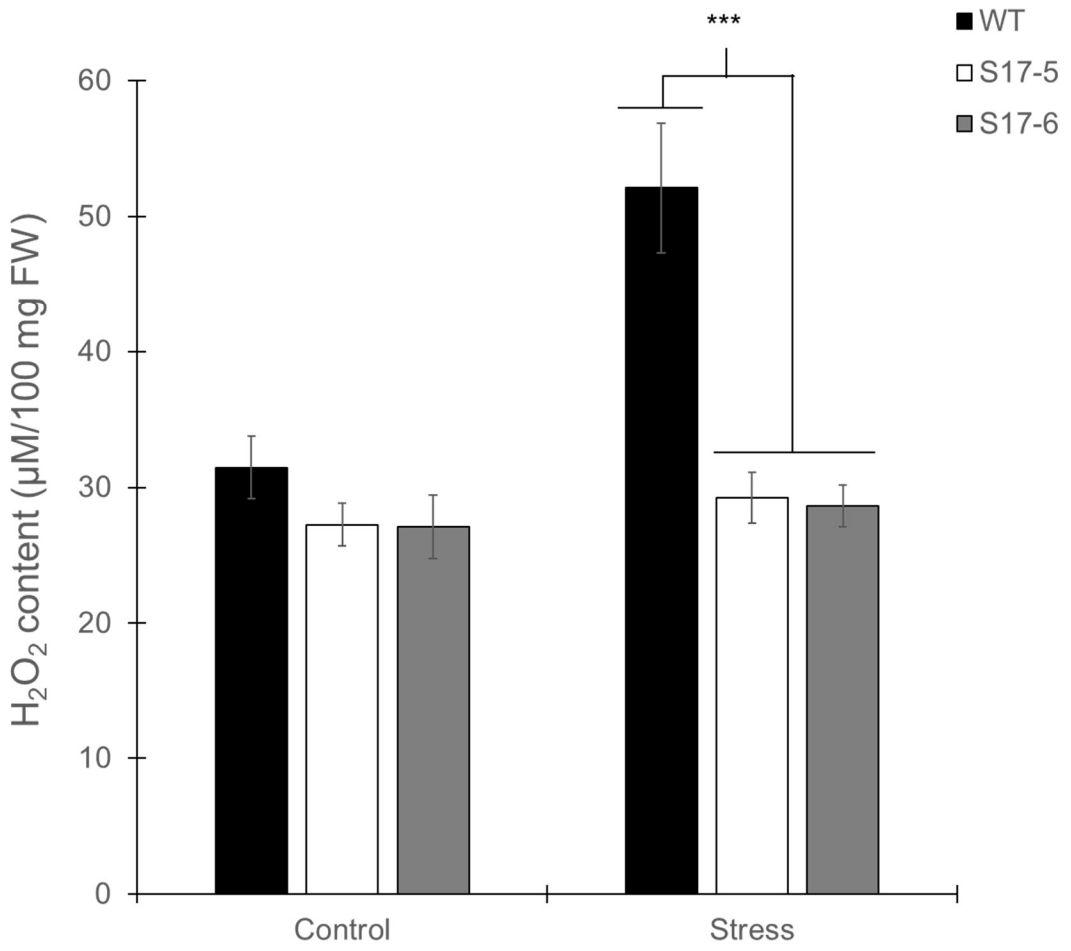


Figure 2.6 Effect of ectopic expression of *AtGRXS17* in stomatal conductance and H_2O_2 content.

a) H_2O_2 content of the WT and *AtGRXS17*-expressing maize plants grown under the optimal condition and 3-days HTD stress. Data shown are Mean ± SE (n=6) and were analyzed using one-way ANOVA. Asterisks indicate a level of significance (***P<0.001).

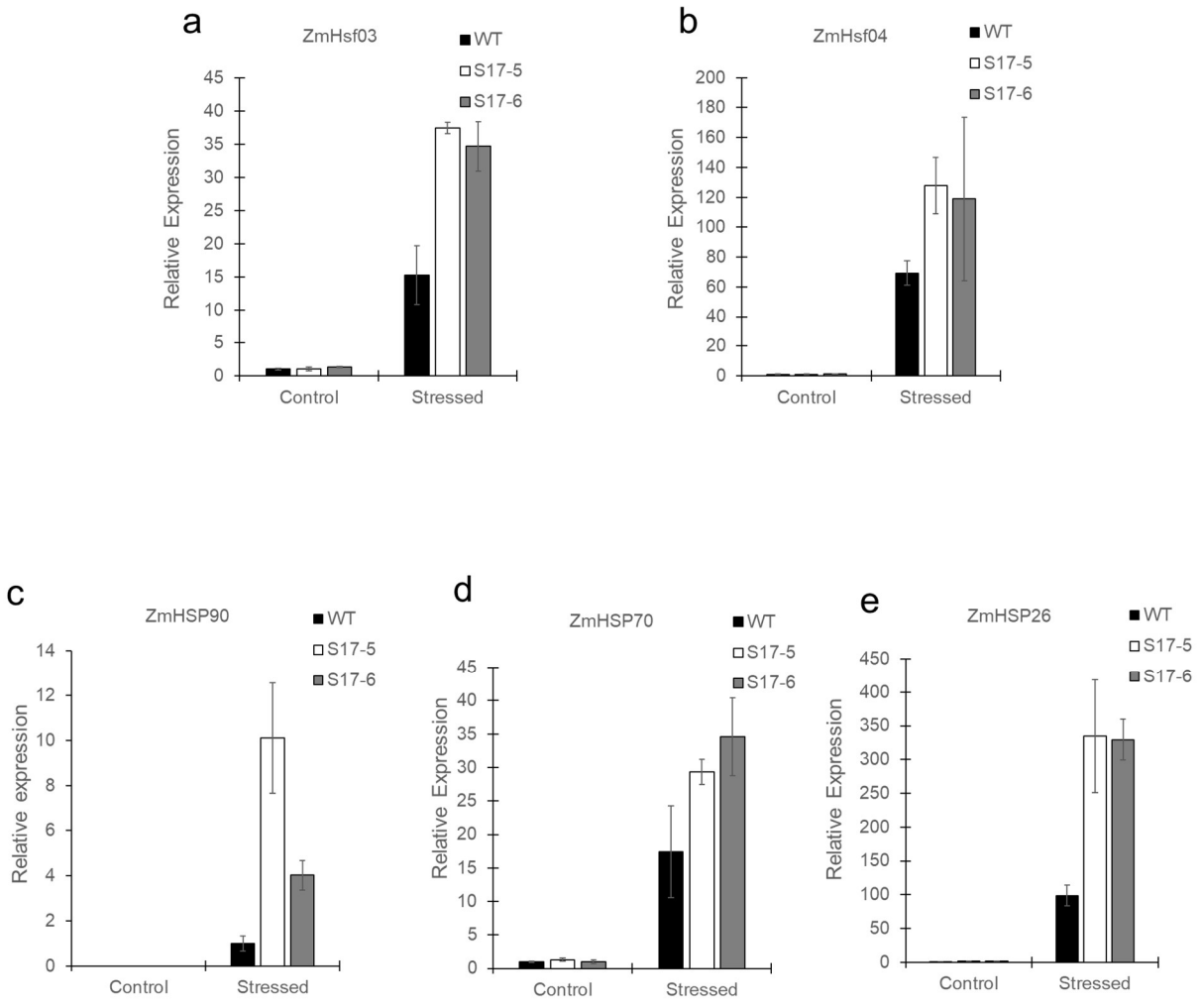


Figure 2.7 A relative comparison of *HSFs* and *HSPs* between *AtGRXS17*-expressing and WT maize plants in response to the HTD stress.

a) *ZmHSF03* **b)** *ZmHSF04* **c)** Heat shock protein, 90 KDa (*ZmHSP90)* **d)** Heat Shock protein, 70 KDa (*ZmHSP70)* **e)** Heat Shock protein, 26 KDa (*ZmHSP26)*

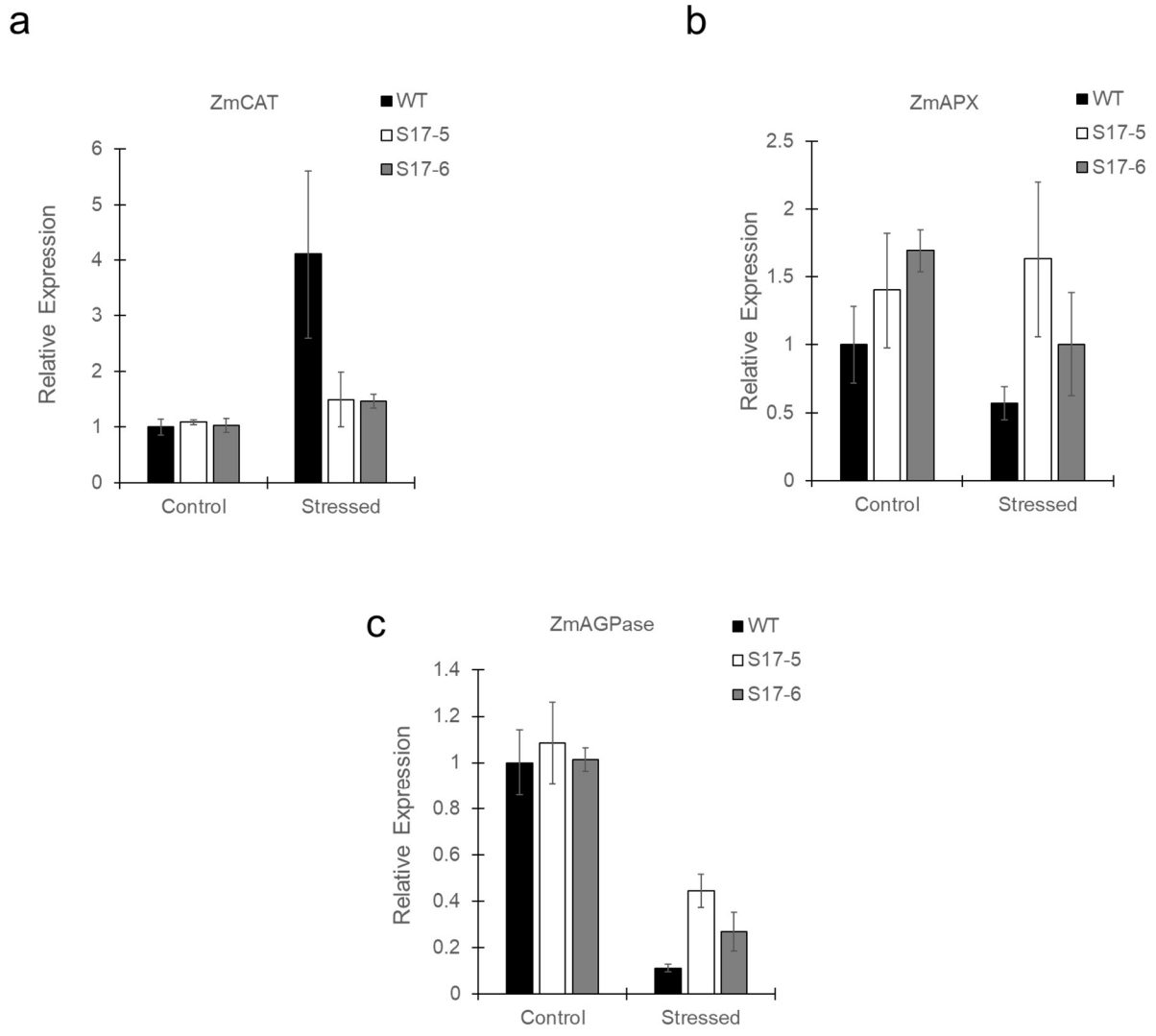


Figure 2.8 A relative comparison of ROS-related genes and *ZmAGPase* between *AtGRXS17*-expressing and WT maize plants in response to the HTD stress.

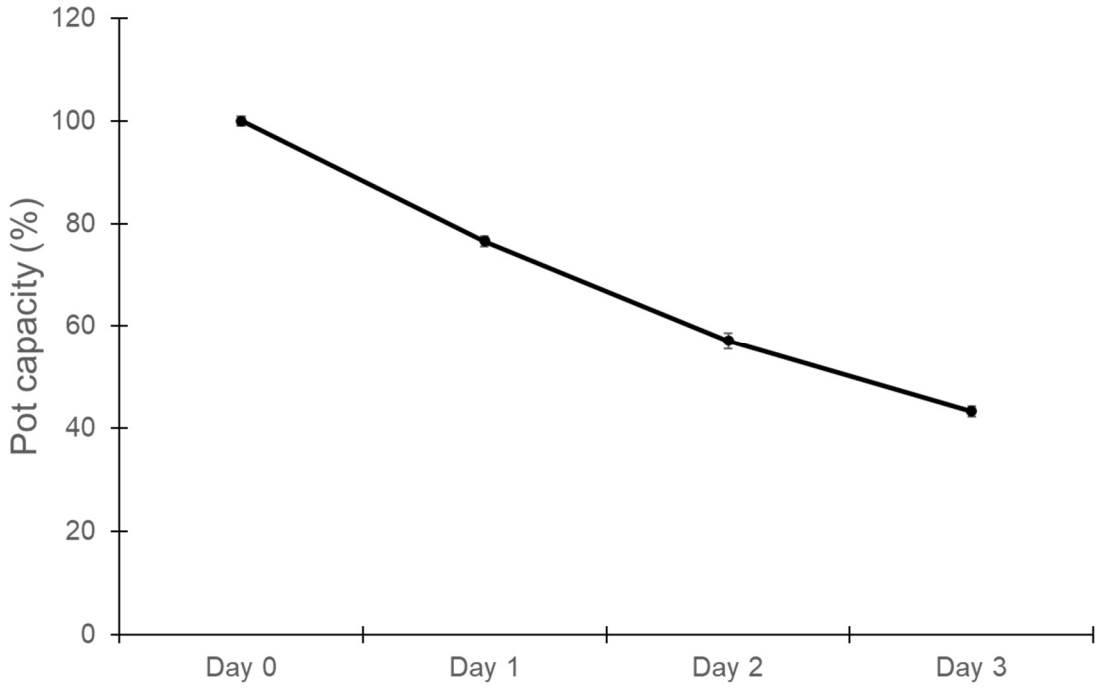
a) Catalase (*ZmCAT1)* **b)** L-ascorbate peroxidase (*ZmAPX*) **(c)** *ADP-glucose pyrophosphorylase (ZmAGPase)*

Table

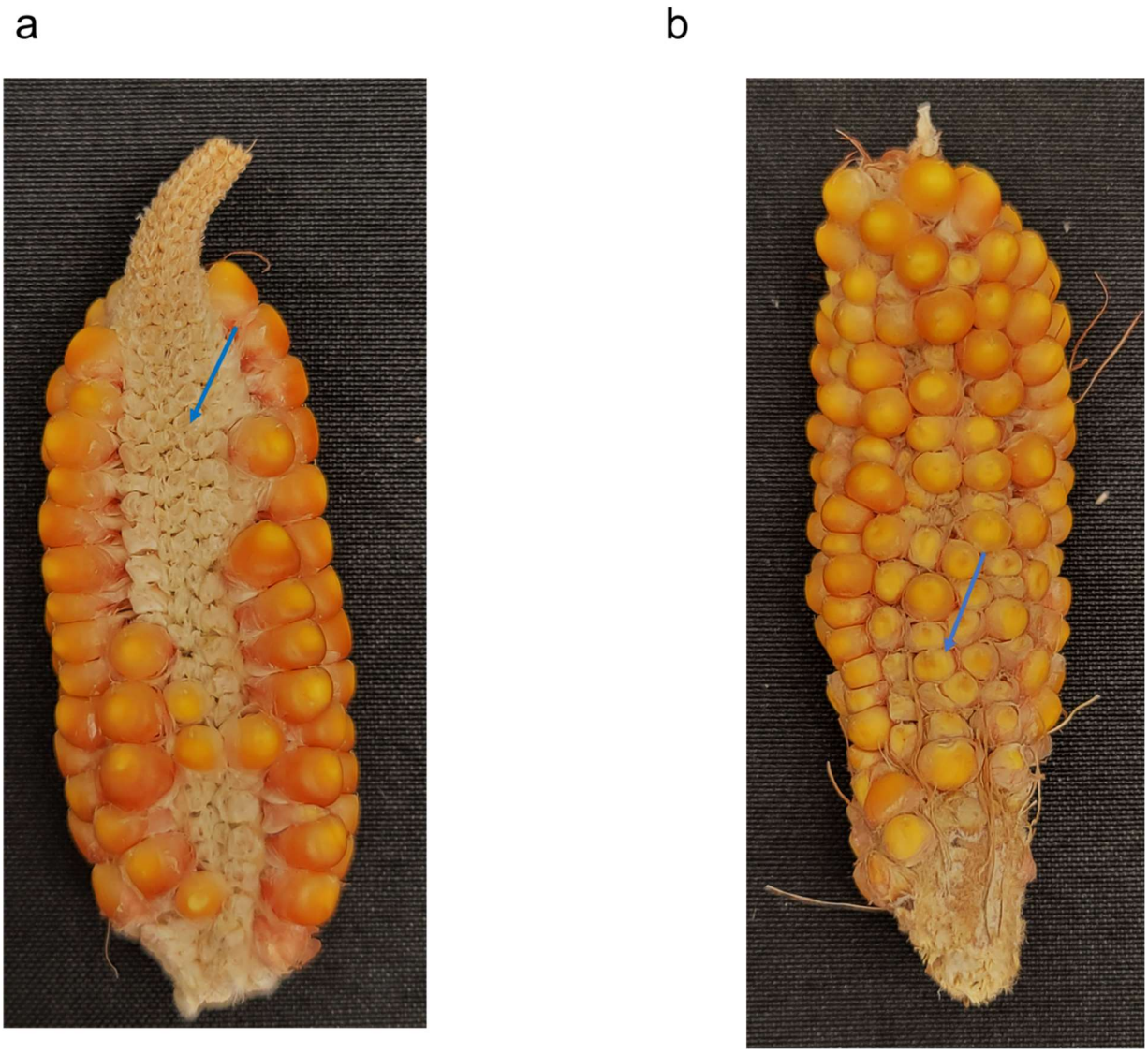
Gene	Gene ID	Primers
Arabidopsis Glutaredoxin 17 (AtGRXS17)	AT4G04950	Forward: AGTCGTGCTTCACTTCTGGG Reverse: CCAACCCTAAGCTTGCAGGA
Maize Glutaredoxin 17 (ZmGRXS17)	GRMZM2G131769	Forward: CCTGAGTCTGCAACTGAGAAG Reverse: CATCACTGGGCTGGAGTTAAT
Heat shock factor (ZmHSF03)	GRMZM2G118047	Forward: GAGCAGGTGCTGTCGTCAAG Reverse: TTGTTGCACAGCTTCTTCATCTG
Heat shock factor (ZmHSF04)	GRMZM2G125969	Forward: AGCAGCAAGACAAGAGGAAGGA Reverse: TGTTCGCTTTCCCCATCACT
Heat shock protein, 90 KDa (ZmHSP90)	GRMZM5G833699	Forward: TTTCGGGTCTGCTCTGAGTG Reverse: CTTGGGCATCTGTTGAGCTTC
Heat Shock protein, 70 KDa (ZmHSP70)	GRMZM2G079668	Forward: AATGGCATCCTCAACGTCTC Reverse: CCTGCACCATCTTCTCAATCT
Heat Shock protein, 26 KDa (ZmHSP26)	GRMZM2G149647	Forward: TGTCCGGAAGCTTCAACGTC Reverse: TTTCCTGGTTGGAGACCGTG
Catalase 1 (ZmCAT1)	GRMZM2G088212	Forward: ACAGGCTGTCGTGAGAAGTG Reverse: TACGGTGTTCATGGGTCACG
L-ascorbate peroxidase (ZmAPX)	GRMZM2G156227	Forward: TAAAACAGGCCTTCCAGGGG Reverse: TGCCTTTCTGGAGATCGCTG
ADP-glucose pyrophosphorylase (ZmAGPase)	GRMZM2G027955	Forward: CAGCCGGGCATCTGATTTTG Reverse: TAAGCCAAGCACTGTGGTGT

Table 2.1 Primers used for the qRT-PCR

Supplementary Figures



Supplementary Figure 2.1. Pot capacity decreased as the HTD stress progressed. On day 3, the pot capacity reduced to 43.3%.



Supplementary Figure 2.2. Effect of HTD stress on the kernel set and kernel size.

a) HTD stress can cause kernel abortion. **b)** HTD stress causes a reduction in the kernel size.

Chapter 3 - Improving blast resistance in the commercial rice cultivar by introducing *Pi9* with cisgenesis method of breeding

Abstract

The selectable markers like antibiotic and herbicide resistance genes are very practical in developing transgenic plants. However, consumers have concerns about the use of such genes in commercial crops because of the perceived potential for unpredictable hazards to human health and environment. Therefore, commercialization of the transgenic crops is tightly regulated. Here, we report the successful incorporation of a rice blast resistance gene *Pi9* into an elite US rice cultivar via a breeding strategy known as cisgenesis. We used a co-transformation system that eliminated the *hygromycin B phosphotransferase (Hpt)* gene as a selection marker from the cisgenic rice line. Out of 18 cisgenic lines, we found only one line to be successfully co-transformed indicating the low success of this transformation method. We analyzed fifty individual plants at T_1 generation after selfing of the T_0 cisgenic line and found four individual rice plants containing the *Pi9* gene without the *Hpt* gene. The blast-resistant cisgenic elite cultivar can help farmers fight off the various strains of the blast-causing agent. Moreover, the cisgenic rice plants demonstrate that plants can be developed that possess novel beneficial genes like disease resistance but lacking any trace of a selectable marker gene.

Introduction

Despite being beneficial to the economy and the environment, GM crops have been questioned about their safety and efficacy [1]. Concerns are raised about the presence of foreign genes in GM crops. Cisgenesis is one of the breeding methods that uses the native genes with the native introns, native promoter, and native terminator in the proper orientation. The production of the plants by the cisgenesis method is considered safe as it does not cause a huge change in the genome of recipient plants. The cisgenesis method is like traditional breeding and hence, the final product is likely to be welcomed by the consumers and environmental lobbyists [2–4]. The use of selectable marker genes (SMGs) is considered undesirable due to public and environmental concerns. The major concerns include the transfer of herbicide resistance genes in weeds and the spread of antibiotic resistance in gut bacteria even though these propositions are not backed by supporting evidence [5]. Moreover, there is no point in keeping them in the plant genome once the transgenic plant is generated [6]. Selectable marker-free products are more likely to get public acceptance for their commercialization [7].

Rice (*Oryza sativa* L.) is a very important staple crop of the world and is a primary source of diet (50-80% of calories daily) for more than 3 billion people worldwide [8,9]. Even though rice production has increased by double in previous decades, rice has been significantly impacted by abiotic and biotic stresses [8]. Rice blast is a destructive rice disease caused by the filamentous fungus *Magnaporthe oryzae* [9,10]. The disease causes a significant yield loss of 10-30% in most rice-growing countries [11,12]. Large-scale use of the fungicide to control *M. oryzae* could result in unwarranted health and environmental issues [13]. Hence, it is desirable to use the cultivar having resistance gene for controlling the blast disease. Approximately, 100 genes that confer resistance to the blast disease have been identified, and 22 of them have been cloned [14].

Developing a resistant variety through traditional breeding is challenging and can be gained for only short-term as genetic variation in the fungus improves fungus's capacity to overcome the resistance within 3-4 years [15,16].

Several attempts have been made to develop the resistance in rice by cloning the resistance genes. One of the cloned genes that show wide spectrum resistance against the blast disease is *Pi9*. *Pi9* is located in chromosome 6 and originated from *O. minuta* from where the gene was introgressed into isogenic line 75-1-127 genotype of *O. indica* [15]. *Pi9* encodes an NBS-LRR protein [16] that inhibits the interaction of host and blast pathogen by activating defense response genes. These genes synthesize secondary metabolites that prohibit the *M. oryzae* infection [17]. *Pi9* has been widely studied for its role in the improvement of the blast resistance rice cultivars [18–21].

The objective of the present work was to generate the marker-free blast-resistant rice cultivar via cisgenesis. We also investigated if the cisgenic expression of the *Pi9* gene affects the grain yield and improves the blast resistance in commercially cultivated rice.

Materials and Methods

Construction of the *Pi9* and selectable marker constructs

Pi9 gene containing its native promoter and terminator was inserted pLJ42 vector (**Figure 3.1A**). The integration of the cassette was confirmed by PCR after transferring into *E. coli.* Plasmids were transformed into *Agrobacterium tumefaciens* LBA4404 and confirmed by PCR. Similarly, *GFP* driven by rice ubiquitin promotor and terminated by NOS terminator was cloned in C- terminal of *Hpt* gene which was driven by (cauliflower mosaic virus) CaMV 35S promotor and terminated by 35S terminator (**Figure 3.1**). The construct was cloned into pRB01 vector and the plasmid was transformed into competent *E. coli* cells by the freeze/thaw method. The integration of the construct was confirmed by PCR and the representative clone was sequenced. The plasmids were transformed into the *Agrobacterium tumefaciens* LBA4404 and confirmed by PCR.

Rice callus was induced from the embryo of the seeds of "Jupiter" cultivar. Rice transformation was conducted using the mix of *Agrobacterium* containing both *Pi9 and Hpt* constructs via the agrobacterium mixture method of co-transformation [22].

DNA isolation and PCR analysis

Rice genomic DNA was isolated from leaf tissue of transgenic and "Jupiter" cultivar using 2% cetyltrimethylammonium bromide (CTAB) and phenol:chloroform: isoamyl alcohol (25:24:1). The integrity of DNA was confirmed by running 1% gel electrophoresis. A total of 20 µl of PCR reaction was carried out using the forward primer 5'- GCCCTGCAAGGACCCTAATA-3' and reverse primer 5'-GATGTTTCGTGGATGAGATC-3' to produce 699 bp amplicon size to confirm the integration of the *Pi9*. Similarly, the presence of *Hpt* was confirmed by amplifying the gene by using forward primer 5'- AAGTTCGACAGCGTCTCCGACC- 3' and reverse

primer 5'- GGCGACCTCGTATTGGGAATCC-3'.

RNA isolation and RT-PCR of *Pi9*

Total RNA was isolated from leaf tissues of cisgenic and wildtype rice using the Direct-zol RNA Miniprep Plus Kits (Zymo Research). 1 µg total RNA was used to synthesize first-strand cDNA using Revert Aid First Strand cDNA synthesis kit (Thermo Scientific™). 2 µl of cDNA was used as a template for RT-PCR. RT-PCR was carried out using the forward primer 5'-TGCCCAACCTTTACCCACACTGTA-3' and the reverse primer 5'-AACATGAGTAGAAACAAATTAGTTTG-3'. *Actin 1* gene was used as the internal control and was amplified using forward primer 5'-CGTCTGCGATAATGGAACTGG-3' and reverse primer 5'-CTGCTGGAATGTGCTGAGAGAT-3'.

GFP expression in rice leaves

In order to further confirm the presence or absence of the *Hpt* gene in the cisgenic crops, the plants were screened for the presence of GFP in the leaves of the rice plants. The rice leaf samples were visualized under the fluorescent microscope with a filter specific to eGFP (Zeiss AX10).

Blast inoculation

Fungal inoculation was performed on the uniformly grown transplant stage rice seedlings by using the spray inoculation method [23]. Two blast strains, 0-507 and 0-508 were used. Briefly, fungal spore concentration was adjusted to 50,000 spores/ml of 0.25% gelatin in 50 ml conical tubes. 3 ml to 4 ml of inoculum were applied per pot using an artist's airbrush (Paasche H no. 1) connected to the compressed air at 20 psi (1.4 bars) inside the dust containment hood. Plants were kept inside the plastic bag to maintain the high humidity. After 24 hours, the plants were

placed in the growth chamber. The plants were grown for 14 days and plant phenotypes were observed.

Agronomic traits

Cisgenic rice lines and wild-type cultivar ‘Jupiter’ were planted in the greenhouse to evaluate the agronomic traits of cisgenic rice. The agronomic traits such as time to bolting, plant height, plant biomass, number of tillers, 1000-grain weight, and grain yield per plant were measured and statistically analyzed by using one-way ANOVA.

Results

Cisgnic rice plant generation and molecular characterization of the *Pi9*

Out of 18 plants analyzed, only one plant got successfully co-transformed indicating low transformation. In the successful co-transformed line, *Pi9-8*, we detected the *Pi9* gene and *hygromycin B phosphotransferase* (*Hpt*) gene through PCR (**Figure 3.2A-C)**. The T_0 plants with both *Pi9* and *Hpt* were selfed and the T_1 seeds were grown. Out of 50 plants analyzed in the T_1 generation, 4 plants were identified with the *Pi9* gene but no *Hpt* gene. We named these 4 plants *Pi9-8-28, Pi9-8-35, Pi9-8-40,* and *Pi9-8-48* (**Figure 3.2D**) and were used for their ability to resist the blast. *Pi9-8-28, Pi9-8-40, Pi9-8-48*, the null segregate, and "Jupiter" cultivar were further studied for the agronomic properties.

***Pi9* expression in T_0 and T_1 generation plants**

Pi9-8 at T_0 generation were studied for the proper expression of the *Pi9*. Pi9-8 had the proper expression of the *Pi9* (**Figure 3.3A**). Similarly, *Pi9-8-28, Pi9-8-40, Pi9-8-47, Pi9-8-48*, the null-segregate, and "Jupiter" at T_1 generation were studied for the expression of the *Pi9*. At T_1 generation, *Pi9* specific bands were visible for *Pi9-8-28, Pi9-8-35, Pi9-8-40,* and *Pi9-8-48* indicating the proper expression of the *Pi9* gene in the segregating lines (**Figure 3.3B**). However, the bands specific to *Pi9* were not visible in null segregates and "Jupiter" (**Figure 3.3B**).

In order to further confirm the absence of marker in cisgenic rice, GFP was tagged along *Hpt* gene and screened in the T_1 generation. GFP was observed in the plants with the *Hpt* gene but was not observed in the plants that had only *Pi9* (**Figure 3.4**).

Effect of cisgenic expression of Pi9 on agronomic traits in rice

To find the effect of cisgenic expression of Pi9 on agronomic traits, WT, null segregate, and three *Pi9* cisgenic lines at T_1 were grown in the greenhouse under controlled condition. The WT and cisgenic lines were indistinguishable from each other phenotypically (**Figure 3.5A**). Several agronomic parameters were evaluated. Most of the plants started flowering between 95 to 100 days after sowing of the seed with most of the plants flowering on day 99 (**Figure 3.5B**). The null-segregate flowered earlier than the rest in average. The cisgenic lines were not different in growth (Figure 4) and in agronomic traits compared to WT and the null segregate (**Figure 3.5C-F)**.

***Pi9* expression improved the plant growth even after blast fungus inoculation**

In order to test the growth after fungal inoculation, the plants were treated with fungal spores using the spray assay method. The plants were grown for 14 days post-inoculation and plant height was observed qualitatively. The cisgenic rice plants showed relatively better growth than the WT when sprayed with both strains of fungus (**Figure 3.6**).

Discussion

Rice blast disease causes severe yield loss and threatens world food security. An effective and well-accepted rice variety needs to be developed for long-lasting resistance against the blast. Selectable marker-free cisgenic rice plants have the potential to be accepted by consumers. Several strategies are used to produce selectable marker-free plants. One of them is co-transformation and segregation in which the gene of interest and selectable marker genes are cloned into two separate vectors. It is expected that gene of interest and selectable marker genes are integrated into unlinked loci and segregate in the next generation [24]. Even though cisgenic and transgenic approaches are based on the same breeding technology, they are fundamentally different. Transgenic methods can use the genes from the alien species whereas cisgenesis uses the genes from the same or closely related species. Currently, the legal status of cisgenic plants is debated; however, consumer acceptance tended to be better when the scientific definition was presented [25].

In this study, we successfully generated cisgenic selectable marker-free rice plants expressing the *Pi9* gene. However, the success of co-transformation was very low. Several strategies of co-transformation have been performed [22,26–28]. Low transformation frequency can be seen in the mixed strain method of co-transformation as the frequency of transformation is dependent upon the successful transformation of two independent transformation events [26]. However, most of these co-transformation procedures had higher efficiency than we found in our research. The co-transformation efficiency ranged from 16-50% [29–31]. Co-transformation success frequency is also found to depend upon the proportion of strains with strains mixing in a 1:1 ratio resulting in 81% of co-transformation frequency [32].

The primer combination for identifying the *Pi9* gene can successfully distinguish between the *Pi9* gene and its homolog. “Jupiter” contains the homolog of the *Pi9* gene which is slightly larger than

the expected 699 bp size. M202, a blast susceptible rice, also produced a larger band of 769 bp when used the same primer pairs [33]. The cisgenic rice consisted of two bands: one corresponds to the expected specific 699 bp size and the other corresponds to the homolog present in the "Jupiter" (**Figure 3.2C**). Furthermore, the band-specific to *Pi9* gene was gel extracted and sequenced. The sequenced 699 bp amplicon was searched in NCBI database and found to be well aligned with *Pi9* sequence in the database as well as the plasmid sequence. When *Pi9* gene and its homolog in "Jupiter" cultivar were aligned, we observed the deletion in *Pi9* gene (**supplementary fig. 3.1**). A phylogenetic tree suggests that the sequence similarity exists between the smaller band (699 bp) and the plasmid sequence (**supplementary fig. 3.1**).

"Jupiter" (*O. sativa* L.) is a medium-grain rice cultivar generated from the cross of high-yielding cultivars 'Bengal'/'Rico1'/3/'Bengal'//'Mercury'/'Rico 1'. The grain yield of "Jupiter" is extremely good. "Jupiter" is resistant to blast race IB-54 and rotten neck blast but susceptible to races IC-17, Ib-49, IG-1, and IE-1K [34]. The presence of the homolog gene in "Jupiter" might provide the resistance against the blast race IB-54 and rotten neck blast. The resistance capability of the cisgenic rice was evaluated by spray assay. Several viruses and fungi cause stunted growth in rice. Infection by *M. oryzae* in rice plants results in a reduction in plant growth rate and leaf area formation [35]. We used two strains of *M. oryzae* that cause the blast disease. Even though we did not observe the blast resistance in the cisgenic rice, we found that the cisgenic rice plant height relatively increased than WT after two weeks of inoculation. However, our study is incomplete. We believe that *Pi9* cisgenic rice should be tested vigorously against other *M. oryzae* strains.

Removing the selection marker and unnecessary transgene can potentially address the biosafety concerns of regulatory bodies. In our study, we used the mixed-strain method to co-transform the elite rice cultivar using the cisgenic approach. Even though the co-transformation frequency was

very low, we successfully generated a marker-free *Pi9* expressing cisgenic rice. One of the ways to improve the co-transformation frequency is by using *Agrobacterium* mixes at different proportions. This system provides an effective and convenient approach to develop cisgenic plants.

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Figures

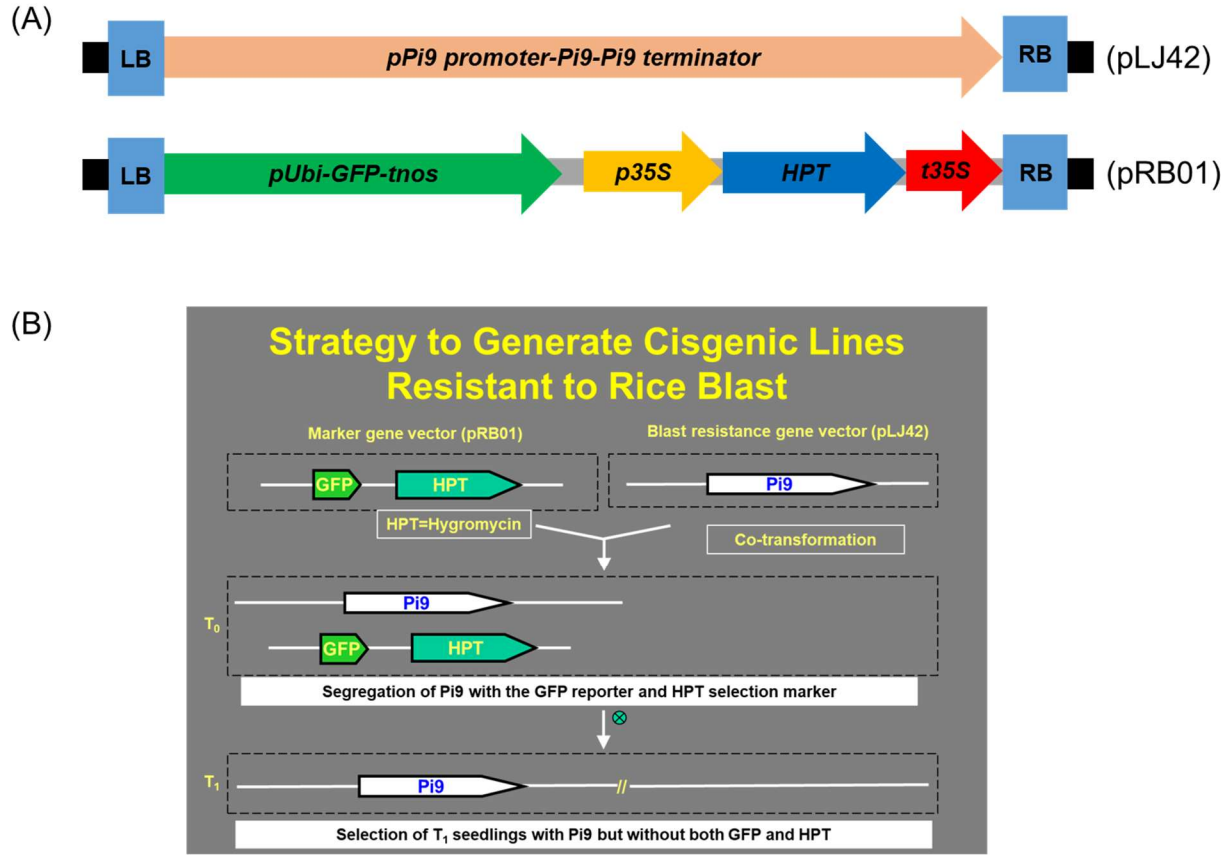


Figure 3.1 Construct design for *Pi9* and *Hpt* gene.

(A) T-DNA region design for *Pi9* gene which is driven by its native promoter and terminator (Upper panel) and T-DNA region design for marker genes (Lower panel). (B) A flowchart to generate the marker-free cisgenic rice by co-transformation approach.

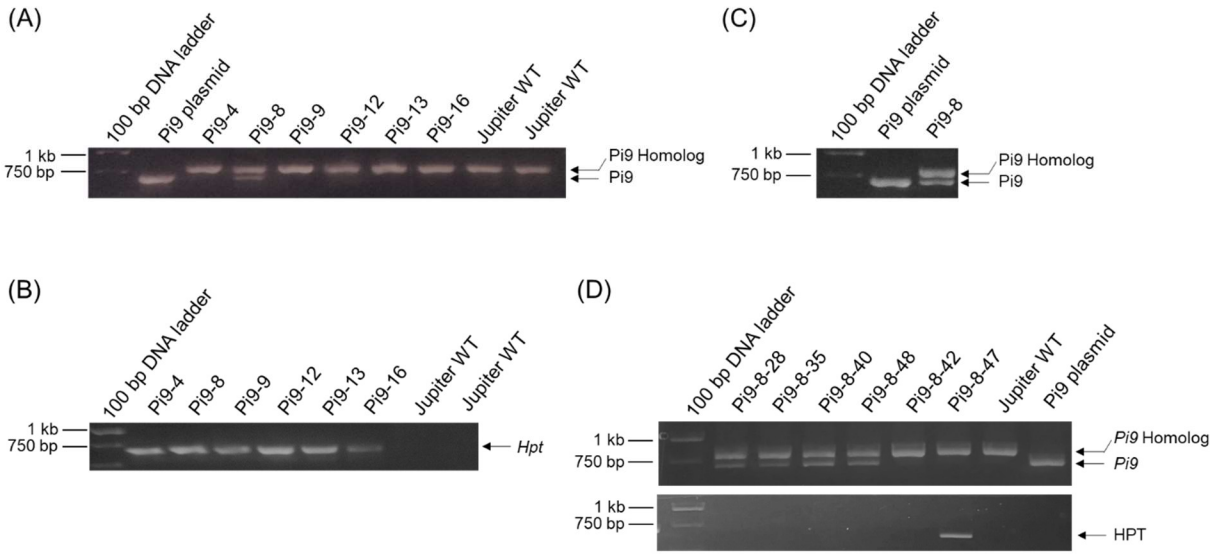


Figure 3.2 . PCR analysis of the cisgenic rice in T0 and T1 generation.

(A) PCR detection of *Pi9* gene (above) and *Hpt* gene (below) at T_0 generation in the genomic level. Cisgenic Pi9-8 shows the double bands (enlarged in the side), the smaller band representing the *Pi9*-specific band and larger band representing the homolog. (B) PCR detection of *Pi9* only segregate at T_1 generation in the genomic level. Pi9-8-28, Pi9-8-35, Pi9-8-40 and Pi9-8-48 show double bands (above) and do not contain the *Hpt* bands indicating the successful generation of the marker-free cisgenic rice plants. Pi9-8-42 lacks both *Pi9* and *Hpt* hence, it is the null segregate.

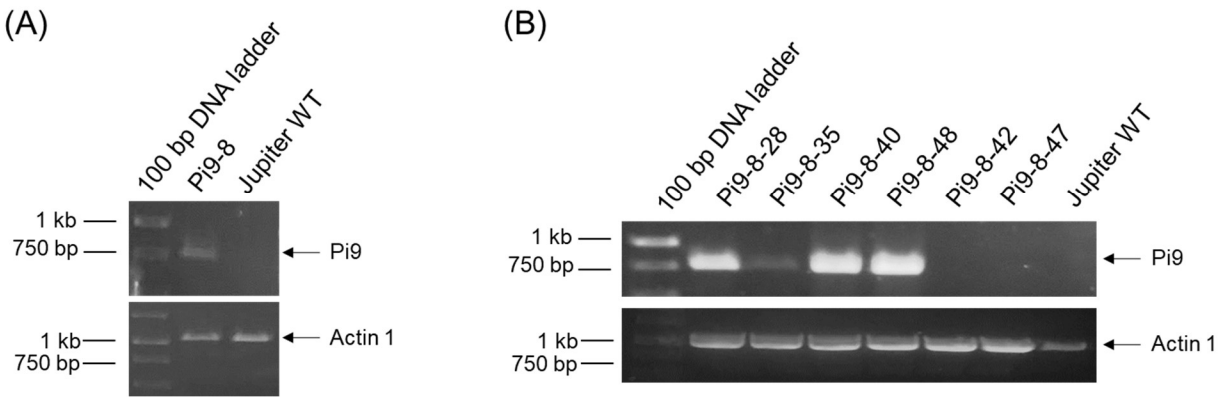


Figure 3.3 Semi-quantitative RT-PCR analysis of the cisgenic rice in T0 and T1 generation. (A) Presence of *Pi9*-specific band in Pi9-8 at T_0 generation. (B) Presence of *Pi9*-specific band in cisgenic marker-free rice plants at T1 generation. *Actin 1* was used as the internal control.

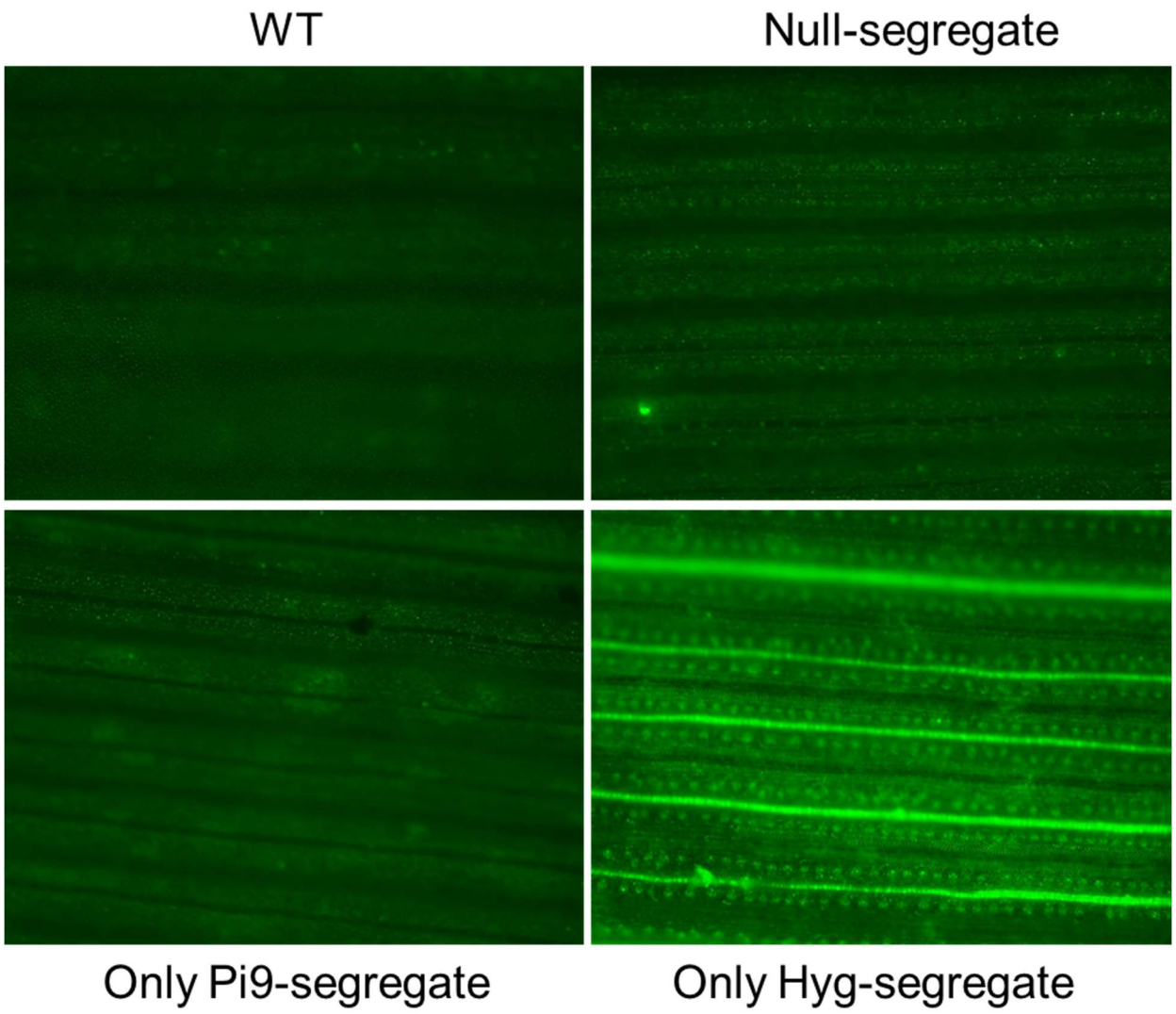


Figure 3.4 Fluorescent microscopy of the rice leaves under the fluorescent microscope at T1 generation.

Figure 4. The GFP is expressed together with *hpt* gene. All the cisgenic plants used for further analysis were *Hpt* free and GFP free.

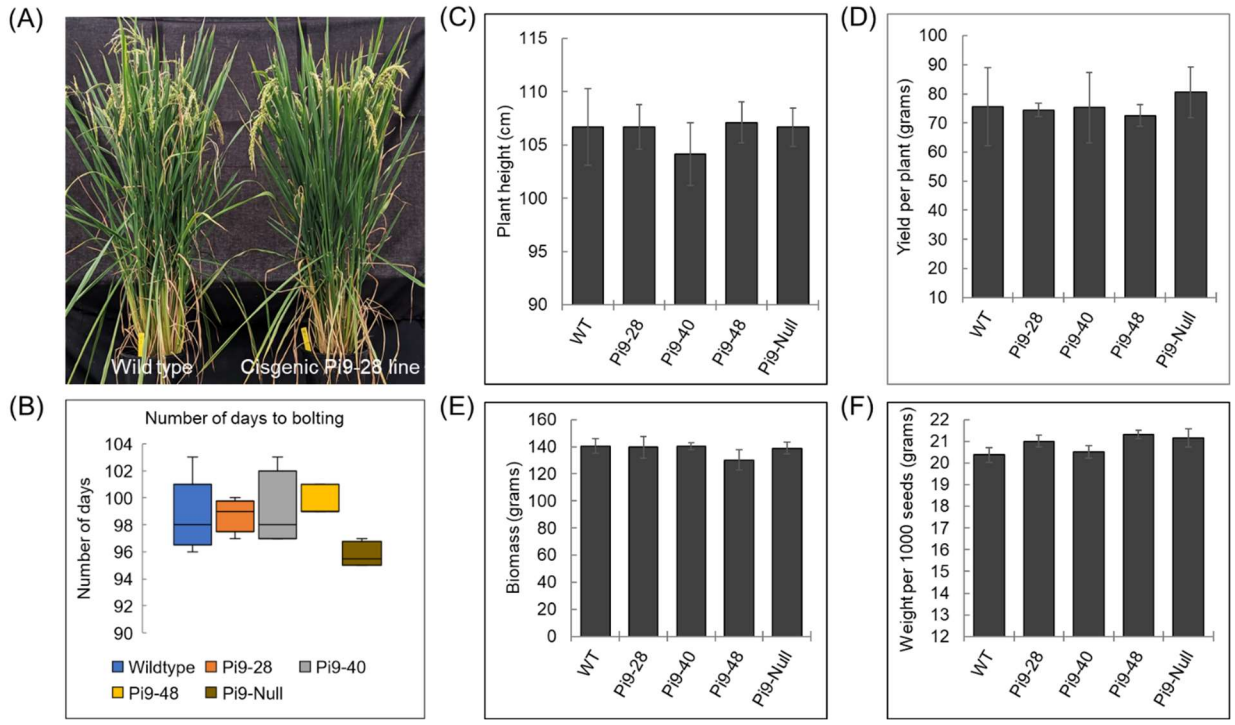


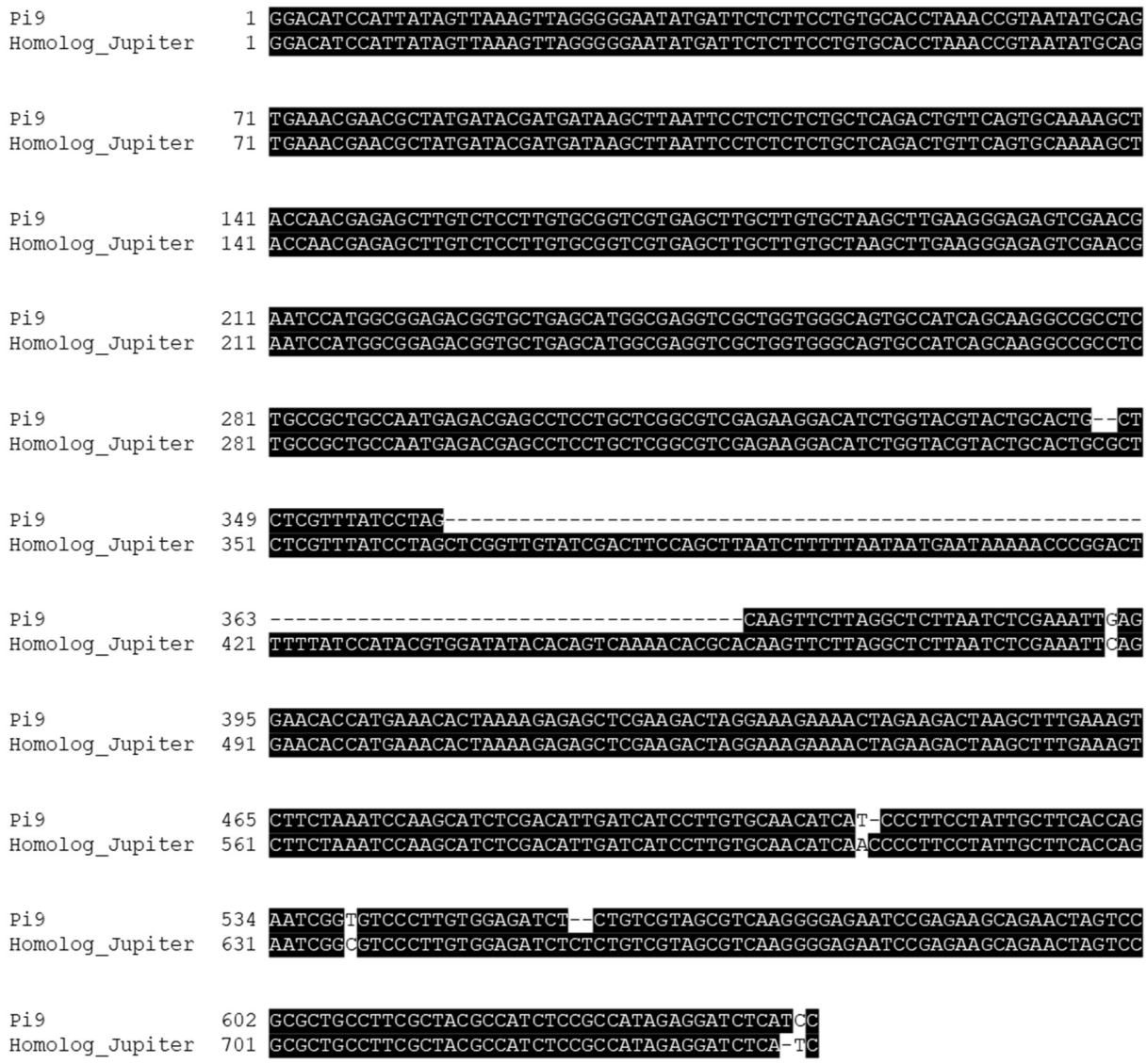
Figure 3.5 Phenotypic analysis of the various agronomic properties between the wildtype, null segregate and three cisgenic lines (n=6).

(A) The cisgenic rice lines were phenotypically indistinguishable from the wildtype. (B) Number of days taken by rice plants to bolt. The null segregate flowered 2 days earlier than the other rice lines including the wildtype, Jupiter. (C) The plant height of the cisgenic rice, wildtype and null segregate were not significantly different from each other. (D) The yield per plant of the cisgenic rice, wildtype and null segregate were not significantly different from each other. (E) The biomass of the cisgenic rice, wildtype and null segregate were not significantly different from each other. (F) The weight per 1000 seeds of the cisgenic rice, wildtype and null segregate were not significantly different from each other.

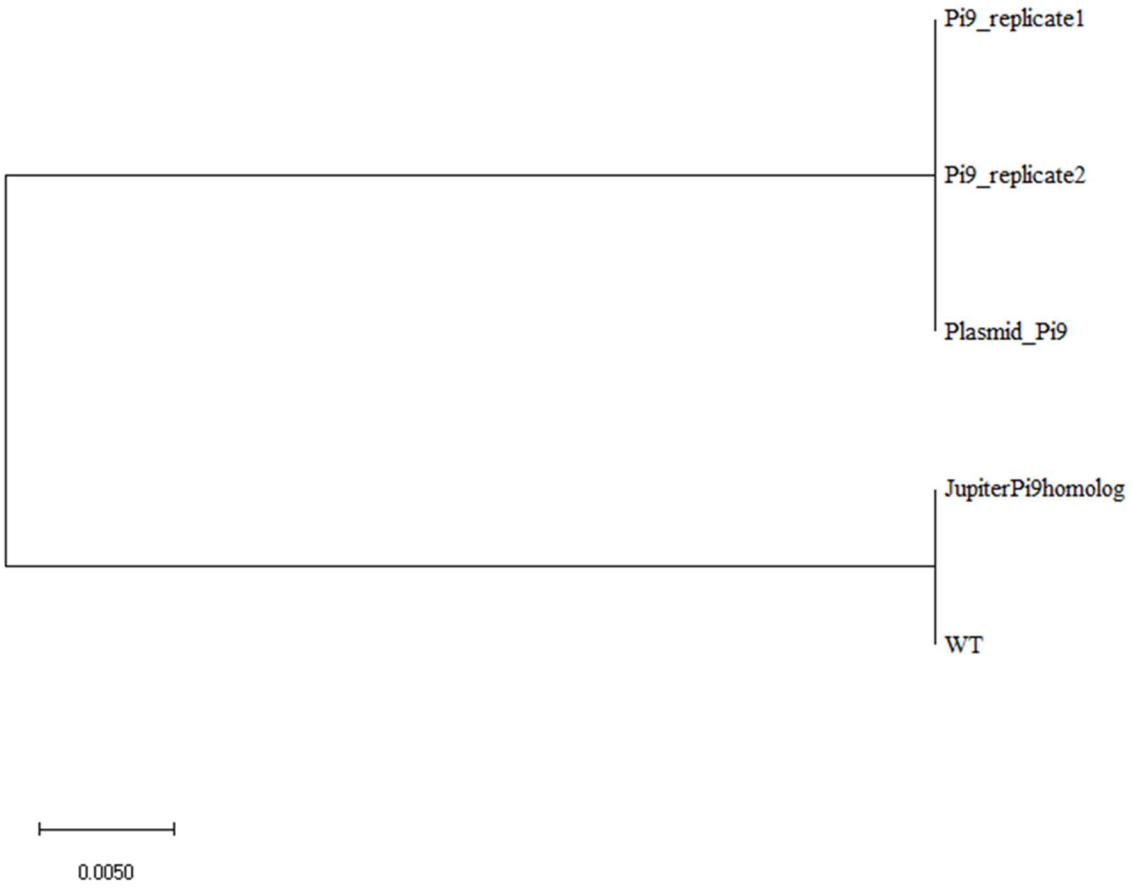
Figure 3.6 Blast disease resistance analysis of the rice plants after two weeks of inoculation.

Figure 6. Rice plants include wildtype, marker-free cisgenic rice and cisgenic rice with marker. The plants containing *Pi9* gene kept growing better than the wildtype after two weeks of inoculation. Two blast strains were used 0-507 and 0-508.

Supplementary Figures



Supplementary figure 3.1. DNA sequence alignment between the *Pi9* homolog gene found in *O. sativa* 'Jupiter' and *Pi9* gene.



Supplementary figure 3.2. A phylogenetic analysis between the amplicon sequence of smaller amplicon (699 bp), large amplicon, *Pi9* homolog of "Jupiter" cultivar and WT "Jupiter" using the primer pairs for amplifying *Pi9* gene portion. The smaller amplicons (Pi9_replicate1 and Pi9_replicate2) were grouped together with the sequence of the plasmid indicating the match between smaller amplicon and plasmid.