THE ROLES OF GLUTAREDOXIN GRXS17 IN IMPROVING CHILLING TOLERANCE IN TOMATO AND DROUGHT TOLERANCE IN RICE VIA DIFFERENT MECHANISMS

by

YING HU

B.S., China Agricultural University, 2006
M.S., China Agricultural University, 2009

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Horticulture, Forestry and Recreation Resources
College of Agriculture

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Abstract

Abiotic stresses, including chilling and drought stresses, are considered to be major limiting factors for growth and yield of agricultural and horticultural crops. One of the inevitable consequences of abiotic stresses is the accumulation of reactive oxygen species (ROS) in plants. ROS can either act as an alarm signal to induce the defense pathway when kept at a low level or cause oxidative damage to various cellular components when increased to a phytotoxic level. Glutaredoxins (GRXs) are members of ROS scavenging system that can maintain the cell redox homeostasis by using the reducing power of glutathione. In this research, we characterized the roles of GRXs in protecting tomato (*Solanum lycopersicum*) from chilling stresses and rice (*Oryza sativa L.*) from drought stresses. Our results indicated that ectopic expression of an *Arabidopsis* gene *AtGRXS17* in tomato could enhance the chilling tolerance by increasing antioxidant enzyme activities and reducing H_2O_2 accumulation to ameliorate oxidative damage to cell membranes and photosystems. Furthermore, *AtGRXS17*-expressing tomato plants had increased accumulation of soluble sugars to protect plant cells from dehydration stress. In rice, silenced expression of a rice glutaredoxin gene *OsGRXS17* was used as a reverse-genetic approach to elucidate the roles of OsGRXS17 in drought stress tolerance. Our results showed that silenced expression of *OsGRXS17* conferred improved tolerance to drought stress in rice. ABA-mediated stomatal closure is an important protection mechanism that plants adapt to a drought stress conditions, and H_2O_2 acts as secondary messenger in ABA signaling to induce the stomatal closure. Silenced expression of *OsGRXS17* gave rise to H_2O_2 accumulation in the guard cells and promoted ABA-mediated stomatal closure, resulting in reduced water loss, higher relative water content, and consequently enhanced drought tolerance in rice. This research provides a new perspective on the functions of GRXs in chilling and drought stress tolerance of tomato and rice, and an important genetic engineering approach to improve chilling and drought stress tolerance for other crop species.

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Major Professor
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Dedication

I dedicate my dissertation work to my mother and father, Shumei Sun and Jianwei Hu, who always encourage me to face difficulties positively and teach me the value of education. I also dedicate this work to my dear sister, Yi Hu, who is my best friend and has never left my side.

A special feeling of gratitude to my husband, Zhao Peng, for his love, support and encouragement; and my son, Lionel Peng, whose innocence and happiness taught me the beauty of life.

Chapter 1 - Introduction

Today, we are living in a world of 7 billion people. The unprecedented challenge facing world agriculture is to produce enough food to meet the food demand of the increasing world population. Because of the global shifting weather patterns, environmental stresses including water scarcity (drought), extreme temperature (heat, freezing and chilling), and contamination of soils by high ion concentration (salt, metals), become major constraints of crop productivity and quality (Fraire-Velázquez and Balderas-Hernández 2013). Therefore, understanding the mechanisms of plant responses to abiotic stresses in physiology, genetics and molecular biology will be very helpful to improve the tolerance of crops to unfavorable environments.

In this dissertation, we use tomato and rice as experimental materials to study how to improve the tolerance in those two crops to environmental stresses. Tomato is one of the most important vegetable crops which is rich in vitamin A, vitamin C and lycopene (Cohen 2002). Because of its tropical origin, it is very sensitive to chilling stress (Saltveit and Morris 1990). Germination, vegetative growth, flowering, fruit set development, ripening and postharvest are all affected by chilling stress (Weiss and Egea-Cortines 2009). Rice is a very important staple food and has high economic and social values (Maclean and Dawe 2002). Rice is typically grown in paddy fields and requires a lot of water to maintain normal growth and development. It is estimated that irrigated rice consumes about 34-43% of the world's total irrigation water (Bouman *et al*. 2007). Increasingly scarce water resources severely limit rice production and cause huge economic losses every year (Bouman et al. 2007). Therefore, it will be very helpful to improve the chilling tolerance in tomato and drought tolerance in rice.

One of the inevitable consequences of abiotic stresses is the accumulation of reactive oxygen species (ROS), which consist of four forms: singlet oxygen ($^1O_2$), superoxide radical (O_2^-

), hydrogen peroxide (H_2O_2) and hydroxyl radical ($HO^{\cdot}$). In plants, most ROS are generated in mitochondria, chloroplasts, and peroxisomes as the result of partial reduction of oxygen during the aerobic metabolic processes such as respiration and photosynthesis (Cruz de Carvalho 2008). Since ROS are continuously produced during the aerobic metabolic processes, under physiological steady state, the level of ROS in plant cells is tightly controlled by a versatile antioxidant system which includes ROS scavenging enzymatic antioxidants such as superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), glutathione reductase (GR), glutathione peroxidase (GPX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR) and non-enzymatic antioxidants such as ascorbate (AA) and glutathione (GSH) (Figure 1.1) (Alscher et al. 1997; Gill and Tuteja 2010).

Reactive oxygen species (ROS) were initially considered as extremely toxic compounds in plant cells and result in oxidative damage to lipids, proteins, and DNA (Apel and Hirt 2004; Hancock et al. 2001). However, research has brought new lines of evidence showing that increases in ROS can serve as an alarm signal, which plays a crucial role in many defense response processes, such as stomata closure, gravitropism, development, hormonal sensing, and responses to biotic and/or abiotic stresses (Bowler et al. 1992; Dat et al. 2000; Noctor and Foyer 1998; Vranová et al. 2002; Willekens et al. 1997). The dual effects of ROS under abiotic stresses depend on overall cellular concentration of ROS. When ROS are kept at a low level under tight control of an antioxidant system, they function as signaling molecules triggering defense responses in plant. If ROS accumulate to a phytotoxic level, uncontrolled oxidative damage to cellular components will be initiated, and subsequently result in cell death (Dat et al. 2000).

ROS have quite a few advantages in serving as signal molecules. First, the ROS level can be changed rapidly and dynamically in response to various environmental stresses. Plants have

evolved a versatile ROS production and scavenging system that can work simultaneously to tightly control the ROS level. A slight tilting balance of ROS production and scavenging could result in the change in ROS level (Bailey-Serres and Mittler 2006). In addition, ROS have been considered to serve as rapid long distance auto-propagating signals in plant (Baxter et al. 2014). Recently developed imaging tools, such as a luciferase reporter gene driven by a rapid ROS-response promoter, allow us to study the distribution of ROS in plants when they suffer from biotic or abiotic stresses. It is revealed that a ROS burst initiated in specific cells could trigger a series of cell-to-cell communication events by a temporal-spatial coordination between ROS and other signals (Miller et al. 2009). As mentioned above, ROS have several forms of molecules with different properties and mobility in cells. For instance, O_2^- is a charged molecule that cannot passively transfer across the membrane, while it can be rapidly converted to H_2O_2 that can passively transfer across membrane (Miller et al. 2010). Therefore, ROS have advantages to serve as signal molecules due to their versatile forms with different properties and mobility. Moreover, as part of cellular signaling networks, ROS signaling is linked with several different pathways such as Ca^{2+}, nitric oxide (NO), protein phosphorylation networks (Besson-Bard et al. 2008; Kobayashi et al. 2007; Ogasawara et al. 2008).

Here I propose the following model for the role of ROS in plants under abiotic stresses (Figure 1.2). When plants are exposed to various abiotic stresses, the level of ROS is changed dynamically by a ROS production/scavenging system. At the beginning of abiotic stresses, although the normal steady-state level of ROS is perturbed by abiotic stresses, the antioxidant system can maintain ROS below a phytotoxic level. If exposure to an abiotic stress is extended, the balance of ROS production and scavenging will tilt towards production, resulting in an increasing level of ROS. Instead of causing immediate damage, the increase in ROS can act as an

alarm signal and induce a plant defense response if kept under a tight control of the ROS scavenging system. However, the continuing abiotic stresses can perturbed equilibrium between ROS production and scavenging and the level of ROS increases dramatically. This controlled production of ROS can cause oxidative damage to lipids, proteins and DNA, and finally result in programmed cell death (Apel and Hirt 2004; Cruz de Carvalho 2008). Therefore, ameliorating oxidative damage through enhancement of *in vivo* levels of antioxidant enzymes is an effective way to protect plants from various abiotic stresses (Table 1.1).

Glutaredoxins (GRXs) are ubiquitous oxidoreducatases of the thioredoxin (Trx) family to maintain the cellular redox homeostasis and regulate redox-dependent signal pathways by utilizing the reducing power of glutathione to catalyze the reversible reduction of disulfide bonds of their target proteins (Fernandes and Holmgren 2004; Rouhier et al. 2008). GRXs can reduce target proteins through a dithiol or monothiol pathway (Figure 1.3) (Rouhier et al. 2008). For the dithiol pathway, GRXs can reduce the disulfide bonds on the target protein into two thiol groups using two active cysteine sites. For the monothiol pathway, GRXs can catalyze the reduction of protein and glutathione mixed disulfide bond and only one cystein site is required. Therefore, it is believed that GRXs maintain the cellular redox homeostasis and regulate redox-dependent signal pathway through a post-translational modification. The functions of GRXs are well studied in bacteria, yeast, animals and humans. They are involved in many processes, such as prevention of apoptosis (Enoksson et al. 2005), dysregulated proliferation (Löfgren et al. 2008) and formation of ion sulfur complex (Lillig et al. 2005). They also play roles in transduction signaling pathways by regulating some transcription factors (Nakamura et al. 1999; Zheng et al. 1998), kinases (Humphries et al. 2002; Ward et al. 1998) and phosphatases (Barrett et al. 1999; Rao and Clayton 2002). They are also important in regulating cell redox homeostasis by targeting some antioxidant enzymes such

as GPX (Björnstedt et al. 1994) and SOD (Klatt and Lamas 2000), in metabolic regulation through enzyme regulation (Cappiello et al. 1996; Lind et al. 1998).

In plants, only a few members of GRXs have been characterized (Rouhier et al. 2004). The first plant GRX was isolated from rice, which shows thioltransferase activity (Minakuchi et al. 1994). Recently, with deeper genomic analysis, increasing number of *GRX* genes is identified in higher plants such as *Arabidopsis thaliana* (31 GRX genes), *Populus trichocarpa* (36 GRX genes) and *Oryzae Sativa* (27 GRX genes) (Nicolas, 2006). Based on the active site sequence, the GRXs of higher plants can be subdivided into four groups (Couturier et al. 2009). GRXs of class I and class II are conserved in all photosynthetic organisms. GRXs of class I have CxxC/S (cys-x-x-cys/ser) active sites and are homologous to *Escherichia coli* GRX1 and GRX3, yeast GRX1 and GRX2, and mammalian GRX1 and GRX2. GRXs of class II have CGFS (cys-gly-phe-ser) active sites and are homologous to *Escherichia coli* GRX4 and yeast GRX2, GRX4 and GRX5 (Herrero and De La Torre-Ruiz 2007). There are four members in GRXs of class II (GRXS14, GRXS15, GRXS16 and GRXS17), which are different in the numbers of GRX domains. GRXS14, GRXS15 and GRXS16 have small molecular weights with only one GRX domain, while GRXS17 are larger with three GRX domains and N-terminal TRX-like homology domain. GRXs of class II are monothiol GRXs that can only reduce the mixed disulfide bond between GSH and target proteins (Lemaire 2004). GRXs of class III are specific to higher plants and have a peculiar CCxx (cys-cys-x-x) active site, a diversified active site. The functions of this class of GRXs are rarely reported. Only two studies showed that they are involved in petal development (ROXY1 and ROXY2) and pathogen responses through jasmonic acid/salicylic acid signaling (ROXY19/GRX480) (Ndamukong et al. 2007; Xing et al. 2005). GRXs of class IV harbor an N-terminal GRX domain with a CxDC/S (cys-x-asp-cys/ser) active site in higher plant. In this study, we are focusing on two

homologous GRXs of class II (AtGRXS17 and OsGRXS17), which have an N-terminal TRX-like domain and three GRX domains (Figure 1.4).

Very few studies are available on the functions of GRXs in plants. It has been proven that GRXs are involved in stress response and redox regulation, petal development, meristem growth, plant-pathogen interactions and iron-sulfur cluster assembly (Knuesting et al. 2015b; Ndamukong et al. 2007; Wu et al. 2012; Xing et al. 2005; Yang et al. 2015). Most studies unveiled the roles of plant GRXs are focusing on abiotic and biotic stress responses. The *Arabidopsis* GRX gene *AtGRXS13* participates in protecting *Arabidopsis* from photooxidative stress by affecting superoxide levels and the ascorbate/dehydroascorbate ratio (Laporte et al. 2011). The *Pteris vittata* glutaredoxin PvGRX5 is involved in the tolerance to arsenic and heat (Sundaram and Rathinasabapathi 2010; Sundaram et al. 2009b). The tomato plants silenced for the expression of *SlGRXS1* are sensitive to oxidative, drought and salt stresses (Guo et al. 2010). The *Arabidopsis* glutaredoxin AtGRXcp and AtGRX4 are involved in oxidative stress responses (Cheng 2008; Cheng et al. 2006). Many previous studies demonstrated that the *Arabidopsis* glutaredoxin AtGRXS17 plays essentials roles in heat stress response and photoperiod response (Cheng et al. 2011a; Knuesting et al. 2015b; Wu et al. 2012). GRXs are also involved in other processes. GRXs of class III have been identified for their functions in petal development and jasmonic acid/salicylic acid signaling (Ndamukong et al. 2007; Xing et al. 2005). Some of GRXs (CXIP1 and CXIP2) are demonstrated to interact with Ca^{2+} transporter and may regulate their activities (Cheng and Hirschi 2003). It is also reported that some GRXs are also involved in reducing dehydroascorbate to ascorbic acid to provide an alternative way to dehydroascorbate reductase (Sha et al. 1997). Another important new discoveries are that poplar GRX C4 could target type II PRX and aldolase by glutathiolation and a poplar glutaredoxin is able to reduce pop Trxh4 indicating a direct

interaction between thioredoxin and glutaredoxin/GSH systems (Gelhaye et al. 2003; Ito et al. 2003; Rouhier et al. 2006).

Nowadays advances in plant molecular biology and plant biotechnology have dramatically improved our capacity for gene discovery, functional characterization of genes, and development of genome editing method. Understanding the mechanisms underlying the abiotic stress tolerance will be greatly helpful in improving the tolerance of plants to abiotic stresses through plant biotechnology techniques. In this dissertation, *AtGRXS17*-expressing tomato plants were used to investigate the function of AtGRXS17 in chilling stress response, and *OsGRXS17* silenced rice plants were used to study the function of OsGRXS17 in drought stress response. Our results suggest that ectopic expression of *AtGRXS17* could enhance the chilling tolerance in tomato by ameliorating oxidative damage while silenced expression of *OsGRXS17* could enhance the drought tolerance in rice via stomatal aperture control.

FIGURES

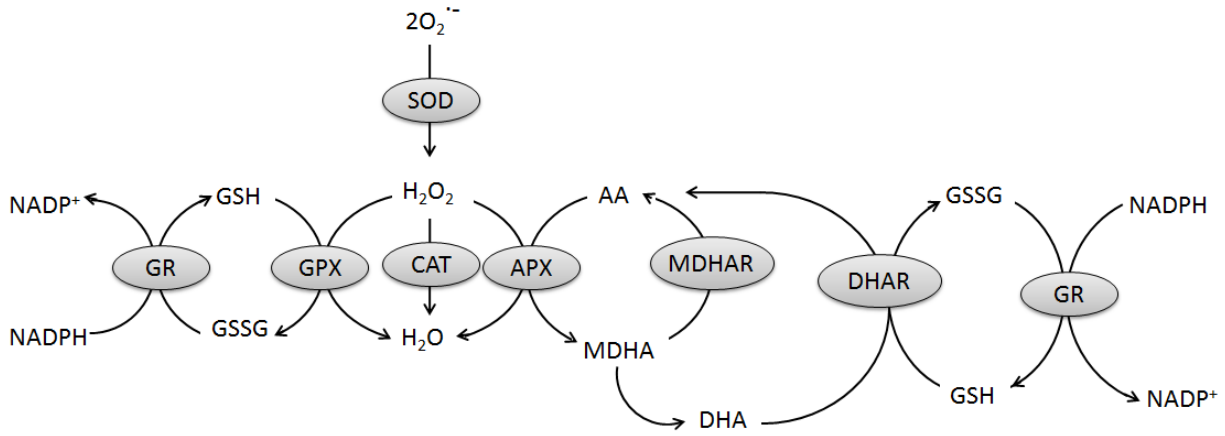

Figure 1. 1 Enzymatic antioxidant system in plants.

Superoxide dismutase (SOD) converts hydrogen superoxide ($O_2^{\cdot-}$) into hydrogen peroxide (H_2O_2). Catalase (CAT) converts H_2O_2 into water. Hydrogen peroxide is also converted into water by ascorbate-glutathione cycle. In this cycle, ascorbate is oxidized into monodehydroascorbate (MDHA). MDHA reductase (MDHAR) reduces MDHA into ascorbate (AA) by using the reducing capacity of NADPH. Dehydroascorbate (DHA) is produced spontaneously by MDHA and is reduced to ascorbate (AA) by DHA reductase (DHAR) with the help of GSH (Reduced glutathione) that is oxidized into GSSG (Oxidized glutathione). Finally, GSSG can be converted into GSH by glutathione reductase (GR). The glutathione peroxidase (GPX) cycle can also converts H_2O_2 into water. H_2O_2 can be reduced into water by GPX with the help of GSH that is oxidized into GSSG. GSSG can be converted into GSH by GR (Gill and Tuteja 2010).

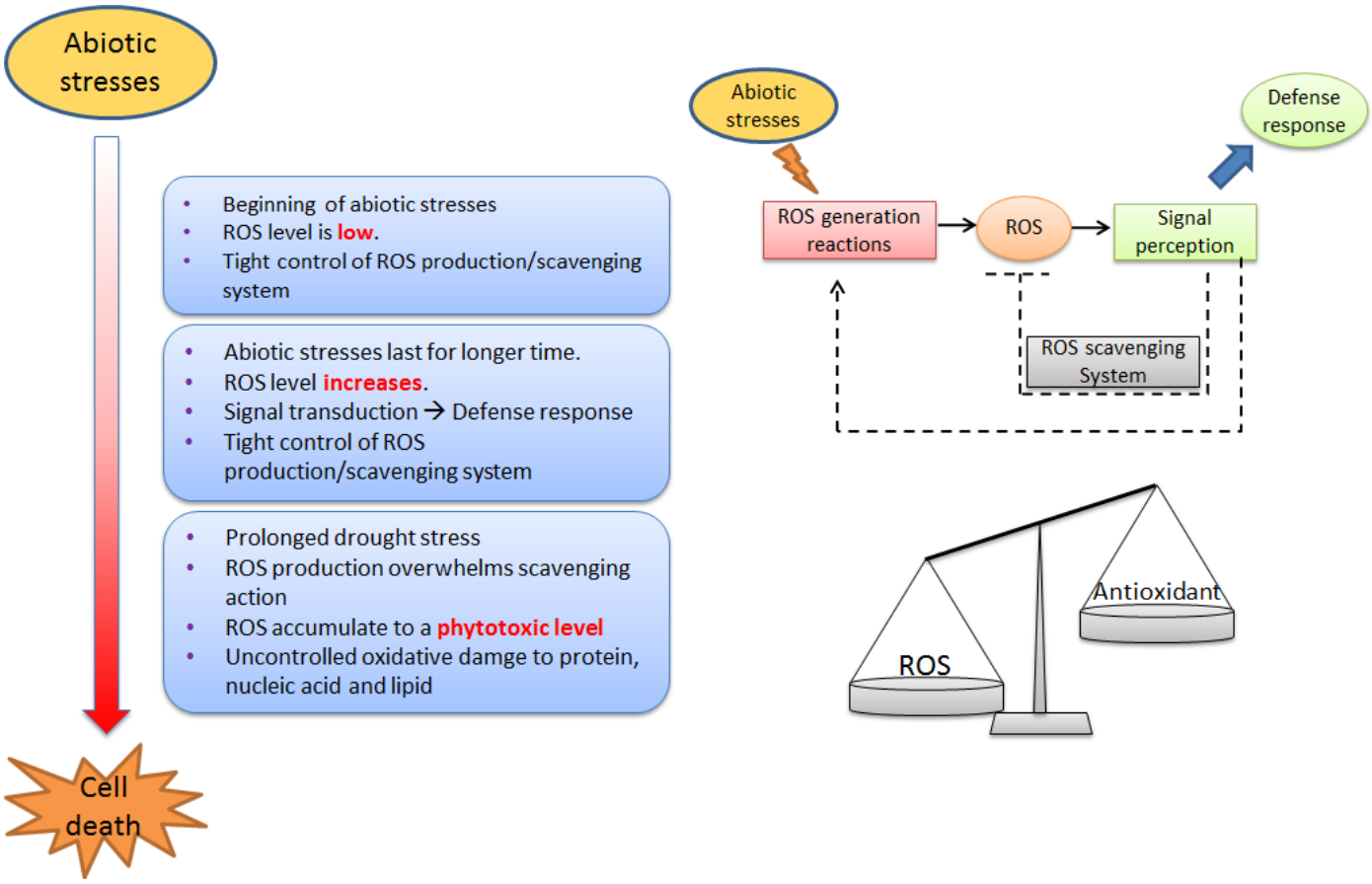

Figure 1. 2 Proposed model for the role of ROS in plant under abiotic stresses.

At the beginning of abiotic stresses, although the normal steady-state level of ROS is perturbed by abiotic stresses, the antioxidant system can tightly control the level of ROS under phytotoxic level. If abiotic stresses are ongoing, the balance of ROS production and scavenging will tilt towards production, resulting in an increasing level of ROS. Instead of causing immediate damage, this rise in ROS can act as an alarm signal and induce a defense response if kept under a tight control of ROS scavenging system. However, the continuous abiotic stresses can change the balance between ROS production and scavenging and the level of ROS will increase dramatically. This controlled production of ROS can cause oxidative damage to lipids, proteins and DNA and finally result in programmed cell death.

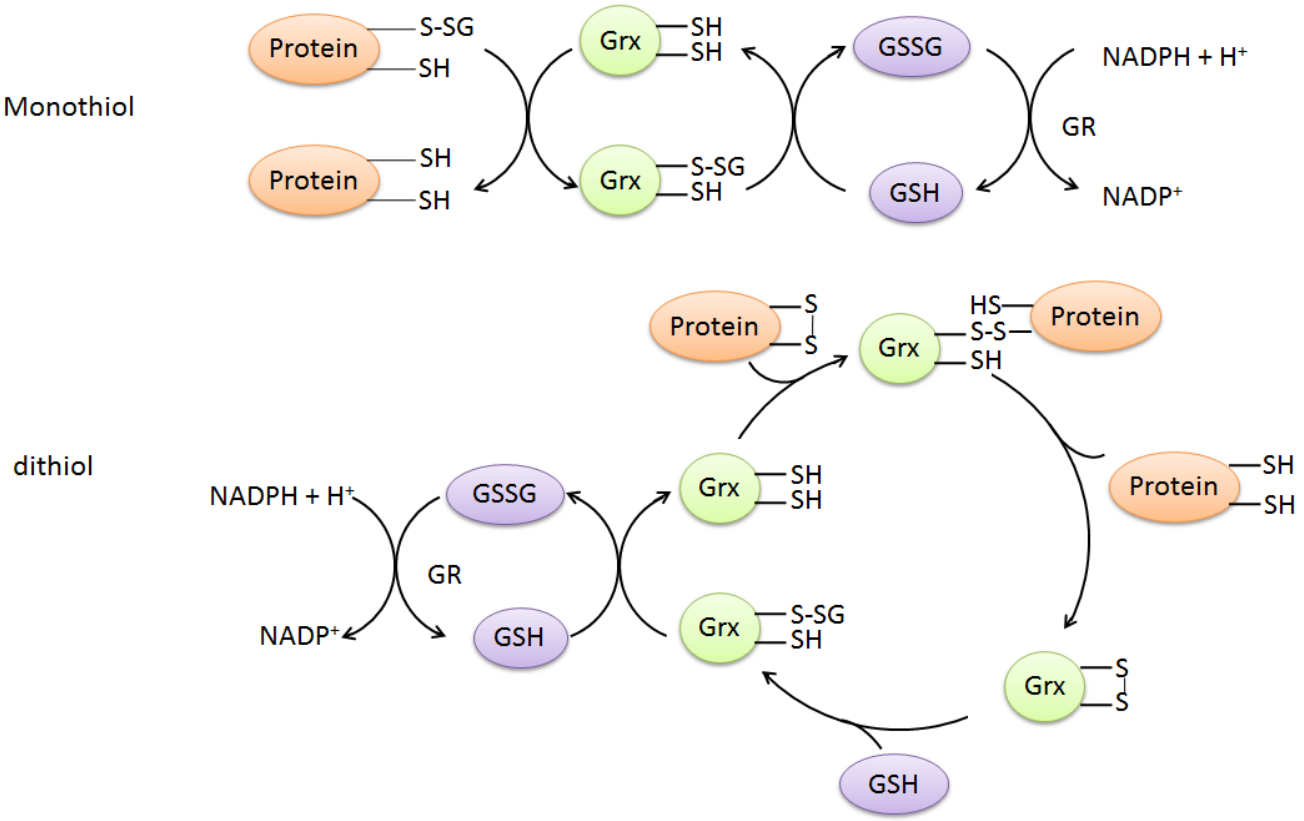

Figure 1. 3 The monothiol and diothiol pathway of glutaredoxin dependent catalyzation.

In the monothiol pathway, only one thiol group of GRXs is required to reduce the mixed disulfide bond between target protein and glutathione, and the glutathionylated GRX is reduced by reduced glutathione (GSH). In the dithiol pathway, two thiol groups of GRXs are required to reduce the disulfide bond in target protein, and the disulfide bond of oxidized GRXs can be reduced to thiol groups by two reduced GSH (Rouhier et al. 2008).

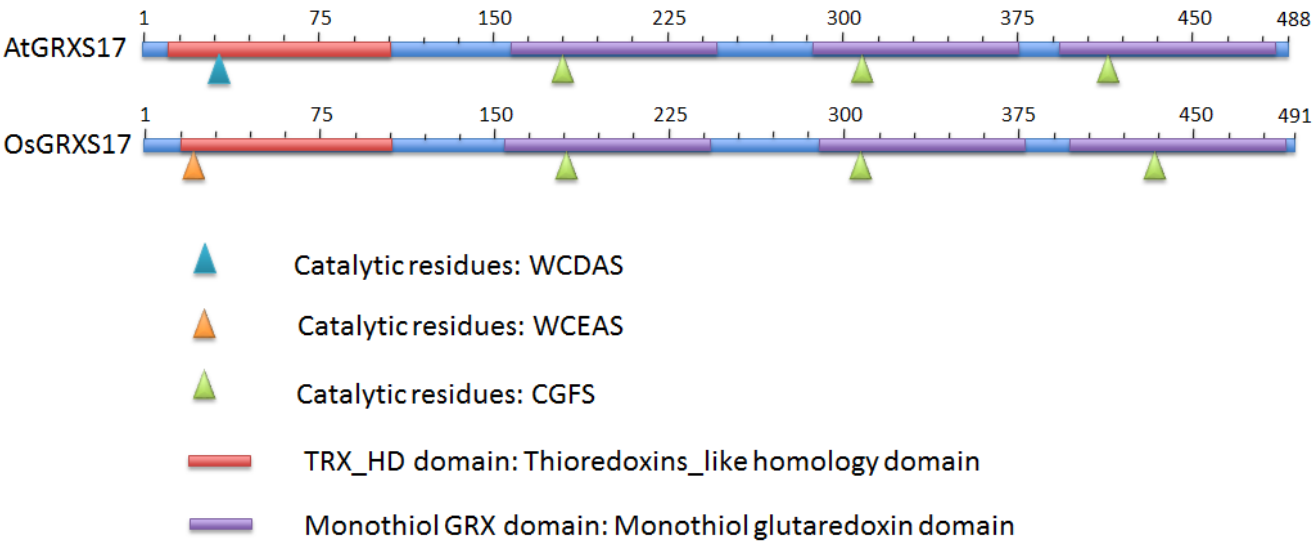

Figure 1. 4 The domain structure of AtGRXS**17 and Os**GRXS**17.**

Both of AtGRXS17 and OsGRXS17 possess an N-terminal TRX_HD domain followed by three GRX domains with CGFS motifs. The differences between AtGRXS17 and OsGRXS17 are active motif in N-terminal TRX_HD domain (AtGRXS17 has WCDAS (Trp-Cys-Asp-Ala-Ser) motif while OsGRXS17 has WCEAS (Trp-Cys-Glu-Ala-Ser) motif) and positions of all active motifs.

Table 1. 1 A brief list of studies showing the roles of ROS scavenging enzymatic and non-enzymatic antioxidants in protection of transgenic plants from abiotic stresses

Antioxidants	**Source**	**Target**	**Response in transgenic plants**	**Reference**
Cu/Zn SOD	*Oryza sativa* L.	*Nicotiana tabacum*	Enhanced tolerance to salt and water stresses	Badawi et al., 2004
Cu/ZnSOD or MnSOD and APX	*Nicotiana tabacum*	*Nicotiana tabacum* cv. Xanthi	Enhanced tolerance to oxidative and less mambrance damage	Kwon et al., 2002
MnSOD	*Pisum sativum*	*Oryza sativa* L. cv. Zhonghua 11	Enhanced drought tolerance	Wang et al., 2005
MnSOD	*Tamarix androssowii*	*Populus davidiana* × *P. bolleana*	Enhanced salt tolerance	Wang et al., 2010
MnSOD	*Arabidopsis thaliana*	*Arabidopsis thaliana*	Enhanced salt tolerance	Wang et al., 2004
FeSOD	*Arabidopsis thaliana*	*Medicago sativa L.*	Enhanced tolerance to photosynthetic oxidative stress	McKersie et al., 2000
MnSOD	*Triticum aestivum*	*Brassica napus*	Enhanced tolerance to aluminum stress	Basu et al., 2001
Cu/ZnSOD and APX	*Manihot esculenta and Pisum sativum*	*Ipomoea batatas (L.) Lam. cv. Yulmi*	Enhanced tolerance to oxidative stress and chilling stress	Lim et al., 2007
APX	*Arabidopsis thaliana*	*Nicotiana tabacum*	Enhanced tolerance to salt, drought and PEG stresses	Badawi et al., 2003
APX	*Oryza sativa* L.	*Arabidopsis thaliana*	Enhanced salt tolerance	Lu et al., 2007
APX	*Oryza sativa* L.	*Oryza sativa* L.	Enhanced chilling tolerance at the booting stage	Sato et al., 2011
APX	*Hordeum vulgare*	*Arabidopsis thaliana*	Enhanced salt tolerance	Xu et al., 2008
CAT	*Zea mays*	*Nicotiana tabacum cv. SR1*	Enhanced tolerance to oxidative stess	Polidoros et al., 2001

CAT	*Escherichia coli*	*Nicotiana tabacum cv. Xanthi*	Enhanced tolerance to photooxidative stress	Miyagawa et al., 2000
CAT	*Triticum aestivum*	*Oryza sativa* L.	Enhanced tolerance to low temperature stress	Matsumura et al., 2002
GR	*Escherichia coli*	*Brassica juncea*	Enhanced tolerance to cadmium stress	Pilon-Smits et al., 2000
GR	*Arabidopsis thaliana*	*Gossypium hirsutum L.* cv. Coker 312	Enhanced tolerance to chilling stress	Kornyeyev et al., 2003
MDAR	*Arabidopsis thaliana*	*Nicotiana tabacum* (SR-1)	Enhanced tolerance to ozone, salt and PEG stresses	Eltayeb et al., 2007
DHAR	*Oryza sativa* L.	*Arabidopsis thaliana*	Enhanced salt tolerance	Ushimaru et al., 2006
DHAR	*Arabidopsis thaliana*	*Nicotiana tabacum*	Enhanced tolerance to ozone and drought stresses	Eltayeb et al., 2006
DHAR	*Arabidopsis thaliana*	*Nicotiana tabacum*	Enhanced tolerance to aluminum stress	Yin et al.,2010
DHAR, GST and GR	*Arabidopsis thaliana and Escherichia coli*	*Nicotiana tabacum*	Enhanced tolerance to salt and cold tolerance	Martret et al., 2011
GST and GPX	*Nicotiana tabacum*	*Nicotiana tabacum L.* cv. Xanthi NN	Enhanced tolerance to oxidative stress	Roxas et al., 2000
GST	*Gossypium*	*Nicotiana tabacum*	Enhanced toerance to oxidative stress	Yu et al., 2003
GST	*Nicotiana tabacum*	*Arabidopsis thaliana*	Reduced salt stress-induced lipid peroxidation	Katsuhara et al., 2005
GST	*Oryza sativa* L.	*Oryza sativa* L.	Enhanced germination and growth under chilling stress	Takesawa et al., 2002
GPX	*Chlamydomonas*	*Nicotiana tabacum cv. Xanthi*	Reduced unsaturated fatty acid hydroperoxides and increased tolerance to oxidative stress	Yoshimura et al., 2004

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Chapter 2 - Tomato Expressing *Arabidopsis* Glutaredoxin Gene *AtGRXS17* Confers Tolerance to Chilling Stress via Modulating Cold Responsive Components

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ABSTRACT

Chilling stress is a production constraint of tomato (*Solanum lycopersicum*), a tropical origin, and chilling-sensitive horticultural crop. The development of chilling tolerant tomato thus has significant potential to impact tomato production. Glutaredoxins (GRXs) are ubiquitous oxidoreductases, which utilize the reducing power of glutathione to reduce disulfide bonds of substrate proteins and maintain cellular redox homeostasis. Here, we report that ectopic expression of *Arabidopsis* GRX gene *AtGRXS17* in tomato conferred tolerance to chilling stress without adverse effects on growth and development. *AtGRXS17*-expressing tomato plants displayed lower ion leakage, higher maximal photochemical efficiency of photosystem II (Fv/Fm) and increased accumulation of soluble sugars compared with wild-type plants after the chilling stress challenge. Furthermore, chilling tolerance was correlated with increased antioxidant enzyme activities and reduced H_2O_2 accumulation. At the same time, temporal expression patterns of the endogenous C-repeat/DRE Binding Factor 1 (*SlCBF1*) and *CBF* mediated-cold regulated (*COR*) genes were not altered in *AtGRXS17*-expressing plants when compared to wild-type plants, and proline concentrations remained unchanged relative to wild-type plants under chilling stress. GFP-AtGRXS17 fusion proteins, which were initially localized in the cytoplasm, migrated into the nucleus during chilling stress, reflecting a possible role of AtGRXS17 in nuclear signaling of chilling stress responses. Together, our findings demonstrate that genetically engineered tomato plants expressing *AtGRXS17* can enhance chilling tolerance and suggest a genetic engineering strategy to improve chilling tolerance without yield penalty across different crop species.

Keywords: Chilling stress, Tomato, Glutaredoxin, Oxidative stress, Tolerance.

INTRODUCTION

Chilling stress, here defined as exposure to temperatures ranging from 0 to 12 °C, adversely affects the growth and development of many crop species of tropical or subtropical origin, limiting agricultural productivity(Cruz et al. 2013). Tomato (*Solanum lycopersicum*) is also subject to chilling stress due to extensive cultivation in temperate regions despite a tropical origin. Germination, vegetative growth, flowering, fruit set development, ripening, and postharvest are all affected by chilling stress (Weiss and Egea-Cortines 2009). In the past two decades, while numerous traditional or molecular breeding efforts have been undertaken to improve tomato chilling tolerance, significant successful progress has not been made due to the complexity of chilling tolerance traits, linkage drag that affects yield and quality, and the lack of quantifiable physiological parameters related to chilling tolerance (Chinnusamy et al. 2004; Fowler and Thomashow 2002). Thus, genetic engineering should be considered as an alternative approach to improve chilling tolerance in tomato.

One of the well utilized gene families to genetically engineer chilling tolerance in tomato is *CBF/DREB1* (C-repeat binding factor/dehydration responsive element binding protein 1) (Thomashow 2010). Plants that are adapted to chilling conditions, including *Arabidopsis*, respond to low temperatures and activate expression of members of the *CBF/DREB1* gene family of transcription factors, which includes *CBF1*, *CBF2*, and *CBF3*. The protein products of the genes, in turn, regulate the expression of *COR* (cold-regulated) genes (Thomashow 2010). *COR* genes constitute the *CBF* regulon, which includes more than 100 genes, and have been shown to be essential for both chilling tolerance and cold acclimation in plants (Chinnusamy et al. 2004; Fowler and Thomashow 2002). Though tomato has a CBF-mediated cold responsive pathway, the tomato *CBF* regulon consists of fewer and less functionally diverse genes than that of *Arabidopsis*

(Carvallo et al. 2011; Zhang et al. 2004). Therefore, ectopic expression of genes involved in cold-responsive pathways of *Arabidopsis*, particularly the *CBFs*, may have a different response and adaptation to cold stress than in tomato (Carvallo et al. 2011; Zhang et al. 2004). Moreover, growth retardation has been attributed to ectopic expression of *CBF* genes in tomato plants, further limiting the effectiveness of ectopic expression of *CBFs* for engineering chilling tolerant tomato (Hsieh et al. 2002; Zhang et al. 2004). Similarly, constitutive expression of *CBFs* in *Arabidopsis* (Kasuga et al. 1999), potato (Pino et al. 2007), and *Brassica napus* (Jaglo et al. 2001) enhances freezing tolerance but induces dwarfism, in part, due to the accumulation of DELLA proteins, a family of nuclear growth-repressing regulatory proteins (Achard et al. 2008).

An inevitable consequence of chilling stress is the accumulation of reactive oxygen species (ROS), which is one of the major factors leading to cold injury (Cruz et al. 2013; Jaspers and Kangasjärvi 2010). Although ROS can act as signal molecules for stress responses, excess ROS causes oxidative damage to various cellular components including membrane lipids, structural proteins, and enzymes, and leads to inhibition of plant growth and development (Gill and Tuteja 2010; Jaspers and Kangasjärvi 2010; Suzuki and Mittler 2006). Therefore, ROS levels must be regulated in plants through the coordination of ROS production and scavenging to manage oxidative damage while maintaining ROS-mediated signaling (Foyer and Noctor 2005; Rouhier et al. 2008).

Glutaredoxins (GRXs) are small ubiquitous oxidoreductases of the thioredoxin (TRX) family and catalyze reversible reduction of disulfide bonds of substrate proteins by using the reducing power of glutathione (GSH) (Rouhier et al. 2008). The family members are present in both prokaryotes and eukaryotes and are necessary for redox buffering, heavy metal detoxification, plant development, plant-pathogen interactions, iron homeostasis, and oxidative stress response

(Cheng et al. 2011a; Lillig et al. 2008; Rouhier et al. 2008; Rouhier et al. 2005; Shelton et al. 2005). In addition, previous studies suggest that individual GRX family members may have multiple functions in plants. For instance, PvGRX5 from *Pteris vittata* plays roles in both arsenic and heat stress tolerance (Sundaram and Rathinasabapathi 2010; Sundaram et al. 2009b). AtGRXS13 from *Arabidopsis* functions in both pathogen and photooxidative stress responses (La Camera et al. 2011; Laporte et al. 2011), and AtGRXS17 has critical functions in regulating cellular ROS metabolism (Cheng et al. 2011b), shoot apical meristem development (Knuesting et al. 2015a), and heat stress tolerance (Wu 2012). Yet, the function of AtGRXS17 in oxidative damage due to chilling stress of plants, how AtGRXS17 affects chilling stress responses, chilling-associated gene expression, plant growth and development, and signaling under chilling stress, is unknown.

In this study, we ectopically expressed *AtGRXS17* in tomatoes to investigate the role of *AtGRXS17* in chilling tolerance. Further, we generated the stress inducible *RD29A*::*AtCBF3*-expressing tomatoes as positive control lines and compared chilling tolerance of the plants to *AtGRXS17*-expressing and wild-type lines. The cellular localization of AtGRXS17 under chilling stress conditions was also characterized by using transient expression in *Nicotiana tabacum*. Temporal expression patterns of the endogenous *SlCBF1* and *CBF* mediated-*COR* genes, as well as physiological and biochemical responses were analyzed to investigate whether ectopic expression of *AtGRXS17* enhanced chilling tolerance through a CBF-independent manner during chilling stress. This work provides an innovative perspective on engineering chilling tolerance in tomato.

MATERIALS AND METHODS

Bacterial strain, plasmid and tomato transformation

AtGRXS17 coding region was cloned into pBICaMV vector driven by the cauliflower mosaic virus (CaMV) *35S* promoter as described previously (Wu 2012). The *AtCBF3* coding region was also cloned into pMDC99 vector driven by a cold-inducible *RD29A* promoter (Feng et al. 2011) to avoid any negative effects on plant growth due to constitutive expression of *CBFs* (Kasuga et al. 1999). Binary plasmids pMDC-AtCBF3 and pBICaMV-AtGRXS17 were introduced into *Agrobacterium tumefaciens* strain LBA4404 using the freeze-thaw method(Holsters et al. 1978) and used for generating stable transgenic lines, respectively. Seeds of tomato *Solanum lycopersicum* L. (cv Rubion) were surface sterilized and germinated on the Murashige and Skoog inorganic salt medium (Murashige and Skoog 1962), and tomato transformation was performed via *Agrobacterium*-mediated transformation method using cotyledon and hypocotyls explants as described (Park et al. 2003).

Growth conditions and tolerance analyses of tomato

T2 generation of *AtGRXS17*-, *AtCBF3*-expressing or wild-type tomato seeds were surface-sterilized, germinated, and grown in pots containing Metro Mix (900) growing medium in a growth chamber maintained at 24 ^{o}C/20 ^{o}C (day/night) under a 16-h photoperiod, and a light intensity maintained at 300 μmol photons m^{-2} s^{-1}. The plants were regularly watered and fertilized on a weekly basis with 20:20:20 fertilizer (Scotts). For the chilling treatment, 4-week-old *AtGRXS17*-, *AtCBF3*-expressing or wild-type seedlings were treated at 4 ^{o}C (day/night) for 3 weeks in a walk-in growth chamber, and then recovered in normal growth conditions as mentioned above for 2

weeks. The electrolyte leakage and chlorophyll fluorescence was tracked during the first 7-day chilling treatment.

For oxidative stress treatment, 7-day-old *AtGRXS17*-expressing and wild-type seedlings grown on the MS media were transferred into the MS medium with or without 20 μM methyl viologen (MV) in magenta boxes and incubated for 14 days. The primary root length was measured after harvest.

RNA Extraction and qRT-PCR

Total RNA was isolated using the Qiagen Plant RNeasy kit (Qiagen, Valencia, California, USA) from leaves of tomato plants according to the manufacturer's instructions. The cDNA was synthesized using the iScript Select cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). One microliter of the reverse transcription reaction solution was used as a template in a 25 μl PCR solution. Real-time qRT-PCR was performed in 25 μl reactions contain 10.5 μl cDNA, 1 μl 10mM of each primer, and 12.5 μl SYBR Green PCR Master Mix (Bio-Rad, Hercules, CA, USA). Analysis was performed using the Bio-Rad IQ3 (Bio-Rad, Hercules, CA, USA). Primer efficiencies were measured and relative expression level was calculated using the comparative Ct method (Wu 2012). *SlPP2ACS* was uses as a normalization control (Lovdal and Lillo 2009). The primers for PCR were listed in the supplementary data (Table 2.1).

Electrolyte leakage and Fv/Fm ratio

Injury to plants was characterized by measuring chlorophyll fluorescence and electrolyte leakage of leaves as described previously (Oh et al. 2009). Chlorophyll fluorescence from the adaxial side of the leaf was monitored using a portable chlorophyll fluorometer (PEA, Hansatech Instruments Ltd., UK). Photochemical efficiency of leaves as determined by chlorophyll fluorescence ratios

(Fv/Fm) was monitored during and after the chilling treatment. Measurements were made during the light cycle on the leaves using the saturation pulse method after 30 min of dark adaption. For electrolyte leakage, tomato leaf samples were incubated in 15 mL of distilled water for 10 h to measure the initial electrolyte leakage using an YSI conductance meter (Model 32, YSI, Inc., Yellow Springs, OH, USA). The samples were subjected to 80 ^{o}C for 2 h to release the total electrolytes and then held at room temperature for 10 h. The final conductivity on the leachate was measured to determine the percent electrolyte leakage from the leaf samples.

Histochemical detection of H_2O_2

H_2O_2 was visually detected *in situ* in the leaves of tomato plants by staining with 3,3'-Diaminobenzidine (DAB) as described previously (Bindschedler et al. 2006; Thordal-Christensen et al. 1997) with modification. Briefly, the terminal leaflet of the first fully expanded leaf was sampled from wild-type and *AtGRXS17*-expressing 4-week-old plants. Leaflets were completely immersed with the DAB solution (1 mg/ml, pH 3.8, added with 0.05% (v/v) Tween 20 and 5% (v/v) 200 mM Na_2HPO_4). The sampled leaves were placed in petri dishes covered with aluminum foil under 4 ^{o}C until brown precipitate was observed (2 to 3 d) and then cleared in boiling ethanol (96%) for 10 min. H_2O_2 accumulation was detected as brown spots after DAB staining. Quantitative analyses of DAB staining were performed using image J analysis (Wu 2012).

Enzyme assays

Superoxide dismutase (SOD) was measured using a modified NBT method (Beyer and Fridovich 1987). Reactions were set up containing 50 mM phosphate buffer (pH 7.8), 13 mM methionine, 75 μM p-nitro blue tetrazolium, 2 μM riboflavin, 10 μM EDTA and 50 μl of enzyme extract. The reaction was initiated by illuminating the samples under a 15 W fluorescent tube. Four samples

containing pure buffer instead of extract were also made. Two of these were placed in the dark and used as the blank and another two samples were placed under the 15 W fluorescent tubes as the control. Both controls and those containing enzyme extract were placed under lights for 10 min. The absorbance of the samples at 560 nm was measured, and one unit of SOD activity was defined as the amount of enzyme that inhibits the NBT photoreduction by 50%. The specific activity of SOD was expressed as unit/mg protein.

Catalase (CAT) activity was measured by using the Amplex Red catalase assay kit (Molecular Probes). In brief, initially reaction mixtures containing 25 µl catalase-containing samples and 25µl 40 µM H_2O_2 were incubated at room temperature for 30 min. Then 50 µl 100 µM Amplex red reagent containing 0.4 U/ml horseradish peroxidase (HRP) was added to each microplate well containing the samples and controls and incubated at 37 ^oC for 30 min under protected from light. CAT activity was determined by measuring the absorbance at 560 nm using a microplate reader. One unit was defined as the amount of enzyme that will decompose 1.0 µM of H_2O_2 per minute at pH 7.0 at 25 ^oC. The specific activity of CAT was expressed as mU/µg protein.

Guaiacol peroxidase (POD) activity was measured by a modified method of Maehly and Chance(Maehly and Chance 1954). The reaction was set up containing 50 mM sodium acetate buffer pH 5.6, 0.2% guaiacol, 0.3% H_2O_2 and enzyme extract. The increase in absorbance due to the oxidation of guaiacol to tetraguaiacol was monitored at 470 nm. One unit was defined as 0.01 absorbance increase per minute at 470 nm. The specific activity of POD was expressed as unit/mg protein.

Proline determination

The proline content was determined using a colorimetric assay as described previously (Ábrahám et al. 2010). Four-week-old plants were treated at 4 ^{o}C and the terminal leaflet of the first fully expanded leaf was sampled from wild-type and *AtGRXS17*-expressing plants were harvested on 0, 1, 3, 5, 7, 12, and 21 days, respectively. One hundred milligram of leaf tissue was homogenized in liquid nitrogen and suspended with 500 µl 3% sulfosalicylic acid (5µl/mg fresh weight). The obtained extraction was centrifuged for 5 min at room temperature with maximum speed. Then 100 µl supernatant of the plant extract to 500 µl reaction mixture (100 µl of 3% sulfosalicylic acid, 200 µl glacial acetic acid, and 200 µl acidic ninhydrin) was added. The tubes were incubated at 96 ^{o}C for 60 min and the reaction was terminated on ice. To extract the samples with toluene, 1 ml toluene to the reaction mixture was added, and the samples were vortexed for 20 s and left on the bench for 5 min to allow the separation of the organic and water phases. The upper organic phase was used for measurement of proline. The absorbance was determined at 520 nm using toluene as reference. The proline concentration was determined using a standard concentration curve and calculated on fresh weight basis (mg/g FW).

Total soluble sugar content determination

The soluble sugar content was determined by anthrone method as described previously (Yemm and Willis 1954). Total soluble sugar was measured by anthrone reagent. Leaf samples (100-400 mg) were homogenized in liquid nitrogen and suspended with 10 ml deionized water. Samples were placed in a water bath for 45 min at 70 ^{o}C, vortexed thoroughly every 15 min and the reaction was terminated on ice. Samples were centrifuged at 3,000 rpm for 10 min and then diluted 10 times. Five milliliter anthrone solution (100 mg anthrone/50 ml sulfuric acid) was added into 2.5 ml diluted supernatant solution. After vortexing thoroughly, samples were place in a water bath

for exactly 10 min at 100 ºC and the reaction was terminated on ice. The absorbance was determined at 630 nm using deionized water as a reference. The total soluble sugar concentration was determined using a standard concentration curve and calculated on fresh weight basis (mg/mg FW).

Time-course analysis of stress-responsive genes

To evaluate the effects of AtGRXS17 on the expression of *dehyrin Ci7*, *dehydrin-lik*e, *proteinase inhibitor*, *glycine rich, SlCBF1, SlCAT1, SlSOD, SlFESOD, SlTPX1,* and *SlTPX2* genes, chilling treatments were applied to 4-week-old *AtGRXS17*-expressing and wild-type plants. For chilling treatment, the plants grown in soil pots were moved to a growth chamber set at 4 ºC. Leaves from wild-type and *AtGRXS17*-expressing tomato plants were harvested and measured after being treated for 0, 4, 8, 24, and 48 h, respectively.

Subcellular localization of AtGRXS17 in plant cells

To investigate the subcellular localization of AtGRXS17 in plant cells under chilling stress, an *Agrobacterium*-mediated transient expression assay was conducted in tobacco leaves (*Nicotiana tabacum*) as described previously (Sparkes et al. 2006). Full-length *AtGRXS17* was fused to the C-terminus of green fluorescent protein (GFP) using a procedure described previously(Cheng et al. 2006). The *GFP-AtGRXS17* construct was made by LR reaction (Invitrogen, Carlsbad, CA) between the binary vector pB7WGF2 (Karimi et al. 2002) and the entry vector carrying *AtGRXS17* (pENTER-4, Invitrogen, Carlsbad, CA). pB7WGF2/GFP-AtGRXS17 was introduced into *A. tumefaciens* LBA4404. A modified green fluorescent protein (smGFP) construct (Free GFP construct) was made by the Cre-loxP recombination system using a procedure described previously(Shigaki et al. 2005). pSK001 construct was generated by inserting a 1.9 kb *SacI*-

HindIII fragment from pBV579 (containing 35S::mCherry::NLS::Tnos) into the unique *Sac*I and *Hind*III sites of pCAMBIA1300. These three constructs were transformed into *A. tumefaciens* LBA4404. *A.* t*umefaciens* cells were cultivated overnight, and 5mL of the culture was pelleted and resuspended with Infiltration medium (250 mg D-glucose, 5 ml MES stock solution, 5 ml Na_3PO_4•$12H_2O$ stock solution, 5 ml 1 M acetosyringone stock solution; make up to 50 ml with ddH_2O.) to 0.1 optical density. *A. tumefaciens* cells were infiltrated into tobacco leaves, and the infiltrated tobacco was kept under constant light for 1.5-2 days. For chilling treatment, at 1.5-2 days post infiltration (DPI), the infiltrated tobacco leaves were detached from tobacco plants, kept in Petri dishes with the moistened filter paper and incubated at 25 or 4 ^oC for overnight, respectively. Images were captured with a confocal laser scanning system (Leica, SP5 X, Leica Microsystems Inc., Buffalo Grove, IL) and fluorescence microscope (Zeiss Axio-Plan, Carl Zeiss Microscopy, Thornwood, NY). The fluorescence signals were detected at 510 nm (excitation at 488 nm) for GFP and at 610 nm (excitation at 587 nm) for mCherry.

RESULTS

AtGRXS17-expressing tomato plants have enhanced chilling tolerance

Thirty plants each of *AtGRXS17*-expressing T2 generation plants from the four independent homozygous lines (*AtGRXS17*-3, -5, -6, and -9) were subjected to chilling stress treatment. The growth and development of *AtGRXS17*-expressing tomato plants were visually indistinguishable from those of wild-type plants before chilling treatment (Figure 2.1a). Wild-type and *AtGRXS17*-expressing tomato plants both wilted after three weeks of chilling treatment (Figure 2.1b), *AtGRXS17*-expressing tomato plants appeared more vigorous in the 2-week recovery period under normal growth conditions (24/20 ^oC day/night cycle) compared with wild-type plants (Figure 2.1c). The relative expression level of *AtGRXS17* of the four tomato lines, *AtGRXS17*-3, -5, -6,

and -9, was analyzed using quantitative real-time PCR (Figure 2.1d). The lowest expression level in *AtGRXS17*-9 was set to 1.0. The enhanced chilling stress tolerance of the transgenic plants *AtGRXS17*-3, -5, and -6 (which show relatively high expression level of *AtGRXS17*) was measured on the basis of electrolyte leakage and chlorophyll fluorescence (Figure 2.1d). *AtGRXS17*-expressing plant leaves had lower electrolyte leakage, indicative of reduced disruption of cell membranes, when compared with wild type plants after the chilling stress treatment (Figure 2.1e). Chlorophyll fluorescence of *AtGRXS17*-expressing plants, as measured by the Fv/Fm ratio (the maximum quantum efficiency of Photosystem II), was higher than that of wild-type plants (Figure 2.1f). After challenge by chilling stress, the growth and yield of the *AtGRXS17*-expressing tomatoes at the reproductive stage were indistinguishable from the wild-type plants under normal growth conditions (Wu 2012).

Ectopic expression of AtGRXS17 affects the activities of ROS scavenging enzymes and the accumulation of H_2O_2 in tomato

H_2O_2 accumulation was assayed using 3, 3'-diaminobenzidine (DAB) staining of leaves from wild-type tomato plants and *AtGRXS17*-expressing tomato lines to examine how the expression of *AtGRXS17* in chilling stressed tomatoes influences H_2O_2 accumulation. In the absence of chilling stress, leaves from wild type and *AtGRXS17*-expressing tomato plants showed minimal DAB staining, indicating low H_2O_2 accumulation (Figure 2.2a, upper row). After 2-3 days of chilling treatment in the dark, substantial brown-staining material was detected in leaves of wild-type plants. In contrast, leaves of *AtGRXS17*-expressing plants showed less brown staining than those of the wild-type plants (Figure 2.2a, lower row), indicating less H_2O_2 accumulation in *AtGRXS17*-expressing tomato plants. Quantitative analysis of DAB stain density on the leaf surface showed

that H_2O_2 accumulation was substantially lower in *AtGRXS17*-expressing leaves compared with those of the wild-type plants after the chilling treatment (Figure 2.2b).

The possible roles of antioxidant enzymes in the transgenic lines were examined by measuring the activities of superoxide dismutase (SOD), catalase (CAT), and guaiacol peroxidase (POD). The activities of SOD, CAT and POD were higher in all *AtGRXS17*-expressing lines than those of wild-type tomato plants in the first 5 days of chilling stress treatment (Figure 2.2c, d, and e). The activity of SOD in *AtGRXS17*-expressing lines and wild-type plants displayed no differences before chilling treatment (Figure 2.2c, 0 d). After chilling treatment, all transgenic lines maintained higher SOD activity steadily in comparison to the wild-type plants that showed dramatically decreased SOD activity over 5 days of chilling treatment (Figure 2. 2c). All transgenic lines showed greater CAT activity than that of the wild-type plants and maintained higher CAT activity over the 5 days under both normal and chilling stress conditions (Figure 2. 2d). All transgenic lines also showed increased POD activity in comparison to the wild-type plants over 5 days under both normal and chilling stress conditions (Figure 2. 2e).

Antioxidant enzyme activities and transcript levels of their respective genes may not correlate tightly (Stitt and Gibon, 2014). The transcription levels of antioxidant enzymes genes *SlCAT1*, *SlSOD*, *SlFESOD*, *SlTPX1* and *SlTPX2* and enzyme activity levels were measured during chilling stress (Figure 2. 3). The *SlCAT1* transcript levels in all *AtGRXS17*-expressing lines were higher than those of wild-type tomato plants before chilling treatment (Figure 2. 3a). During chilling stress, the *SlCAT1* transcript levels in both wild-type and *AtGRXS17*-expressing lines were increased in first 8 h treatment and then returned to resting levels at 48 h (Figure 2. 3a). The overall expression patterns and levels of *SlSOD*, *SlFESOD*, *SlTPX1* and *SlTPX2* were very similar in both wild-type and *AtGRXS17*-expressing lines, showing slightly increased transcripts in first 8 h and

then decreasing within 48 h under chilling stress. Variation of gene transcript levels among *AtGRXS17*-expressing lines was observed (Figure 2. 3b-e). These results indicate that *AtGRXS17* expression in chilling stressed tomatoes affects activity and stability, but not the transcript level of the antioxidant enzymes.

AtGRXS17-expressing tomato plants have improved tolerance to oxidative stress.

AtGRXS17-expressing and wild-type tomato seedlings were grown in MS media with or without methyl viologen (MV), a pro-oxidant herbicide that stimulates formation of destructive ROS within chloroplasts, to determine if the selected transgenic lines have improved tolerance to oxidative stress (Foyer and Noctor 2009). *AtGRXS17*-expressing tomato seedlings displayed more vigorous growth visually and had longer primary root growth as compared to wild-type seedlings at 14 d of growth, indicating that AtGRXS17 ameliorates defective root growth and development due to MV treatment (Figure 2. 4).

Effect of ectopic expression of AtGRXS17 on the accumulation of proline and soluble sugars under chilling stress.

An increase in proline content was observed in both wild-type and *AtGRXS17*-expressing tomato plants upon exposure to chilling stress. Proline content was not different between wild type and *AtGRXS17*-expressing tomato plants during the first 12 days of chilling treatment. However, after 21 days of chilling treatment, the proline content of wild-type plants was higher than that of transgenic plants (Figure 2.5a), indicating that relatively higher proline accumulation did not contribute to the enhanced chilling tolerance of *AtGRXS17*-expressing tomato plants. On the other hand, soluble sugar content in tomato leaves from wild-type and *AtGRXS17*-expressing plants had no difference in total soluble sugar content before chilling treatment and sugar content in all

AtGRXS17-expressing lines as compared with wild-type tomato plants, exhibiting 2- to 3-fold elevated content after 5 days of chilling treatment (Figure 2. 5b).

AtGRXS17 accumulates in the nucleus during chilling stress.

The subcellular localization of AtGRXS17 protein in plant cells with or without chilling stress treatment was examined using a version of the *AtGRXS17* gene that was fused to the C-terminus of green fluorescent protein (GFP) gene and transiently expressed under the control of the cauliflower mosaic virus (CaMV) *35S* promoter (35S::GFP-AtGRXS17) in tobacco leaf epidermal cells. A vector harboring 35S::mCherry::NLS was used as a control for nuclear localization in transient co-expression assays (Figure 2. 6a, b middle). GFP gene was also expressed under the control of the *35S* promoter as a control for free GFP localization (Figure 2. 6a, b, lower panel). Under normal growth conditions (25 ^{o}C), the GFP-AtGRXS17 fusion protein was primarily detected in the cytoplasm and the nuclei (Figure 2. 6a left, upper panel). Eighty-five percent (85 out of 101) of the cell cells had fluorescence signals detected in the cytoplasm with weaker signals in the nucleus, while 15.8% (16 out of 101) cells having stronger signals detected in the nucleus (Figure 2. 6c). In response to chilling stress (overnight at 4 ^{o}C), the GFP-AtGRXS17 fusion protein emitted strong fluorescence signals in the nuclei (Figure 2. 6b left, upper panel). Forty-two percent (86 out of 207) cells having fluorescence signals detected in the cytoplasm with weaker signals in the nuclei, while 58.4% (121 out of 207) cells had stronger signals detected in the nuclei (Figure 2. 6c), indicating that AtGRXS17 accumulates in the nucleus during chilling stress. In contrast, free GFP was localized in nuclei independent of chilling treatment (Figure 2. 6a and b lower panel and Figure 2. 6c).

Ectopic expression of AtGRXS17 in tomato does not alter expression patterns of SlCBF1 and CBF target genes under chilling stress.

The effect of *AtGRXS17* ectopic expression on the response of *SlCBF1* and four *CBF* target genes (*dehydrin Ci7*, *dehydrin-like*, *proteinase inhibitor*, and *glycine-rich*; Table 2.S1) to chilling stress in tomato plants was measured. *SlCBF1* expression levels in both wild-type and *AtGRXS17*-expressing lines peaked at 4 h after chilling stress and returned rapidly to resting levels (Figure 2. 7a). No differences in overall expression patterns and levels were observed between wild-type and *AtGRXS17*-expressing lines, although variation of gene expression levels among *AtGRXS17*-expressing lines was observed as either higher or lower *SlCBF1* expression level compared to wild-type plants (Figure 2. 7a). *Dehydrin Ci7* and *Dehydrin-like* expression levels in both wild-type and *AtGRXS17*-expressing lines were elevated and peaked at 24 h after chilling stress and then decreased at 48 h (Figure 2. 7b and c). *Glycine-rich* expression remained at a constant level and then increased at 48 h in both wild-type and *AtGRXS17*-expressing lines, while *Proteinase inhibitor* expression levels in both wild-type and *AtGRXS17*-expressing lines were increased slowly for the first 8 h and then decreased at 24 h (Figure 2. 7d and e, respectively). Real time qRT-PCR analysis showed that the overall expression patterns and levels of *SlCBF1* and four *CBF* target genes are similar between wild-type and *AtGRXS17*-expressing lines after chilling stress, indicating that the chilling tolerance in *AtGRXS17*-expressing tomato plants does not depend on the CBF pathway.

DISCUSSION

AtGRXS17 is a global and conserved heat stress responsive factor, which confers thermotolerance in both yeast and plant species (Wu et al. 2012). In tomato, while endogenous *SlGRXS17* was also expressed in all tissues, *SlGRXS17* expression was not induced by applications of chilling stress

for different time periods (Wu 2012). However, ectopically expressed *AtGRXS17* in tomato modulates a number of cold responsive components to suppress chilling induced oxidative damage and may enhance chilling tolerance in a manner independent of CBF.

ROS are known to accumulate during various abiotic stresses, causing damage to macromolecules and ultimately to cellular structure (Fridovich 1986; Imlay and Linn 1988; Jaspers and Kangasjärvi 2010; KJA 1987; Møller et al. 2007). On the other hand, ROS are key regulators of growth, development and defense pathways (Mittler et al. 2004). Thus, the ROS and redox state must be tightly regulated by ROS-scavenging and ROS-producing systems. GRXs are oxidoreductase enzymes that are capable of mediating reversible reduction of their substrate proteins in the presence of glutathione (GSH) (Garg et al. 2010), therefore maintaining and regulating the cellular redox state and redox-dependent signaling pathways (Lillig et al. 2008). Many studies demonstrated that glutaredoxins play roles in regulating redox homeostasis (Rouhier et al. 2002; Rouhier et al. 2003; Sha et al. 1997; Tsukamoto et al. 2005). Our results suggested that *AtGRXS17*-expressing tomato plants showed improved chilling tolerance as compared to the wild-type tomato plants, partially due to preventing photo-oxidation of chlorophyll and reducing the oxidative damage under chilling stress. Furthermore, our MV treatment data provide direct evidence that AtGRXS17 relieves the defective growth of primary roots correlated with increased accumulation of ROS. *AtGRXS17*-expressing tomato plants displayed higher antioxidant enzyme activities, less H_2O_2 accumulation in the *in vitro* test of tomato leaves under chilling stress, more vigorous growth and significantly longer root length as compared to the wild-type plants under oxidative stress, indicating that AtGRXS17 is an important component of the cellular ROS-scavenging/antioxidant system.

To adapt to various environmental stresses, plants have evolved specific enzymatic antioxidants such as CAT, SOD and POD to protect them from oxidative stress. Interestingly, *AtGRXS17*-expressing tomato plants maintained significantly higher activities of CAT, SOD and POD steadily as compared with wild-type tomato plants over 5 days under chilling stress, while expression levels and patterns of those antioxidant genes in both wild-type and *AtGRXS17*-expressing lines were nearly identical. The results revealed that AtGRXS17-mediated chilling tolerance was associated with increased activities and stabilities, rather than transcript levels, of the antioxidant enzymes. It is possible that AtGRXS17 is able to protect CAT, SOD and POD as target proteins by thiol-disulfide exchange. Previous studies indicated that there are several proteins identified as GRX targets, especially in response to oxidative stress conditions. The bovine Cu, Zn SOD, as nonplant GRX targets, have been found to be glutathiolated and are thus potential targets of GRX for deglutathiolation (Klatt and Lamas 2000). It was also reported that CAT is one of GRX-interacting proteins targeted by Poplar GRX C1 (Rouhier et al. 2005). Recently, numerous proteins have been identified as GRX substrate proteins by using Reversibly Oxidized Cysteine Detector (ROCD) (Lee et al. 2010). CAT isozyme 2 and SOD belong to these targeted proteins (Lee et al. 2010). Another implication is that AtGRXS17 may protect CAT, SOD and POD indirectly by increasing the production of total soluble sugars under chilling stress. It was reported that soluble sugars interact with proteins and membranes through hydrogen bonding, thereby preventing protein denaturation (Koch 1996). In fact, we found that *AtGRXS17*-expressing tomato plants had a significantly higher amount of soluble sugars as compared with wild-type plants, which may contribute to protection of CAT, SOD and POD under chilling stress.

Proline is a known protectant in abiotic stress and oxidative damage (Hoque et al. 2008; Matysik et al. 2002; Smirnoff and Cumbes 1989). Interestingly, our results suggest that the proline

content of transgenic lines was lower than that of wild-type plants after 21-days chilling treatment. Despite this, both follow a similar trend as proline content was increased in both wild-type and *AtGRXS17*-expressing tomato plants over the treatment period. It is possible that proline accumulation is induced by ROS signaling. In *Arabidopsis*, the expression of *AtP5CS2* was up-regulated by ROS that correspondingly resulted in accumulation of proline (Fabro et al. 2004). It is also reported that proline accumulation is induced by H_2O_2 in rice seedlings (Uchida et al. 2002). Therefore, the lower proline content may be due to less ROS accumulation in the transgenic plants. Unlike proline, soluble sugar content was higher in the *AtGRXS17*-expressing plants during chilling stress. Soluble sugars act as osmoprotectants to prevent cellular membrane damage caused by dehydration (Anchordoguy et al. 1987; Shalaev and Steponkus 2001). It is also reported that soluble sugars contribute to ROS scavenging by supporting NADPH-producing metabolic pathways (Couée et al. 2006). For instance, glucose has been shown to protect certain mammalian cell types from cytotoxicity of H_2O_2 (Averillbates and Przybytkowski 1994). Besides, glucose is involved in synthesis of antioxidant compounds as well as acts as a carbonic precursor for some amino acids, which are involved in synthesis of glutathione (Noctor and Foyer 1998; Smirnoff and Cumbes 1989). Proteomic analyses have identified a large number of TRX and GRX target candidates that are involved in sugar and starch synthesis (Meyer et al. 2009). Thus, AtGRXS17-mediated regulation of the sugar content may partially contribute to the chilling stress tolerance.

The subcellular localization changes of AtGRXS17 are consistent with the changes observed during heat stress (Wu et al. 2012). This translocation could be caused by the accumulation of ROS under stress conditions. Protein nuclear translocation in response to ROS has been intensively studied in mammalian cells (Kim et al. 2012). The nuclear pool of DJ-1, the protein that protects neurons from oxidative stress, dramatically increases after treatment with

H_2O_2, whereas, the nuclear translocation of DJ-1 can be blocked by applying antioxidants (Kim et al. 2012). In addition, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a protein which plays roles in apoptosis and oxidative stress, is translocated to the nucleus in response to H_2O_2 treatment (Dastoor and Dreyer 2001). Interestingly, AtGRXS17 does not have predicted nuclear targeting signals; therefore, AtGRXS17 nuclear translocation may be facilitated by other proteins under chilling stress.

One possible importance of translocation of AtGRXS17 into the nucleus is that AtGRXS17 may interact with some transcription factors. Previous studies have demonstrated that glutaredoxins can interact with transcription factors in plants. For example, the *Arabidopsis* ROXY1, a CC-type GRX, has been reported to control petal development by interacting with TGA transcription factors (Li et al. 2009). Similarly, the maize ROXY1 ortholog MSCA1 also interacts with FEA4, a maize TGA transcription factor, and the interaction may contribute to the maize shoot apical meristem regulation (Pautler et al. 2015; Yang et al. 2015). Furthermore, the yeast ortholog protein of AtGRXS17, GRX3, has been reported to interact with Aft1, a transcription factor that regulates iron homeostasis (Pujol-Carrion et al. 2006). Our previous studies also suggest that AtGRXS17 may interact with heat shock transcription factors (HSFs), which play an important role in thermotolerance under heat stress conditions (Wu et al. 2012). Recently, AtGRXS17 has been shown to interact with a nuclear transcription factor, NF-YC11/NC2α to maintain its redox state and this interaction may contribute to the shoot apical meristem maintenance (Knuesting et al. 2015b). All of these studies indicate that the glutaredoxins could play important roles in the nucleus through interaction with transcription factors. Another possible importance of translocation of AtGRXS17 into the nucleus is that the protein may function to protect DNA from ROS toxicity under abiotic stress conditions. ROS are a major source of DNA

damage (Imlay and Linn 1988), which leads to malfunctions or complete inactivation of encoded proteins (Sharma et al. 2012). Therefore, AtGRXS17 may translocate to nuclei to interact with transcription factors that activate expression of stress-related genes or further protect DNA from ROS damage under chilling stress conditions.

The CBF/DREB1 pathway is a well-characterized cold response pathway (Knight and Knight 2012), and various approaches have been proposed to improve cold (freezing and/or chilling) tolerance by manipulating *CBF* expression across different species (Morran et al. 2011; Xu et al. 2011; Yang et al. 2011). Although the induction of *CBFs* is one of the predominant responses to cold stress, over-accumulation of CBFs disrupts the regular biological processes of plants (Chinnusamy et al. 2007). Indeed, constitutive expression of either *AtCBF3* or *SlCBF1* in both *Arabidopsis* and tomato plants results in stunted growth and a significant yield penalty (Kasuga et al. 1999; Zhang et al. 2004). Even though the stress inducible *RD29A* promoter to minimize the negative effects on plant growth was used in this study, the *AtCBF3*-expressing tomato plants still displayed stunted growth and reducing yield under normal growth conditions [24/20 ^{o}C (day/night)] (unpublished data). The lack of stunted growth in plants expressing *AtGRXS17* suggests that manipulation of AtGRXS17 may be a useful alternative to CBFs to improve chilling tolerance across different species.

Unlike *Arabidopsis*, which includes cold-induced expression of three homologous *CBFs* (*CBF1*, *2* and *3*) and a large and diverse *CBF* regulon, the *CBF1* ortholog (*SlCBF1*) in tomato is the only cold-inducible *CBF*. In addition, transcriptome analysis using a cDNA microarray covering approximately 25% of the tomato genome found only four candidate genes for a CBF regulon induced by chilling stress and over-expression of *SlCBF1* or *AtCBF3* in tomato plants (Zhang et al. 2004). Our results demonstrate that ectopic expression of *AtGRXS17* in tomato does

not alter expression patterns and levels of *SlCBF1* and those four *CBF* target genes compared to wild-type plants under chilling stress, indicating AtGRXS17-mediated chilling tolerance may not be associated with part of the CBF-dependent transcriptional pathway. Several studies have also reported that chilling tolerance in plants is attributed to cold responsive pathways other than the CBF cold responsive pathway. For instance, HOS9, an *Arabidopsis* homeodomain transcription factor, is involved in maintaining freezing tolerance through a constitutive pathway instead of CBF cold responsive pathway (Zhu et al. 2004). The mutations in ESKIMO1 confer enhanced freezing tolerance by regulating salt and osmotic stress or ABA responsive genes rather than those of the CBF regulon.

Taken together, our results suggest that ectopic expression of *AtGRXS17* enhances the chilling tolerance of tomato plants. Minimized photo-oxidation of chlorophyll, reduced oxidative damage of cell membranes, increased activities of antioxidant enzymes such as CAT, SOD and POD, accumulation of osmoprotectant soluble sugars, and reduced accumulation of H_2O_2 are all associated with the enhanced chilling tolerance. Due to the conserved function of GRXs in plant species, manipulation of GRXs across different species may be a useful approach to improve tolerance to chilling stress without adverse effects on plant growth and development.

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FIGURES

a

WT AtGRXS17-3 AtGRXS17-5 AtGRXS17-6 AtGRXS17-9

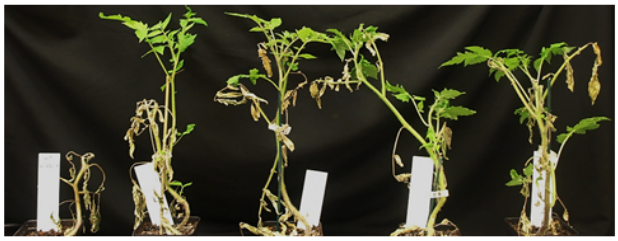
Before chilling treatment

b

WT AtGRXS17-3 AtGRXS17-5 AtGRXS17-6 AtGRXS17-9

After chilling treatment

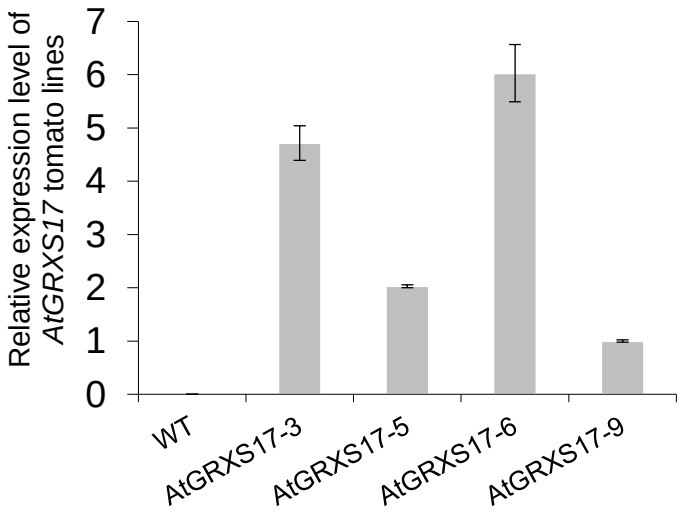
c



WT AtGRXS17-3 AtGRXS17-5 AtGRXS17-6 AtGRXS17-9

Recovery after chilling treatment

d



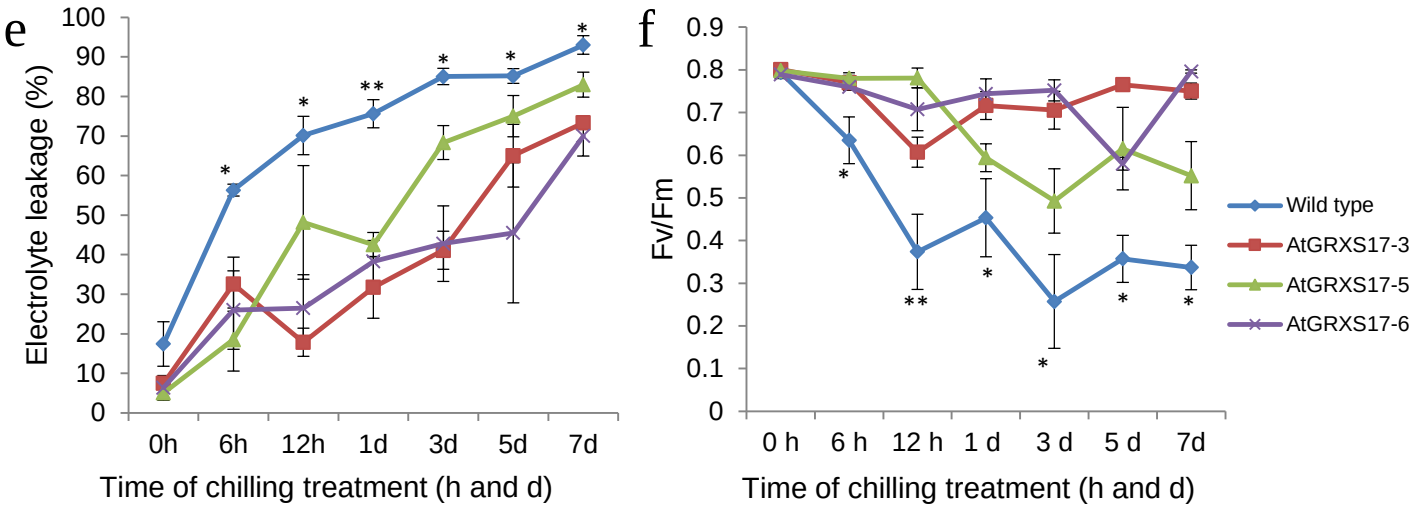
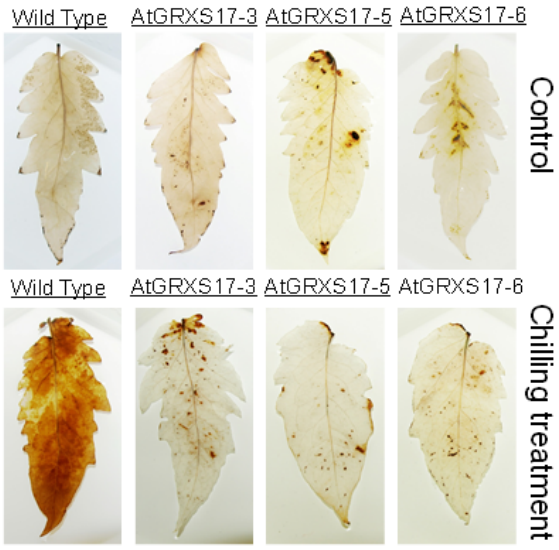


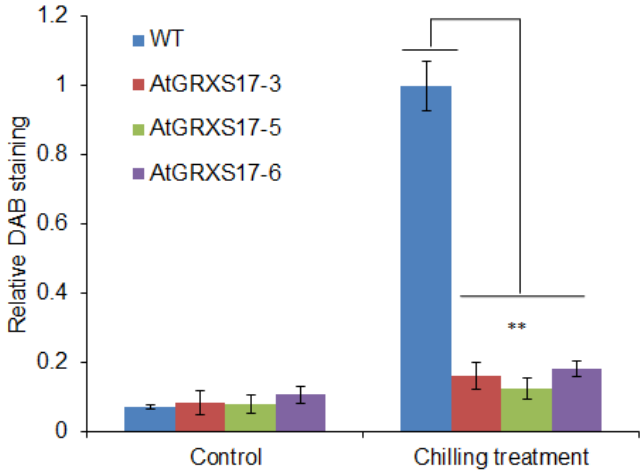
Figure 2. 1 qRT-PCR analysis of T2 transgenic tomato plants and effects of ectopic expressing *AtGRXS17* on tomato chilling tolerance.

Four-week-old *AtGRXS17*-expressing and wild-type tomato plants grown at 24 ˚C/20 ˚C (day/night) were transferred to 4 ˚C. (a) Phenotype of 4-week-old *AtGRXS17*-expressing and wild-type tomato plants before chilling treatment. (b) Phenotype of *AtGRXS17*-expressing and wild-type plants treated under 4 ˚C (day/night) for 3 weeks. (c) Two-week recovery after 3-week-chilling treatment. (d) Relative expression level of *AtGRXS17* in four independent transgenic tomato lines (*AtGRXS17*-3, *AtGRXS17*-5, *AtGRXS17*-6, and *AtGRXS17*-9). The leaves (the bottom second leaves) were sampled after 0 h, 6 h, 12 h, 1 d, 3 d, 5 d, and 7 d chilling treatment, respectively, and electrolyte leakage and chlorophyll fluorescence were analyzed. Electrolyte leakage (e) and Chlorophyll fluorescence (f) of *AtGRXS17*-expressing and wild-type plants during chilling treatment. Data represent means ± SD from three independent biological replicates and were analyzed using student t test. Asterisks (*, **) represent statistically significant differences between wild-type and *AtGRXS17*-expressing lines (*$P < 0.05$, **$P < 0.01$).

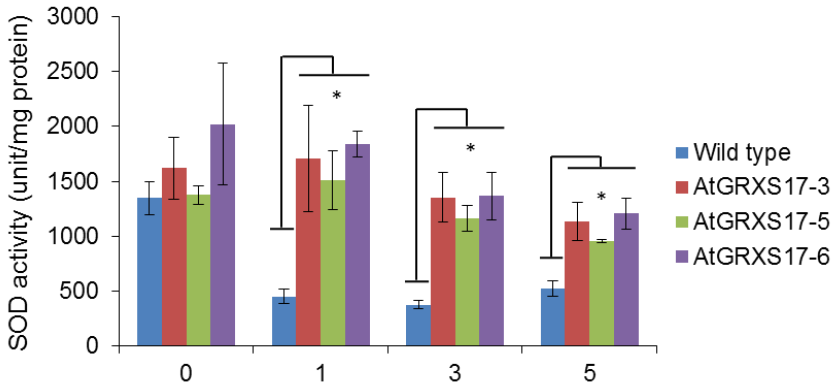
a


Wild Type
AtGRXS17-3
AtGRXS17-5
AtGRXS17-6
Control
Wild Type
AtGRXS17-3
AtGRXS17-5
AtGRXS17-6
Chilling treatment

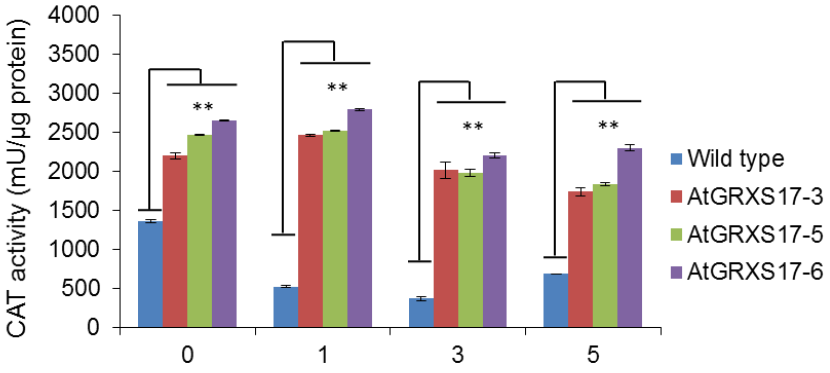
b


Relative DAB staining
1.2
1
0.8
0.6
0.4
0.2
0
WT
AtGRXS17-3
AtGRXS17-5
AtGRXS17-6
**
Control
Chilling treatment

c


SOD activity (unit/mg protein)
3000
2500
2000
1500
1000
500
0
*
*
*
0
1
3
5
Wild type
AtGRXS17-3
AtGRXS17-5
AtGRXS17-6

d


CAT activity (mU/µg protein)
4000
3500
3000
2500
2000
1500
1000
500
0
**
**
**
**
0
1
3
5
Wild type
AtGRXS17-3
AtGRXS17-5
AtGRXS17-6

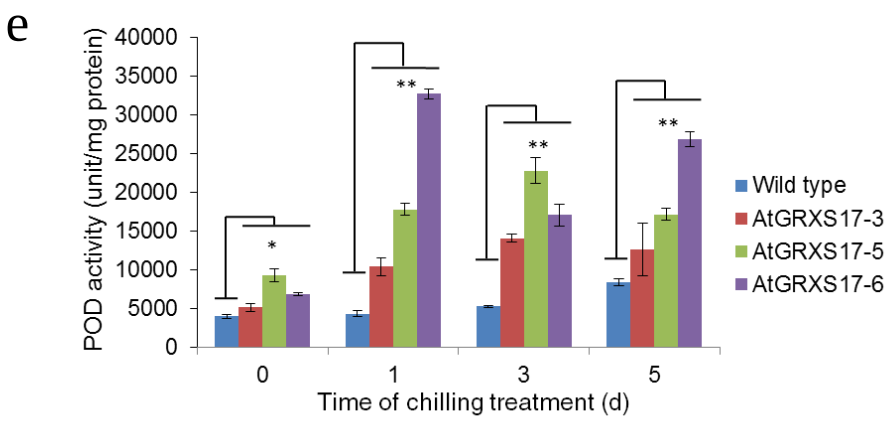


Figure 2. 2 Effects of AtGRXS17 on H_2O_2 accumulation and the activities of ROS scavenging enzymes.

(a) DAB staining in the terminal leaflet of the first fully expanded leaf of wild-type and *AtGRXS17*-expressing 4-week-old plants with or without chilling stress treatment. (b) Quantitative analysis of DAB staining. The relative intensity of DAB staining leaves was analyzed after being transformed to 256 grey scale images. Data are expressed as relative values based on wild-type plants treated at 4 ^{o}C as reference sample set as 1.0. Error bars represent the means ± SD (n = 3). The effects of *AtGRXS17* expression on Superoxide Dismutase (SOD) (c), catalase (CAT) (d), and Guaiacol peroxidase (POD) (e) activity under normal and chilling stress conditions. Data represent means ± SD from three independent biological replicates and were analyzed using student *t* test. Asterisks (*,**) represent statistically significant differences between wild-type and *AtGRXS17*-expressing lines (*$P < 0.05$, **$P < 0.01$).

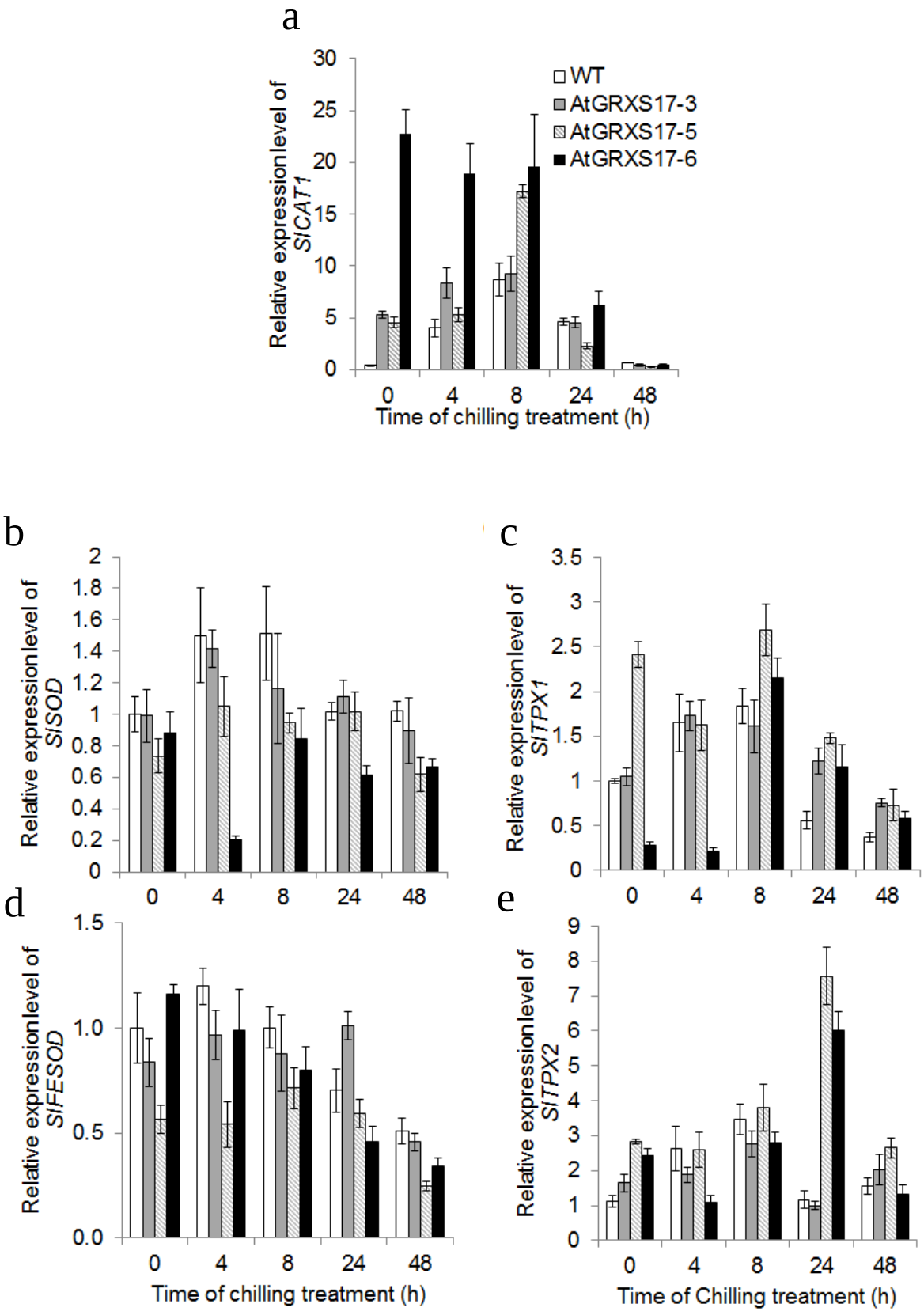

a
Relative expression level of SlCAT1
WT
AtGRXS17-3
AtGRXS17-5
AtGRXS17-6
Time of chilling treatment (h)
b
Relative expression level of SlSOD
c
Relative expression level of SlTPX1
d
Relative expression level of SlFESOD
Time of chilling treatment (h)
e
Relative expression level of SlTPX2
Time of Chilling treatment (h)

Figure 2. 3 Effects of *AtGRXS17* expression on temporal expression patterns and levels of genes encoding ROS scavenging enzymes under chilling stress.

Relative mRNA levels of *SlCAT1* (a), *SlSOD* (b), *SlTPX1* (c), *SlFESOD* (d), and *SlTPX2* (e) in 4-week-old wild-type and *AtGRXS17*-expressing tomato plants were analyzed after being treated at 4 ^{o}C for 0, 4, 8, 24, and 48 h, respectively. Data represent means ± SD from three independent biological replicates.

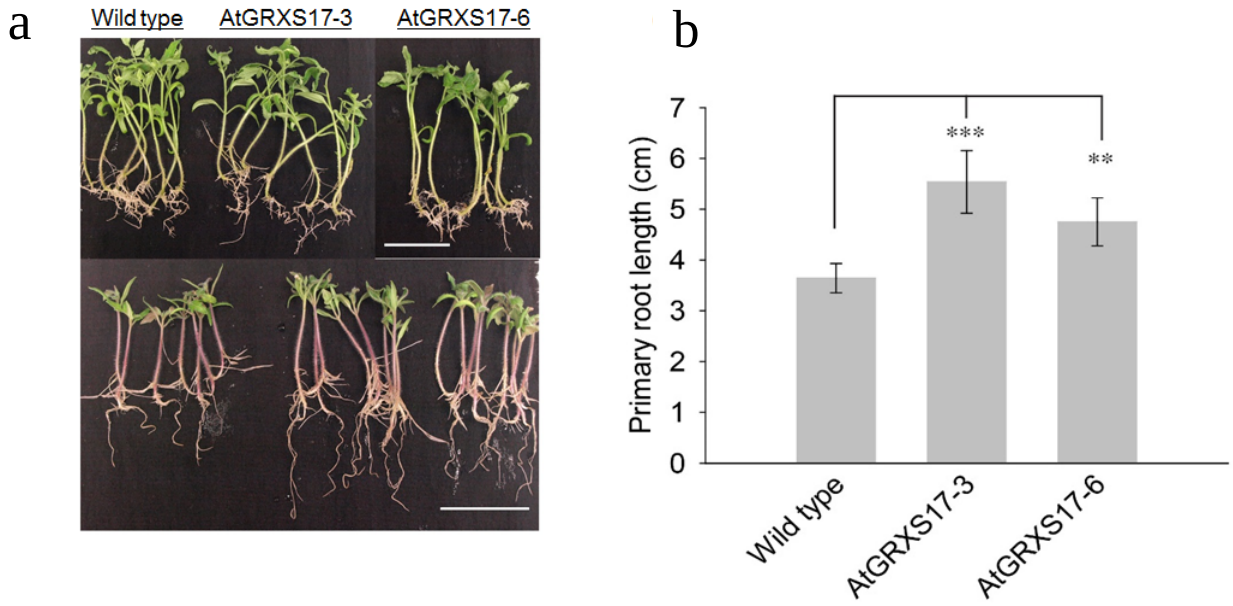
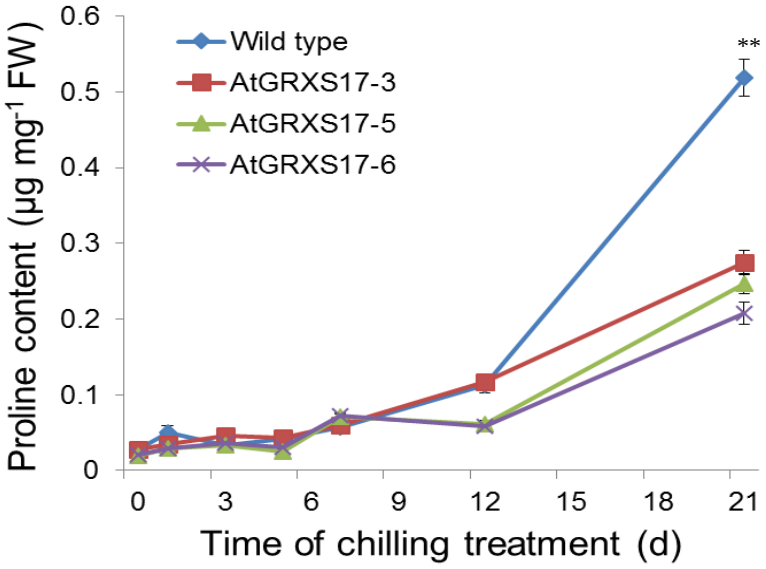


Figure 2. 4 Response to oxidative stress in *AtGRXS17*-expressing tomato plants.

(a) *AtGRXS17*-expressing and wild-type tomato seedlings treated with oxidative herbicide MV (methyl viologen). Seven-day-old *AtGRXS17*-expressing and wild-type tomato seedlings were transferred onto MS media with (lower panel) or without (upper panel) 20 µM MV and incubated for 14 d. Bars = 5 cm. (b) Root length of wild-type and *AtGRXS17*-expressing tomato seedlings that were treated by 20 µM MV. Data represent means ± SD from eight independent biological replicates and were analyzed using student *t* test. Asterisks (**, ***) represent statistically significant differences between wild-type and *AtGRXS17*-expressing lines (**$P < 0.01$, ***$P < 0.001$).

a


Wild type
AtGRXS17-3
AtGRXS17-5
AtGRXS17-6
Proline content (μg mg-1 FW)
Time of chilling treatment (d)
0
0.1
0.2
0.3
0.4
0.5
0.6
0
3
6
9
12
15
18
21
**

b

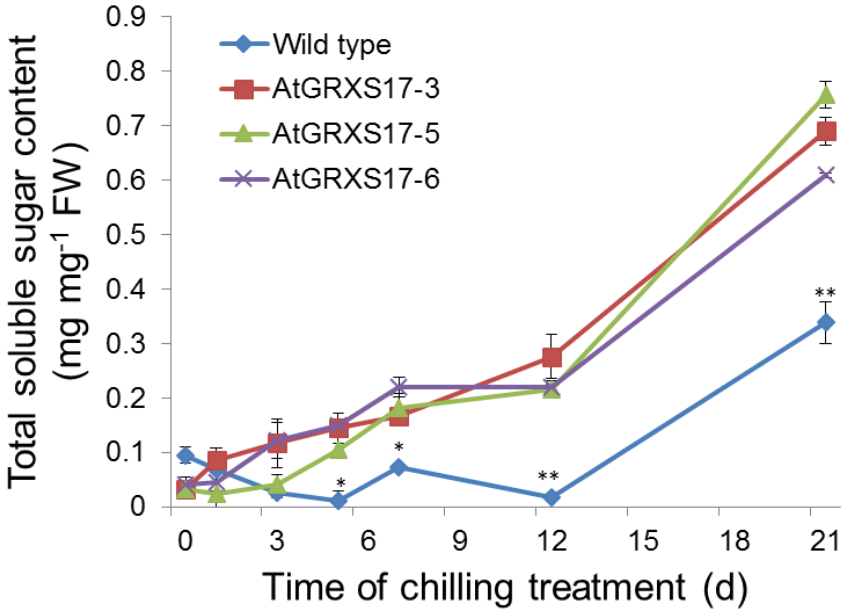
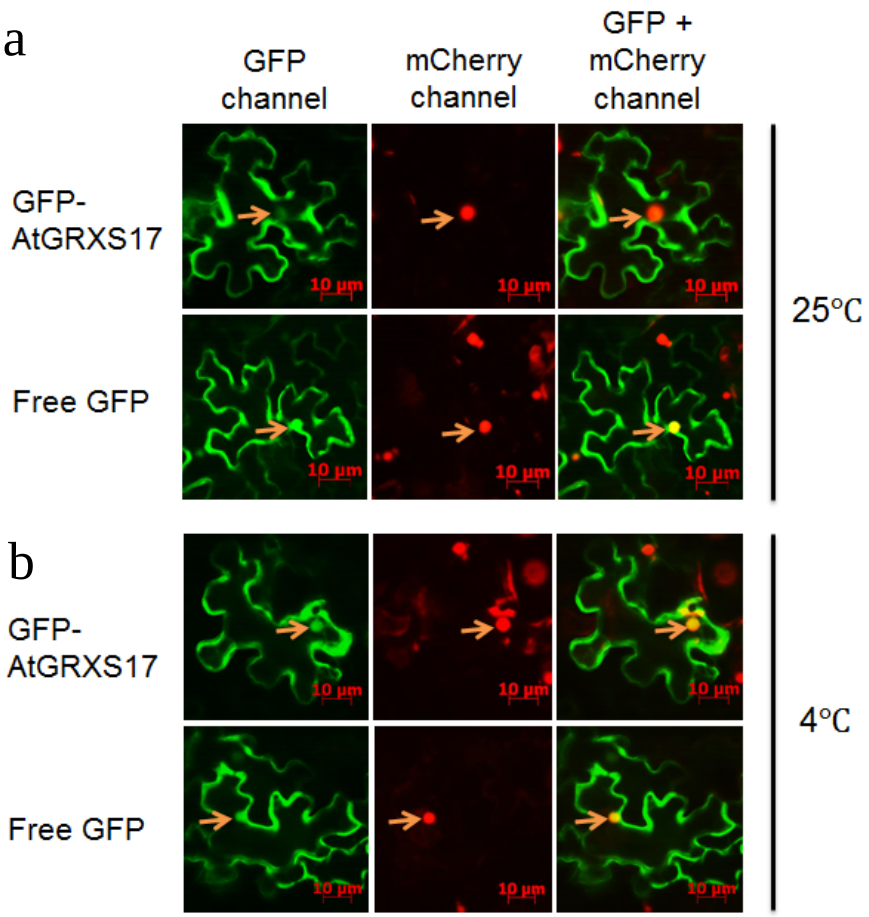

Wild type
AtGRXS17-3
AtGRXS17-5
AtGRXS17-6
Total soluble sugar content (mg mg-1 FW)
Time of chilling treatment (d)
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
0
3
6
9
12
15
18
21
*
*
**
**

Figure 2. 5 Effects of ectopic expressing *AtGRXS17* in tomato on proline and soluble sugar accumulation under chilling stress.

(a) The proline accumulation of wild-type and *AtGRXS17*-expressing tomato plants after being treated at 4 ^{o}C for 0, 1, 3, 5, 7, 12, and 21 days, respectively. (b) The total soluble sugar accumulation of wild-type and *AtGRXS17*-expressing tomato plants after being treated at 4 ^{o}C for 0, 1, 3, 5, 7, 12, and 21 days, respectively. Error bars represent the means ± SD (n = 3) and were analyzed using student *t* test. Asterisks (*,**) represent statistically significant differences between wild-type and *AtGRXS17*-expressing lines (*$P < 0.05$, **$P < 0.01$).


a
GFP channel
mCherry channel
GFP + mCherry channel
GFP-AtGRXS17
Free GFP
25°C
b
GFP-AtGRXS17
Free GFP
4°C
10 μm

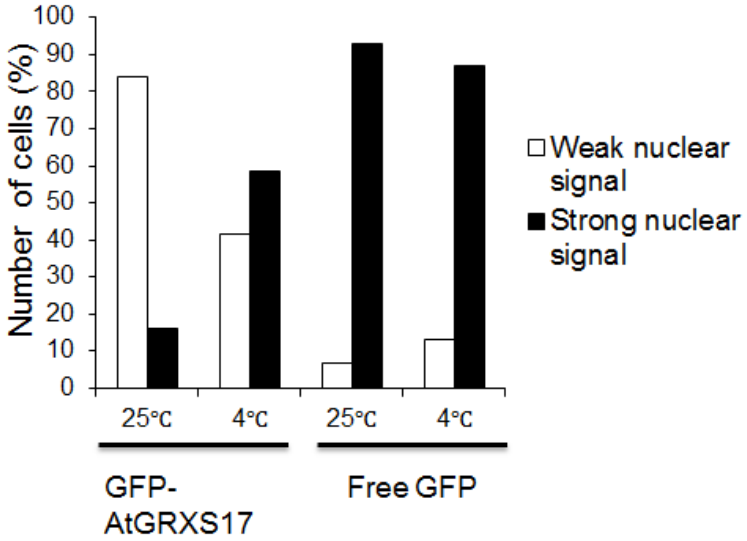
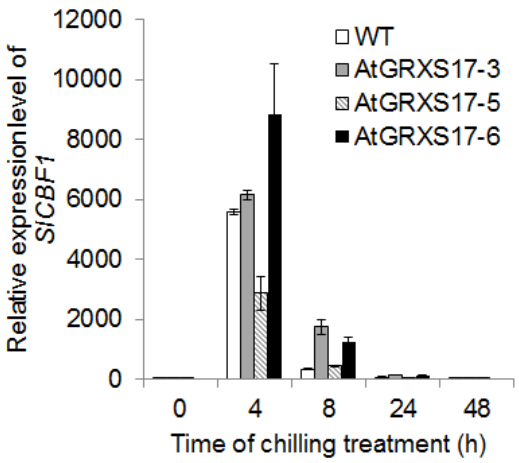

c
Number of cells (%)
100
90
80
70
60
50
40
30
20
10
0
25°C
4°C
25°C
4°C
GFP-AtGRXS17
Free GFP
Weak nuclear signal
Strong nuclear signal

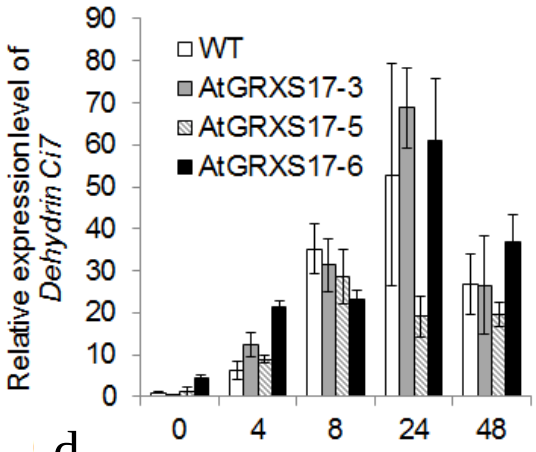
Figure 2. 6 Subcellular localization of AtGRXS17.

(a) Transient expression of *GFP-AtGRXS17* and free *GFP* in tobacco epidermal cells were imaged after being treated at 25 ºC overnight, respectively. Scale bars = 10μm. The arrows highlight the nuclei. (b) Transient expression of *GFP-AtGRXS17* and free *GFP* in tobacco epidermal cells after being treated at 4 ºC overnight. (c) The numbers of cells (%) with weak nuclear signals or strong nuclear signals. AtGRXS17::GFP: 16 cells with GFP signal and 85 cells without GFP signal in the nucleus at 25 ºC. Free GFP: 95 cells with GFP signal and 7 cells without GFP signal in the nucleus at 25 ºC. AtGRXS17::GFP: 121 cells with GFP signal and 86 cells without GFP signal in the nucleus at 4 ºC. Free GFP: 27 cells with GFP signal and 4 cells without GFP signal in the nucleus at 4 ºC.

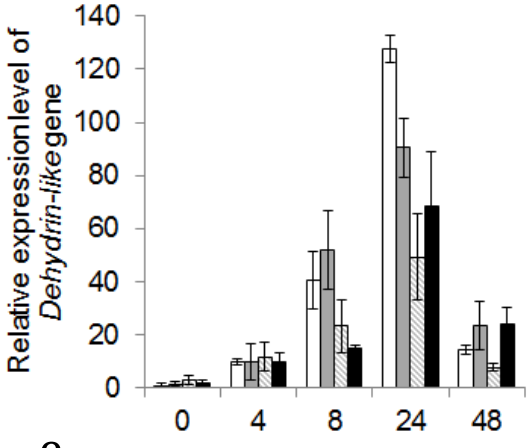
a


Relative expression level of SlCBF1
12000
10000
8000
6000
4000
2000
0
WT
AtGRXS17-3
AtGRXS17-5
AtGRXS17-6
0
4
8
24
48
Time of chilling treatment (h)

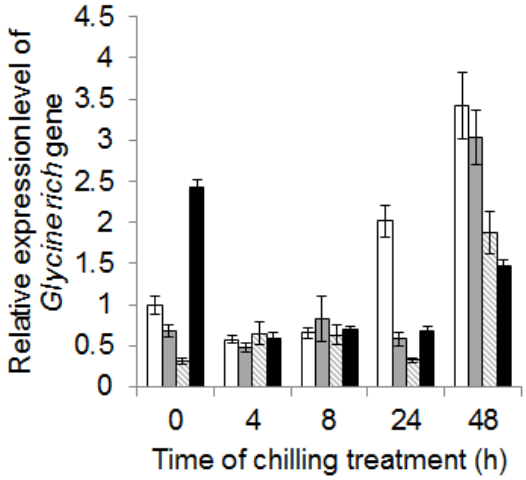
b


Relative expression level of Dehydrin Ci7
90
80
70
60
50
40
30
20
10
0
WT
AtGRXS17-3
AtGRXS17-5
AtGRXS17-6
0
4
8
24
48

c


Relative expression level of Dehydrin-like gene
140
120
100
80
60
40
20
0
0
4
8
24
48

d


Relative expression level of Glycine rich gene
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
0
4
8
24
48
Time of chilling treatment (h)

e

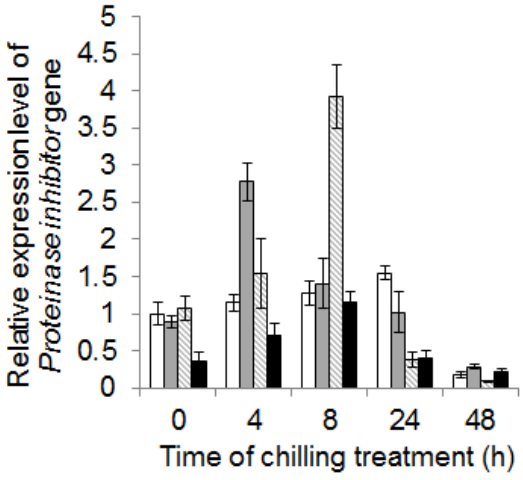

Relative expression level of Proteinase inhibitor gene
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
0
4
8
24
48
Time of chilling treatment (h)

Figure 2. 7 Expression patterns and levels of *SlCBF1* and four *CBF* target genes affected by *AtGRXS17* expression under chilling stress.

Relative mRNA levels of *SlCBF1* (a), *dehyrin Ci7* gene (b), *dehydrin-like* gene (c), *glycine rich* gene (d), and *proteinase inhibitor* gene (e) in four-week-old wild-type and *AtGRXS17*-expressing tomato plants were analyzed after being treated at 4 ^{o}C for 0, 4, 8, 24, and 48 h, respectively. Data represent means ± SD from three independent biological replicates.

Table 2. 1 Primers used for qRT-PCR

Gene name	Accession #	Primer sequence
SlCBF1	AY034473.1	Forward: GCTGGCAGGAAGAAGTTTCG Reverse: GAGTTGGAGGAAGCAGGGATAG
SlPP2Acs **(housekeeping**	AY325818	Forward: CGATGTGTGATCTCCTATGGTC Reverse: AAGCTGATGGGCTCTAGAAATC
AtGRXS17	NM_116733	Forward: AGGTGAGTTAATTGGAGGATGT Reverse: TCGGATAGAGTTGCTTTGAGAT
Dehydrin Ci7	AK224734	Forward: GAACCCAAGGAGGAGGAAA Reverse: TCTTCTTCTTGATCTTCTGTCCAT
Dehydrin-like	AI775935	Forward: CATATCAATAAGCGGAGGAGAG Reverse: GAAAGCAGTTAAACACAAGATAGA
Proteinase inhibitor	BT012682	Forward: ATCGTTTCAAGGGACCAT Reverse: AATCCACGGCAATTACCA
Glycine rich	NM_001247128	Forward: CTACTTCTGAAGAGGATTCCAAGA Reverse: CACCACCACCACCACTAC
SlCAT1	NM_001247898	Forward: ATTGCTGCTGGAAACTATCCTGAG Reverse: GGTCCAATACGGTGTCTCTGAGTA
SlSOD	NM_001247840	Forward: CGGTGTGGTTGGTTTGAC Reverse: AAAGTGTTGGCAAGTGTGTA
SlFESOD	NM_001246860	Forward: GCATACAAACCTGAAGACAAA Reverse: TGACACCAACTTCTCCATAA
SlTPX1	L13654	Forward: GGTCTGTTCCAATCCGATG Reverse: CACAATGCTTCCTGATTTCAC
SlTPX2	NM_001247715	Forward: CCATCAATGACAACACCACAA Reverse: CGGTTCGGCATAGAATTGTT

Chapter 3 - Silenced Expression of a Rice Glutaredoxin Gene, *OsGRXS17*, Enhances Drought Tolerance and Increases ABA Sensitivity in Rice

ABSTRACT

Drought is the biggest constraint of growth and development in rice, often resulting in reduced yields. Here we report that silenced expression of *OsGRXS17*, a rice monothiol glutaredoxin gene, conferred hypersensitivity to exogenous abscisic acid (ABA) and improved tolerance to drought stress in rice. Expression of *OsGRXS17* was high in the roots and leaves and up-regulated by drought and PEG treatment, as revealed by quantitative real-time RT-PCR analyses. Under drought stress, *OsGRXS17*-silenced rice plants displayed reduced water loss rate and stomatal conductance, higher relative water content and improved survival compared with wild-type rice plants. *OsGRXS17*-silenced rice plants exhibited enhanced sensitivity to ABA induced stomatal closure, which is associated with enhanced ABA-induced H_2O_2 production in the guard cells of these transgenic plants. In addition, *OsGRXS17*-silenced rice plants also accumulated more H_2O_2 in the root tips and exhibited hypersensitivity to ABA in the seed germination and post-germination growth. This ABA-mediated inhibition in *OsGRXS17*-silenced rice plants was released by addition of reduced glutathione (GSH). Using global metabolite profiling analysis, less abundance of amino acids, amino acid derivatives and peptide and more abundance of phospholipids were observed in *OsGRXS17*-silenced rice plants under drought stress. Collectively, our data suggest that silenced expression of *OsGRXS17* improves drought tolerance in rice by promoted ABA-mediated stomata closure, which provide an important genetic engineering approach to improve crop drought tolerance.

Key words: Drought stress, rice, glutaredoxins, ABA sensitivity, ROS

INTRODUCTION

Rice (*Oryza sativa L.*), as a global staple food, has high economic and social values (Maclean and Dawe 2002). Rice has a high water requirement, and therefore drought is the biggest constraint of growth and development in rice, often resulting in reduced yields. The elucidation of underlying mechanisms of plant adaptation under drought will be greatly helpful in accelerating development of new varieties with enhanced drought tolerance.

One of inevitable consequences of drought stress is the accumulation of reactive oxygen species (ROS), which is the result of partial reduction of oxygen and consists of four forms: singlet oxygen ($^1O_2$), superoxide radical (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical ($HO^\cdot$) (Cruz de Carvalho 2008). In plants, this increase of ROS is tightly controlled by a versatile antioxidant system which can prevent cellular oxidation and maintain the redox homeostasis (Bowler et al. 1992; Noctor and Foyer 1998; Willekens et al. 1997). Although ROS are considered as extremely toxic compounds and result in oxidative damage to lipids, proteins and DNA, research has shown that they also act as alarm signals which can induce the defense pathway and acclimatory response, protecting plants from changing environments (Dat et al. 2000; Vranová et al. 2002). The dual effect of ROS under abiotic stresses depends on overall cellular amount. When ROS are kept at a low level, under tight control of antioxidant system, they function as signaling molecules triggering defense responses in plants. If ROS accumulate to a phytotoxic level, uncontrolled oxidative damage to cellular components will be initiated, finally result in cell death (Dat et al. 2000).

Under drought stress, ABA-mediated stomata closure is one of the most important protection mechanisms of plants to reduce water loss (Schroeder et al. 2001; Zhu 2002). Early studies demonstrated that ABA triggers stomatal closure by activating Ca^{2+} channels at the plasma

membrane (Hamilton et al. 2000). In recent years, many studies show that ABA-induced H_2O_2, synthesized in the guard cell by a membrane bound NADPH oxidase, functions as a second messenger to mediate stomata closure by activating plasma membrane Ca^{2+} channels (Kwak et al. 2003b; Pei et al. 2000a; Zhang et al. 2001). *Arabidopsis thaliana* double mutants in rbohD and rbohF, two NADPH oxidases, show impaired ABA-induced ROS generation in guard cells, reduced Ca^{2+} channel activation and impaired ABA-induced stomatal closure (Kwak et al. 2003b). *Arabidopsis thaliana* mutants in ABI1 and ABI2, two negative regulators in ABA signal transduction pathway, show hypersensitivity to ABA and also disruptions in ABA-induced ROS production and Ca^{2+} channel activation in guard cell (Murata et al. 2001).

ROS also act as significant second messengers in regulating root growth and seed germination in the ABA signaling pathway. *Arabidopsis thaliana rbohD/F* double mutants have reduced ROS content and show insensitivity to ABA-mediated inhibition of seed germination and root growth (Kwak et al. 2003b). An *Arabidopsis thaliana* ABA overly sensitive mutant, *abo6*, accumulates more ROS in mitochondria and exhibits sensitivity to ABA-mediated inhibition of seed germination, primary root growth and stomatal opening (He et al. 2012). The accumulation of ROS was found in the root tips of another *Arabidopsis thaliana* ABA overly sensitive mutant, *abo8*, which shows hypersensitivity to ABA in root growth. This ABA induced ROS accumulation in root tips play a role in regulating auxin homeostasis and *PLETHORA1* (*PLT*) expression (Yang et al. 2014).

It is well established that glutaredoxins (GRXs) are ubiquitous oxidoreducatases of the thioredoxin (Trx) family to maintain the cellular redox homeostasis and regulate redox-dependent signal pathway by utilizing the reducing power of glutathione to catalyze reversible reduction of disulfide bonds of their target proteins (Fernandes and Holmgren 2004; Rouhier et al. 2008). GRXs

are already known to be involved in many processes in humans and animals. For instance, they play roles in transduction signaling pathway by regulating some transcription factors (Zheng, 1998; Nakamura, 1999), kinases (Ward, 1998; Humphries, 2002) and phosphatases (Rao, 2002; Barrett, 1999). They are also important in regulating cell redox homeostasis by targeting some antioxidant enzymes such as glutathione peroxidase (Bjornstedt, 1994) and superoxide dismutase (Klatt, 2000), in metabolic regulation through enzyme regulation (Cappiello, 1996; Lind, 1998). The first plant GRX was isolated from rice, which shows thioltransferase activity (Minakuchi, 1994). Recently, with deeper genomic analysis, increasing number of *GRX* genes are identified in higher plants such as *Arabidopsis thaliana* (31 GRX genes), *Populus trichocarpa* (36 GRX genes) and *Oryzae Sativa* (27 GRX genes) (Nicolas, 2006). Based on the active site sequence, the GRXs of higher plants can be subdivided into four groups (Couturier, 2009a). Grxs of class I and class II have CxxC/S and CGFS active sites, respectively, which are conserved in all photosynthetic organisms. Grxs of class III are specific to higher plants and have a peculiar CCxx active site, a diversified active site. Grxs of class IV harbor an N-terminal GRX domain with a CxDC/S active site in higher plants and CPxC active site in green algae fused to two domains of unknown function in the C-terminal. Very few studies are available concerning the functions of GRXs in plants compared with other organisms, especially in stress response and redox regulation. Recently, many studies unveiled the roles of plant GRXs in stress responses. The *Arabidopsis* AtGRXS13 participates in protecting Arabidopsis from photooxidative stress (Laporte, 2012). The *Pteris vittata* PvGRXS14 is involved in the tolerance to arsenic and heat stress (Sundaram and Rathinasabapathi 2010; Sundaram et al. 2009a). The tomato plants silenced for the expression of *SlGRXS16* are sensitive to oxidative and salt stresses (Guo, 2010). The *Arabidopsis* AtGRXS14 and AtGRXS15 are involved in oxidative stress responses (Cheng, 2006; Cheng, 2008). Recently

many studies demonstrated that the *Arabidopsis* AtGRXS17 plays essentials roles in heat stress response and photoperiod response (Wu et al., 2012; Cheng et al., 2011; Knuesting, 2015).

In this study, we used silenced expression of *OsGRXS17* to examine the functions of OsGRXS17 in drought stress. The *OsGRXS17*-silenced rice plants showed enhanced tolerance to drought stress and ABA hypersensitivity in terms of seed germination and postembryonic development compared to wild-type plants. These studies allow elucidation of OsGRXS17 roles in linking ABA and H_2O_2 signaling in stomatal closure, seed germination and postembryonic growth and provide an important genetic engineering approach to improve crop drought tolerance.

MATERIALS and METHODS

RNAi Plasmid Construction and Rice transformation

The *OsGRXS17* RNAi vector was generated by Gateway technology (Invitrogen, Carlsbad, CA). The 398-bp of the *OsGRXS17* gene comprising nts 1,291 to 1689 at the 3' ends of the coding sequence and 3' UTRs was amplified using an forward primer: 5'-CACCAGGGATCGTTGCGAAAGAAA-3' and reverse primer: 5'-AGCAAACTCGATGGTCGACGGATG-3' as the RNAi-targeted region of *RNAi-OsGRXS17* and subcloned into the pENTR/D-TOPO vector (Invitrogen, Carlsbad, CA). After verification by DNA sequencing, the Gateway™ cassette was introduced the pANDA vector (Miki and Shimamoto 2004) by the LR recombination reaction. Recombination between pENTR vectors and destination vectors were performed according to the manufacturer's instructions (Invitrogen, Carlsbad, CA).

The verified plasmid DNAs were introduced into *Agrobacterium tumefaciens* LBA 4404 using the freeze-thaw method (Holsters et al. 1978). Mature seed-derived callus from rice (*Oryza sativa L. Japonica*) cv. Nipponbare was used for *Agrobacterium*-mediated transformation (Park et al. 2001). After inoculating with *A. tumefaciens*, callus was transferred to regeneration medium for 4-10 weeks at 25 ˚C under a 16-h photoperiod. The regenerated shoots were transferred to rooting medium for four more weeks, then established in soil.

Plant materials and Growth conditions

T2-generation *OsGRXS17* RNAi or wild-type rice seeds were surface sterilized and geminated on MS medium with or without 1% (w/v) hygromycin (for the selection of transgenic plants) for 7 days and the 7-d-old seedlings were transferred into soil in small pots (1.9” x 1.9” x 2.37”) with

holes in the bottom containing Baccto premium potting soil (Michigan Peat Company, Houston, Texas, USA), in a growth chamber with a 16 h light (28 ˚C)/ 8 h dark (22 ˚C) photoperiod. The pots were kept in the flat-bottom trays containing water. For drought treatment, the four-week-old rice seedlings with three leaves appeared were exposed to drought stress treatments. Each pot was filled with the same amount of soil to provide similar soil humidity. The water was withheld from the trays for 11 days and then the stressed plants were re-watered to allow the wilted rice plants to recover. After 14 days of recovery, the survival rates (%) were calculated from the numbers of surviving plants per total tested plants. The plants were scored as viable if one or more new leaves appear. For seed germination and post embryonic growth test, 100 seeds per each line were surface sterilized and grown on Murashige and Skoog (MS) agar medium containing with or without 5 µM ABA, with or without 300 µM reduced glutathione (GSH) and with or without 5 µM IAA. To break the dormancy, the seeds were incubated at 4 ˚C for 2-3 days and then transferred to growth chamber with a 16 h light (28 ˚C)/ 8 h dark (22 ˚C) photoperiod. The seeds were scored as germinated when the radicles has emerged by 1mm. The seed germination was followed by 7 days and calculated from the numbers of germinated seeds per total tested seeds. After 7 days of ABA, GSH or IAA treatment on MS agar medium, the shoot length, root length and total biomass were measured for post embryonic growth assay.

Physiological measurement

The measurement of relative water content (RWC) was performed as described previously (Ascenzi and Gantt 1999). Briefly, on the fifth day of withholding water treatment, the first fully expanded leaves were detached and the fresh weight (FW) was measured immediately. Then the leaves were completely immerged in the double distilled water overnight for rehydration. The turgid weight (TW) was measured after blotting the rehydrated leaves. Finally, the dry weight

(DW) was measured after drying in an oven at 80 ˚C overnight. The relative water content (RWC) was calculated as follows: RWC= (FW-DW) / (TW-DW).

The measurement of water loss rate was performed by placing the detached first fully expanded leaves on a laboratory bench and recording their fresh weight for 0, 1, 2, 3, 4, and 5h. It is expressed as percentage of initial fresh weight.

The stomatal conductance (mmol $m^{-2}s^{-1}$) was measured in the first fully expanded leaf of four-week-old wild-type plants and *OsGRXS17*-silenced plants, using a portable porometer (SC-1 Leaf porometer, Decagon Devices, Pullman, WA, USA) in the auto mode for 30 seconds. The stomatal conductance was followed after being treated under drought stress for 0, 1, 3, 5, 7, 9 and 11 days.

To measure the stomata density, first fully expanded leaves of four-week-old wild-type and *OsGRXS17*-silenced rice seedlings grown in the growth chamber were sampled. Imprints were made by coating the adaxial surface with clear nail polish. After a few minutes, the clear tape was used to peel off the nail polish and this was mounted onto a microscope slides. The stomata density (number of stomata per unit area) was counted from three random areas on the leaf under a light microscope (Olympus CH30; Olympus, Tokyo, Japan).

H_2O_2 Assays

H_2O_2 was visually detected in leaves and root tips of rice plants by *in situ* staining with 3, 3'-Diaminobenzidine (DAB) as described previously with modification (Thordal-Christensen et al. 1997). The first fully expanded leaves detached from four-week-old wild-type and *OsGRXS17*-silenced rice plants were vacuum-infiltrated in 0.01% Tween 20 for 5 mins and then treated with 100 µM ABA for 3h. The sampled leaves were collected and incubated in DAB solution (1 mg/mL,

pH 3.8; Sigma-Aldrich) for 24 h at room temperature in darkness. The leaves were then de-colorized in boiling ethanol (96%) for 10 min before photographing. For the root tips staining, the seven-day-old rice seedlings was incubated in DAB solution (0.1 mg/mL, pH 3.8; Sigma-Aldrich) for 2h at room temperature in darkness. Then the root tips were rinsed by ddH_2O for three times and mounted on microscope slides for photographing. Quantitative analyses of DAB staining were performed using image J analysis (Wu 2012).

The H_2DCFDA staining assay was performed as previously to detect H_2O_2 production in the guard cell (Huang et al. 2009). The first fully expanded leaves from four-week-old wild-type and *OsGRXS17*-silenced rice plants were vacuum-infiltrated in 0.01% Tween 20 for 5 min and then incubated in 2 % (w/v) cellulose RS (Yakult Honsha Co., Tokyo) at 37˚C for 5 h to facilitate peeling off the epidermal layers. The peeled epidermal strips were incubated in the loading buffer (10 mM Tris-HCl, 50 mM KCl at pH 7.2) and then transferred to the staining buffer (loading buffer containing 50 mM H_2DCFDA) for 20 min. The stained epidermal strips were washed in the ddH_2O for 3 times to remove the excess H_2DCFDA and mounted on microscope slides to detect with a Zeiss LSM 780 laser-scanning confocal microscope (Carl Zeiss SAS, Jena, Germany) using following parameter settings: excitation 488 nm, emission 546 nm, 2% laser power percent, 16 Bit depth, image size 1024 x 1024 pixels, scanning speed 7. Fluorescence was analyzed using ImageJ software. Thirty to fifty guard cells are observed per treatment for three independent replicates.

Scanning electron microscopy

The first fully expanded leaves detached from four-week-old wild-type and *OsGRXS17*-silenced rice plants treated with 100 µM ABA or drought for 3h were used. The leaf segments (0.5 cm) were cut from the middle of the leaf, fixed by 2.5% glutaraldehyde in 0.1 M phosphate buffer at pH7.2. The samples were then rinsed 3 times in distilled water, dehydrated in ethanol series (30,

50, 70, 80, 95%), and rinsed 3 times in 100% ethanol. The samples were finally critical point dried using hexamethyldisilazane (HMDS) (Bray et al. 1993; Cao et al. 2007). The dehydrated samples were then sputter-coated with gold and used for stomatal observation by using a scanning electron microscope (Hitachi S-3500N, Tokyo, Japan). Thirty to fifty guard cells were observed per treatment for three independent replicates.

RNA extraction and qRT-PCR

Total RNA was isolated from young leaves, stems, roots (two-week-old rice plants), mature leaves, stems, roots (two-month-old rice plants) and panicles using the Qiagen Plant RNeasy kit (Qiagen, Valencia, California, USA) and on-column DNA digestion according to the manufacturer's instructions. The cDNA was transcripted from 1 µg quantity of total RNA using iScript™ Select cDNA Synthesis Kit (Bio-Rad, Munich, Germany). The qRT-PCR was carried out in a total volume of 10 µL containing 4.2 µL the reverse-transcribed product, 0.4 µL 10 mM of each primer, and 5 µL SYBR Green PCR Master Mix (Bio-Rad Laboratories). The PCR was performed with a Bio-Rad CFX-96 real time system (Bio-Rad, Hercules, California, USA). Primer efficiencies were measured and relative expression level was calculated using the comparative Ct method (Wu 2012). TFIIAγ5 was used as the internal control to normalize the samples. The primers used for qRT-PCR were listed in the supplementary data (Table 3.S1).

DNA gel-blot analysis

Rice genomic DNA was extracted from four-week-old rice seedlings using CTAB method as described previously (Murray and Thompson 1980). HindIII-digested genomic DNA (30 µg) was separated by electrophoresis on 0.8% (w/v) agarose gel, and blotted onto a nylon membrane (Zeta-probe GT membrane, Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's

instructions. The probe for the *hpt* gene was isolated from a pIPKB007 vector by PCR amplification. The membranes were prehybridized at 65 ˚C in 7% SDS and 0.25 M Na_2HPO_4 for 2 h and then hybridized overnight at 65 ˚C in the same solution containing the probe labeled with the enzyme horseradish peroxidase (ECL Direct™ Nucleic Acid Labeling and Detection System, Amersham Biosciences, USA) for 10-12 h at 42 ˚C. Membrane was washed twice for 40 min each with 20 mm Na_2HPO_4 and 5% SDS at 65 °C and then washed twice again for 30 min each with 20 mm Na_2HPO_4 and 1% SDS at 65 °C. Finally, the membrane was wrapped in Saran Wrap and exposed to X-ray film (Fuji Film Medical Systems, Stamford, Connecticut, USA) for 1–2 h.

Metabolite profiling analysis

First fully expanded leaves from four-week-old wild type and *OsGRXS17*-silenced rice plants were collected after 0 (0D), 5 (5D) and 8 (8D) days of drought stress treatment. Four biological replicates from each line were collected at each time point, lyophilized and kept at -80 ˚C until extraction. 100 mg of lyophilized samples was used for global metabolomics analysis (Metabolon Inc., Durham, North Carolina, USA).

RESULTS

OsGRXS17 Expression profile and subcellular localization

To examine the function of the *OsGRXS17* gene in *planta*, the expression of *OsGRXS17* under various treatments, including drought, polyethylene glycol (PEG), Indole-3-acetic acid (IAA), high salinity (NaCl) and abscisic acid (ABA), was analyzed by quantitative RT-PCR (Figure 3.1b-f). The increase in *OsGRXS17* transcript levels occurred 2 hours after drought stress treatment and maintained stable for 6 hours, which is in agreement with public microarray data (RicePLEX) (Figure 3.1b). The PEG and ABA treatment slightly induced the expression of *OsGRXS17* after 24

hours of treatment (Figure 3.1c, d). The expression of *OsGRXS17* was slightly induced after 2 hours of IAA and salt treatment, and then decreased to the initial level (Figure 3.1e, f). The expression of this gene in different rice plant tissues was also analyzed by quantitative RT-PCR under normal growth conditions (Figure 3.1a). The results suggested both young and mature leaves and roots had relatively higher expression of *OsGRXS17* than that in mature and young stems and young panicles.

In order to check the subcellular localization of OsGRXS17, the cDNA was fused in frame to the C-terminal of green fluorescent protein (GFP) gene and transiently expressed under the control of the cauliflower mosaic virus (CaMV) *35S* promoter (*P35S:GFP:OsGRXS17*) in tobacco leaf epidermal cells and rice protoplasts (Figure 3.2). A vector harboring *P35S:mCherry:NLS* (the mCherry red fluorescent protein linked to a nuclear localization signal) was expressed under the control of the 35S promoter as an indicator for the nuclear localization in transient co-expression assays (Figure 3.2a, b right). GFP gene was also expressed under the control of the 35S promoter as a control for free GFP localization (Figure 3.2a, b lower panel). Confocal imaging of GFP reveals that the GFP-OsGRXS17 fusion protein localized in both cytoplasm and nuclei in both tobacco leaf epidermal cells and rice protoplasts (Figure 3.2a, b, left and middle).

Generation of OsGRXS17-silenced rice plants

The induction of *OsGRXS17* expression by drought stress suggested that this gene might be involved in drought stress tolerance. To examine the function of *OsGRXS17*, an RNAi construct that express short hairpin RNAs derived from *OsGRXS17* gene under the control of the maize ubiquitin (*Ubi*) promoter was transformed into *Oryza sativa L. Japonica* cv. Nipponbare and more than 20 independent transgenic lines were generated. The transgenic rice lines were confirmed by reverse transcriptase (RT)-PCR analysis using primers of *hpt* gene (Figure 3.3a) and the relative

expression level of *OsGRXS17* was analyzed using quantitative real-time PCR (Figure 3.3b). The qRT-PRC results showed that three transgenic lines (*OsGRXS17* RNAi 7, 8 and -13) had highly reduced levels of transgene expression whereas one transgenic line (*OsGRXS17* RNAi-6) has slightly reduced level of transgene expression compared with the wild type. Those four transgenic rice lines were selected to further analysis of response to drought stress. The copy number of T-DNA insertions of those transgenic lines was confirmed by Southern blot analysis using a *hygromycin (hpt)* gene-specific probe (Figure 3.S1). The phenotype of the *OsGRXS17*-silenced rice plants was indistinguishable from the wild-type plants at both vegetative and reproductive stage under normal growth conditions (Figure 3.3c, d). *OsGRXS17*-silenced rice plants exhibited the unchanged the agronomic traits such as main panicle number per plant and grain number of main panicle compared with wild-type rice plants (Figure 3.S2).

Silenced expression of OsGRXS17 can improve drought tolerance

The induction of *OsGRXS17* expression by drought and PEG stresses suggested that this gene might be involved in drought stress tolerance. Thirty homozygous plants each of four *OsGRXS17*-silenced lines (*OsGRXS17* RNAi-6, 7, 8 and 13) and wild-type plants were used for drought tolerance testing. Before withholding water treatment, *OsGRXS17*-silenced rice plants were visually indistinguishable from those of wild-type plants (Figure 3.4a, 0d). After withholding water for 9 days, leaves of *OsGRXS17* RNAi-7, 8 and 13 rice plants still remained green and turgid, while wild-type and *OsGRXS17* RNAi-6 rice plants wilted (Figure 3.4a, 9d). Wild-type and *OsGRXS17*-silenced rice plants were rewatered to recover after 11 days of drought stress treatment when they were all wilted (Figure 3.4a, 11d). After 6 days of rewatering, 22.6% - 75% of *OsGRXS17*-silenced rice plants regrew, while only 7-8% of wild type plants were survived (Figure 3.4a, 6 days after rewatering; Figure 3.4b). These results indicated that three *OsGRXS17*-silenced

lines (*OsGRXS17* RNAi-7, 8 and 13) were more drought tolerant than wild-type and *OsGRXS17* RNAi-6).

To study the physiological mechanism of drought tolerance in *OsGRXS17*-silenced rice plants, we further examined the water status in leaves of wild-type and *OsGRXS17*-silenced rice plants. Under drought stress conditions, *OsGRXS17*-silenced rice plants lost less water (Figure 3.4c) and maintained higher water content than wild-type plants (Figure 3.4d). Interestingly, the stomatal conductance was lower in *OsGRXS17*-silenced plants than that of wild-type plants under both normal and drought stress growth conditions (Figure 3.4e). These results indicated that the enhanced drought tolerance of *OsGRXS17*-silenced plants was due to an increased ability of maintaining water.

Enhanced sensitivity to ABA induced stomatal closure in OsGRXS17-silenced rice plants

Since the *OsGRXS17*-silenced rice plants showed reduced water loss rate and stomatal conductance, the potential effect of silenced expression of *OsGRXS17* on stomatal closure was investigated. First we checked the stomatal density and the results showed that there was no significant difference in stomatal density between *OsGRXS17*-silenced rice plants and wild-type plants (Figure 3.5b). Then we further checked stomatal aperture of *OsGRXS17*-silenced rice plants and wild-type plants under normal growth condition using scanning electron microscope. The results indicated that 74.2% of stomata were completely open in wild type plants, whereas 54.3%, 42.5%, 38.5% and 35.7% of stomata were completely open in *OsGRXS17* RNAi-6, 7, 8 and 13 rice plants, respectively. On the other hand, only 2% of stomata were completely closed in wild type plants, whereas 13%, 22.5%, 25.6% and 21.4% of stomata were completely closed in *OsGRXS17* RNAi-6, 7, 8 and 13 rice plants, respectively. Also, 24.2% of stomata were partially

open in wild type plants, whereas 32.6%, 35%, 35.9%, 42.8% of stomata were partially closed in *OsGRXS17* RNAi-6, 7, 8 and 13 rice plants, respectively (Figure 3.5a, b). Those results indicated that silenced expression of *OsGRXS17* could induce the stomatal closure under normal growth conditions. In addition, we further evaluated whether the wild type and *OsGRXS17*-silenced rice plants have the different response in stomatal closure in the presence of ABA. The results suggested that 23.2% of stomata were completely open in wild type rice plants, whereas 20.7%, 18.4%, 14.3% and 12.2% of stomata were completely open in *OsGRXS17* RNAi-6, 7, 8 and 13 rice plants, respectively. Moreover, 46.4% of stomata were partially open in wild type rice plants, whereas 20.7%, 26.3%, 28.6%, 24.39% of stomata were partially open in *OsGRXS17* RNAi-6, 7, 8 and 13 rice plants, respectively. Also, 30.4% of stomata were completely closed in wild type rice plants, whereas 58.6%, 55.3%, 57.1% and 63.4% of stomata were completely closed in *OsGRXS17* RNAi-6, 7, 8 and 13 rice plants, respectively (Figure 3.5a, b). These results indicated that ABA treatment resulted in a higher rate of stomatal closure in *OsGRXS17*-silenced rice plants than that in wild-type plants.

Increased ABA induced stomatal closure in OsGRXS17-silenced rice plants due to H_2O_2 accumulation

Prior studies demonstrated that ABA-induced H_2O_2 synthesized in the guard cell is involved in mediating stomata closure by activating plasma membrane Ca^{2+} channels (Kwak et al. 2003a; Pei et al. 2000b; Zhang et al. 2001). Since OsGRXS17 plays a role in maintaining the cellular redox homeostasis, we hypothesized that more accumulation of H_2O_2 in the guard cell due to silenced expression of *OsGRXS17* may contribute to increased ABA induced stomatal closure in *OsGRXS17*-silenced rice plants. We assayed H_2O_2 accumulation by using 3, 3'-diaminobenzidine (DAB) staining of leaves from wild-type and *OsGRXS17*-silenced rice plants (Figure 3.6a). In

absence of ABA addition, the leaves of *OsGRXS17* RNAi-7,8 and 13 plants displayed more brown spotting staining than those of *OsGRXS17* RNAi-6 and wild-type pants, indicating more H_2O_2 accumulation in leaves of *OsGRXS17* RNAi-7,8 and 13 (Figure 3.6a, upper panel). In the presence of 100 µmol/L ABA, the DBA staining in leaves was increased in both wild-type and *OsGRXs17*-silenced lines plants, but *OsGRXS17* RNAi-7,8 and 13 had stronger staining in leaves compared to that of *OsGRXS17* RNAi-6 and wild-type plants (Figure 3.6a, lower panel). The intensity and quantity of brown spots was analyzed by image J after transforming the pictures to 256 grey scale and the statistical analysis showed that H_2O_2 content was significantly higher in *OsGRXS17* RNAi-7, 8 and 13 compared to that of *OsGRXS17* RNAi-6 and wild-type plants (Figure 3.6b).

By using a fluorescence dye, 2', 7'- dichlorodihydrofluorescein diacetate (H_2DCFDA), we found that *OsGRXS17* RNAi-7, 8 and 13 lines had stronger fluorescence signal in the guard cell compared to *OsGRXS17* RNAi-6 and wild-type plants in the absence of ABA addition, indicating more accumulation of H_2O_2 in the guard cell of *OsGRXS17* RNAi-7, 8 and 13 without ABA treatment (Figure 3.6c, upper panel). In the presence of 100 µmol/L ABA, the fluorescence signal in the guard cell was enhanced in both wild-type and *OsGRXS17*-silenced lines, but *OsGRXS17* RNAi-7, 8 and 13 had much stronger fluorescence signal in the guard cell compared to *OsGRXS17* RNAi-6 and wild-type plants, indicating dramatically increased accumulation of H_2O_2 in the guard cells than those of *OsGRXS17* RNAi-6 and wild-type plants with ABA treatment (Figure 3.6c, lower panel). Quantitative analysis of fluorescence intensity showed that H_2O_2 accumulation in the guard cell was higher in *OsGRXS17* RNAi-7, 8 and 13 compared with those of *OsGRXS17* RNAi-6 and wild-type plants with or without ABA treatment (Figure 3.6d).

Increases in ABA sensitivity of OsGRXS17-silenced rice plants in seed germination and postembryonic growth

We tested the seed germination and postembryonic growth of T2 homozygous *OsGRXS17*-silenced rice lines in response to ABA. When seeds were planted in MS medium, the seed germination of *OsGRXS17* silenced rice lines was slightly delayed than that of wild-type rice plants during the process of germination. By contrast, when seeds were planted in MS medium supplemented with 5 µM ABA, *OsGRXS17* RNAi-7, 8, and 13 showed hypersensitivity to ABA-induced inhibition of seed germination compared to *OsGRXS17* RNAi-6 and wild-type rice plants (Figure 3.7b; Figure 3.S3). The results indicated that the wild-type rice plants and *OsGRXS17* RNAi-6 showed 65% and 45% of germination rate while *OsGRXS17* RNAi line-7, 8, and 13 had only about 5%, 10% and 20% of germination rate at 4d after seed germination (Figure 3.7b). Such a hypersensitivity of *OsGRXS17* RNAi lines was also examined at the postembryonic stage (Figure 3.7a). At 9d after seed germination, the shoot and root length and the total biomass of wild-type plants and *OsGRXS17* RNAi lines were measured. The results showed that all transgenic lines had no difference in shoot and root growth compared with the wild type seedlings on MS without ABA supplementation while *OsGRXS17* RNAi-7, 8 and 13 had significantly shorter roots and shoots and less total biomass than wild-type plants on ABA supplemented MS media.

Increased ABA inhibition of seed germination and postembryonic growth in OsGRXS17 silenced rice plants due to H_2O_2 accumulation

One ROS scavenger, Glutathione (GSH), was used to determine whether ROS acts as secondary messenger of ABA-induced inhibition of seed germination and postembryonic growth. The results indicate that the seed germination, shoot and root length and total biomass were similar for wild-

type and *OsGRXS17* RNAi-6, 7, 8 and 13 rice seedlings on MS medium with or without 300 µM GSH (Figure 3.7). As mentioned above, 5 µM ABA could significantly inhibit the seed germination and postembryonic growth of *OsGRXS17* RNAi-6, 7, 8 and 13 rice plants, while the seed germination, shoot and root length and total biomass of those four transgenic lines were similar with those of wild-type plants on MS medium supplemented with 5 µM ABA and 300 µM GSH. Those results indicated GSH can recover the ABA induced inhibition of seed germination and postembryonic growth.

We used 3, 3'-diaminobenzidine (DAB) staining to examine ROS accumulation with or without ABA treatment in primary root tips (Figure 3.8). In absence of ABA, the primary root tips of *OsGRXS17* RNAi-7,8 and 13 plants had more brown spotting staining than those of *OsGRXS17* RNAi-6 and wild-type pants (Figure 3.8a, upper panel). In the presence of ABA, the DBA staining in primary root tips was increased in both wild-type and *OsGRXS17* RNAi lines plants, but *OsGRXS17* RNAi-7,8 and 13 had stronger staining in primary root tips compared to those of *OsGRXS17* RNAi-6 and wild-type plants (Figure 3.8a, lower panel). The intensity and quantity of brown spots was analyzed by image J after transforming the pictures to 256 grey scale and the statistical analysis showed that H_2O_2 content was significantly higher in *OsGRXS17* RNAi-7,8 and 13 compared to those of *OsGRXS17* RNAi-6 and wild-type plants (Figure 3.8b). Those results indicate that *OsGRXS17* RNAi-7, 8 and 13 rice plants accumulate more ROS in primary root tips than wild-type and *OsGRXS17* RNAi-6 rice plants and ABA treatment enhances ROS content in primary root tips.

Expression of ABA responsive genes in OsGRXS17-silenced rice plants

To elucidate if silenced expression of *OsGRXS17* increases ABA sensitivity at gene expression level, we analyzed ABA responsive genes by qRT-PCR. Application of ABA increases the levels

of *LEA3* and *RAB16A* mRNAs in aerial tissue of wild-type and *OsGRXS17*-silenced rice plants. However, the transcripts of *LEA3 and RAB16A* accumulated to higher level in *OsGRXS17*-silenced rice plants with 100 µM ABA treatment (Figure 3.9). These results indicate that silenced expression of *OsGRXS17* enhances ABA sensitivity at the mRNA accumulation level, which is in accordance with increased ABA sensitivity of *OsGRXS17*-silenced rice plants in seed germination and postembryonic growth.

Changes of the metabolite profiles in OsGRXS17-silenced rice plants

To compare the difference in metabolite profiles of wild type and *OsGRXS17*-silenced rice plants in response to drought stress, four biological replicates from each line sampled at full hydration (0d of drought stress treatment) and partial dehydration (5d and 8d of drought stress treatment) were analyzed using global metabolite profiling analysis. A total of 350 metabolites were detected, including amino acids (33.1%), lipids (19.4%), nucleotide (14%), carbohydrates (11.7%), cofactors, prosthetic groups and electron carriers (7.7%), peptide (6.9%), secondary metabolites (6.3%), xenobiotics (0.6%) and hormone (0.3%) (Figure 3.S5).

The results indicated that drought stress had a dramatic effect on metabolite profiling of wild type and *OsGRXS17*-silenced rice plants. There were 57% and 51% of metabolites showed significant drought-related difference ($p < 0.05$) in WT 8D vs WT 0D and RNAi 8D vs RNAi 0D, respectively (Table 3.S2). Of 350 detected metabolites, 26 metabolites exhibited more than five-fold increases in abundance in either wild type or *OsGRXS17*-silenced rice plants in response to dehydration, which has been demonstrated to be induced under drought stress and play crucial roles in drought stress tolerance, such as abscisic acid, proline, tryptophan and tryptamine. However, the silenced expression of *OsGRXS17* had a small effect on metabolite profiling of wild type and *OsGRXS17*-silenced rice plants. Only 2%- 25% of metabolites exhibited significant

genotype-related difference ($p < 0.05$) within treatment (WT vs RNAi) (Table 3.S2). Although most metabolites changed in a similar pattern in WT and *OsGRXS17*-silenced rice plants under drought stress, some trend changes in metabolites did suggest genotype-related difference with or without drought stress treatment.

In comparing the metabolite profiles between wild type and *OsGRXS17*-silenced rice plants at full hydration, 27 metabolites showed significantly altered abundances ($p<0.05$), in which 14 metabolites were more abundant in wild type and 13 exhibited a greater abundance in *OsGRXS17*-silenced rice plants, indicating that some changes of metabolite profiles is related with silenced expression of *OsGRXS17* (Table 3.S2). Under partial dehydration conditions, a total of 32 and 89 metabolites with significantly altered abundance ($p<0.05$) were detected after 5 days and 8 days of drought stress treatment, respectively. Amino acids, amino acid derivatives and peptides are more abundant in wild type, while phospholipids are more abundant in *OsGRXS17*-silenced rice plants under partial dehydration condition (Table 3.S3, 4). Under full hydration condition, the difference in abundance of amino acids and amino acids derivatives was small. Of the detected 119 amino acids and amino acid derivatives, only 6 metabolites were significantly greater ($p<0.05$) in *OsGRXS17*-silenced rice plants, whereas 6 metabolites showed significant abundance ($p<0.05$) in wild type at full hydration. After 5 days of drought stress treatment, the number of amino acids and amino acids derivatives with significant abundance ($p<0.05$) in *OsGRXS17*-silenced rice plants was five, whereas this number in wild type rose to ten. After 8 days of drought stress treatment, only one showed significant abundance ($p<0.05$) in *OsGRXS17*-silenced rice plants, whereas 40 metabolites were significantly abundant in wild type including 11 amino acids and 29 amino acid derivatives (Figure 3.10a, Table 3.S3). Of the detected 24 detected peptide, wild type had an increasing number of peptide with significant abundance ($p<0.05$) compared to *OsGRXS17*-

silenced rice plants after 5 days and 8 days of drought stress treatment, including gamma-glutamyls and dipeptides (Figure 3.10b, Table 3.S4). Another obvious difference in metabolite profiles between wild type and *OsGRXS17*-silenced rice plants was the abundance of phospholipids in *OsGRXS17*-silenced rice plants. *OsGRXS17* RNAi rice plants accumulated more phospholipids after 8 days of drought stress treatment, such as 1-linoleoyl-GPC (18:2), 1-linolenoyl-GPC (18:3)*, and 1-palmitoyl-2-linoleoyl-GPC (16:0/18:2) (Figure 3.S6, Table 3.S5). These results indicated that decreased abundance of amino acids, amino acid derivatives and peptide and increased abundance of phospholipids in *OsGRXS17*-silenced rice plants under drought stress is related with their phenotype of drought tolerance.

DISCUSSION

Glutaredoxins play important roles in maintaining the cellular redox homeostasis and regulate redox-dependent signal pathway (Cao et al. 2007; Fernandes and Holmgren 2004; Rouhier et al. 2008). Quite a few glutaredoxins have been reported for their roles in regulating stress responses by over-expression of those genes in plant (Cheng et al. 2011a; Knuesting et al. 2015a; Laporte et al. 2011; Sundaram et al. 2009a; Wu 2012). Therefore, it is expected that silenced expression of *OsGRXS17* can cause the increase of ROS level in plants and *OsGRXS17*-silenced rice plants will be more drought sensitive compared to wild-type plants. However, our results are contrary to this hypothesis. This finding provides a new prospective on understanding the function of glutaredoxins in plants.

OsGRXS17-silenced rice plants had increased H_2O_2 accumulation in rice guard cells and more closed stomata compared to wild-type rice plants without ABA treatment (Figure 3.5c; Figure 3.6c, d). It might be one reason why *OsGRXS17*-silenced rice plants exhibited less stomatal

conductance before drought stress treatment. With ABA treatment, the H_2O_2 content was significantly increased in guard cells of *OsGRXS17*-silenced rice plants compared to wild-type rice plants which are consistent with increased expression level of *OsGRXS17* under ABA treatment (Fig. 3.1d; Fig. 3.6c, d). This increased H_2O_2 content in the guard cells resulted in more closed stomata, less water loss rate and reduced stomatal conductance, and finally enhanced drought tolerance in *OsGRXS17*-silenced rice plants. These results indicated that OsGRXS17 might be involved in regulating H_2O_2-induced stomatal closure via its ability to modulate H_2O_2 homeostasis in guard cells. Previous studies had demonstrated that ROS has dual effects on plant responses to abiotic and biotic stresses, which can be phytotoxic to plant organelles at high concentration and also functions as secondary messenger at low concentration environment (Dat et al. 2000; Vranová et al. 2002). Therefore, even though *OsGRXS17*-silenced rice plants accumulated more ROS than wild-type rice plants, suggesting that *OsGRXS17*-silenced rice plants suffered from constitutive oxidative stress, this accumulation of ROS might not be high enough to cause phytotoxic damage to plant organelles but alternatively resulted in increased sensitivity to ABA-induced stomatal closure. Many studies have been demonstrated that H_2O_2 can function as a secondary messenger to mediate stomatal closure. In tobacco, suppressed expression of dehydroascorbate reductase (DHAR) gene exhibited an increased level of H_2O_2 in the guard cell, a lower percentage of open stomata and decreased stomatal conductance compared to DHAR over-expressing tobacco plants under normal growth conditions, indicating that changes in H_2O_2 homeostasis in the guard cells alter stomatal movement (Chen and Gallie 2004). It was also reported that H_2O_2 functions as secondary messenger of DST, a transcription factor that binds to the promoter region of H_2O_2 homeostasis genes, to induce the stomatal closure through a ABA independent pathway and the rice *dst* mutants were found to be more drought and salt tolerant compared to wild-type rice plants

(Huang et al. 2009). However, an *Arabidopsis* mutant lacking a *Arabidopsis* glutathione peroxidase (AtGPX3), one of antioxidant enzymes, also showed higher accumulation of H_2O_2 in the guard cells but higher rate of water loss rate and more sensitivity to drought stress which is inconsistent to our results (Miao et al. 2006). It is possible that AtGPX3 acts as both ROS scavenger and oxidative signal transducer in ABA and drought stress signaling and loss-of-function of AtGPX3 impaired oxidative signal transduction pathway. As a consequence, *atgpx3* mutant is drought sensitive though the guard cells of *atgpx3* mutant have even more accumulation of H_2O_2.

H_2O_2 has been proven to be essential for the root growth. The *rhd2* mutant lacking of an NADPH oxidase showed short root hairs compared to wild-type *Arabidopsis* due to decreased Ca^{2+} channel activities, indicating the positive role of H_2O_2 in regulating root growth (Foreman et al. 2003). The primary root length of single mutant *rbohF* and double mutant *robhD/robhF* was significantly reduced compared with that of wild type (Kwak et al. 2003b). In our study, we do not find significant difference in primary root length between *OsGRXS17*-silenced rice plants and wild type under normal growth conditions. It is possible that the root growth may be inhibited when ROS content is below an oxidative level that allows ROS signaling. In *OsGRXS17* -silenced rice plants, the root growth is not affected by silencing the expression of *OsGRXS17* as the ROS content could be above this oxidative level.

Although H_2O_2 is required for root growth, H_2O_2 has also been considered to serve as second messenger in ABA signaling to regulate seed germination and primary root growth (He et al. 2012; Kwak et al. 2003b). The double mutant *rbohD/rbohF* are insensitive to ABA-mediated inhibition of seed germination and primary root growth (Kwak et al. 2003b). Recently, an ABA overly sensitive mutant *abo6* lacking of a DEXH box RNA helicase accumulated more H_2O_2 in

the root tips with or without ABA treatment and showed hypersensitivity to ABA in seed germination and primary root growth (He et al. 2012). Another ABA overly sensitive mutant *abo8-1* deficient in a pentatricopeptide repeat (PPR) protein also had more accumulation of ROS in the root tips and are sensitive to ABA in seed germination and primary root growth (Yang et al. 2014). Our results showed that *OsGRXS17*-silenced rice plants exhibited hypersensitivity to ABA in terms of seed germination and postembryonic development compared to wild-type rice plants. At the meantime, ABA stimulated the accumulation of H_2O_2 in the root tips of *OsGRXS17*-silenced rice plants (Fig. 3.8), leading the inhibition of seed germination and primary root growth. After adding the ROS scavenging reagent GSH, ABA sensitivity in both seed germination, shoot and primary root growth of *OsGRXS17*-silenced rice plants was recovered to a level similar to that of the wild-type. These results indicated that silenced expression of OsGRXS17 enhances ABA-mediated root growth due to increased accumulation of ABA-induced H_2O_2 in the root tips and GSH could restore ABA sensitivity in both seed germination and primary root growth by reducing H_2O_2 accumulation. The biological mechanism that over-accumulation of H_2O_2 by ABA treatment in the root tips inhibits the seed germination and primary root growth remains elusive. It was reported that both ABA overly sensitive mutants, *abo6 and obo8-1*, have reduced auxin transport capacities and auxin levels, indicating that ROS are an important regulator of auxin homeostasis in regulating root growth. However, our results indicated that adding exogenous auxin could not recover the ABA-mediated inhibition of seed germination and primary root growth (Figure 3.S4). It is possible that Ca^{2+} channels activities but not auxin transport capacities and auxin levels were increased by ROS in the presence of ABA in *OsGRXS17*-silenced rice plants. The function of Ca^{2+} as a downstream signaling component of H_2O_2 to regulate root growth has been demonstrated in the *Arabidopsis rhd2* mutant lacking of an NADPH oxidase, in that the reduced ROS content

resulted in decreased Ca^{2+} channel activities, which further leaded to short root hairs compared to wild type.

The pairwise comparison of metabolite profiling between wild type and *OsGRXS17*-silenced rice plants could provide a high throughput way to identify the candidate metabolites that contribute to the drought tolerance in *OsGRXS17*-silenced rice plants. However, most metabolites exhibited similar changing patterns under the drought stress between wild type and *OsGRXS17*-silenced rice plants. That is possible that the effects of drought stress overwhelm the effects of silenced expression of *OsGRXS17* on changes of abundance of metabolites. Our results demonstrated that there were 69% and 57% of metabolites that showed significant drought-related difference ($p < 0.05$) in WT 8d vs WT 0d and RNAi 8d vs RNAi 0d, respectively, indicating a huge impact of drought stress on metabolism in both wild type and *OsGRXS17*-silenced rice plants. However, the difference in abundance of metabolites between wild type and *OsGRXS17*-silenced rice plants under the same treatment (the same time point of drought stress treatment) is small. The most obvious differences are more abundance of amino acids, amino acid derivatives and peptides in wild type than those in *OsGRXS17*-silenced rice plants. Accumulation of amino acids has been observed in many plant species under abiotic or biotic stresses due to breakdown of proteins under stress conditions (Lugan et al. 2010). In many species, increased amount of amino acids under abiotic or biotic stresses indicate the cell damage, while in some other species accumulation of amino acids can protect plants from the damages resulted from abiotic or biotic stresses(Lugan et al. 2010; Widodo et al. 2009). It is likely that since wild type suffered from drought stress earlier than *OsGRXS17*-silenced rice plants after withholding water due to less ABA-mediated stomata closure (Figure 3.5), amino acids, amino acid derivatives and peptides may be derived from breakdown of damaged proteins and accumulate in a large amount in wild type after 8 days of

drought stress treatment. Whereas *OsGRXS17*-silenced rice plants are still turgid and green (Figure 3.4), less drought stress damage may result in less abundance in amino acids, amino acid derivatives and peptides. Phospholipids, the main backbone of the cell membrane, are considered to increase under stresses to serve as precursor of production of secondary molecules (Xiong et al. 2002). Our results indicated that *OsGRXS17*-silenced rice plants accumulated more phospholipids than wild type after 8 days of drought stress treatment, which could transmit the drought stress signals to the downstream secondary molecules and consequently induce the defense response.

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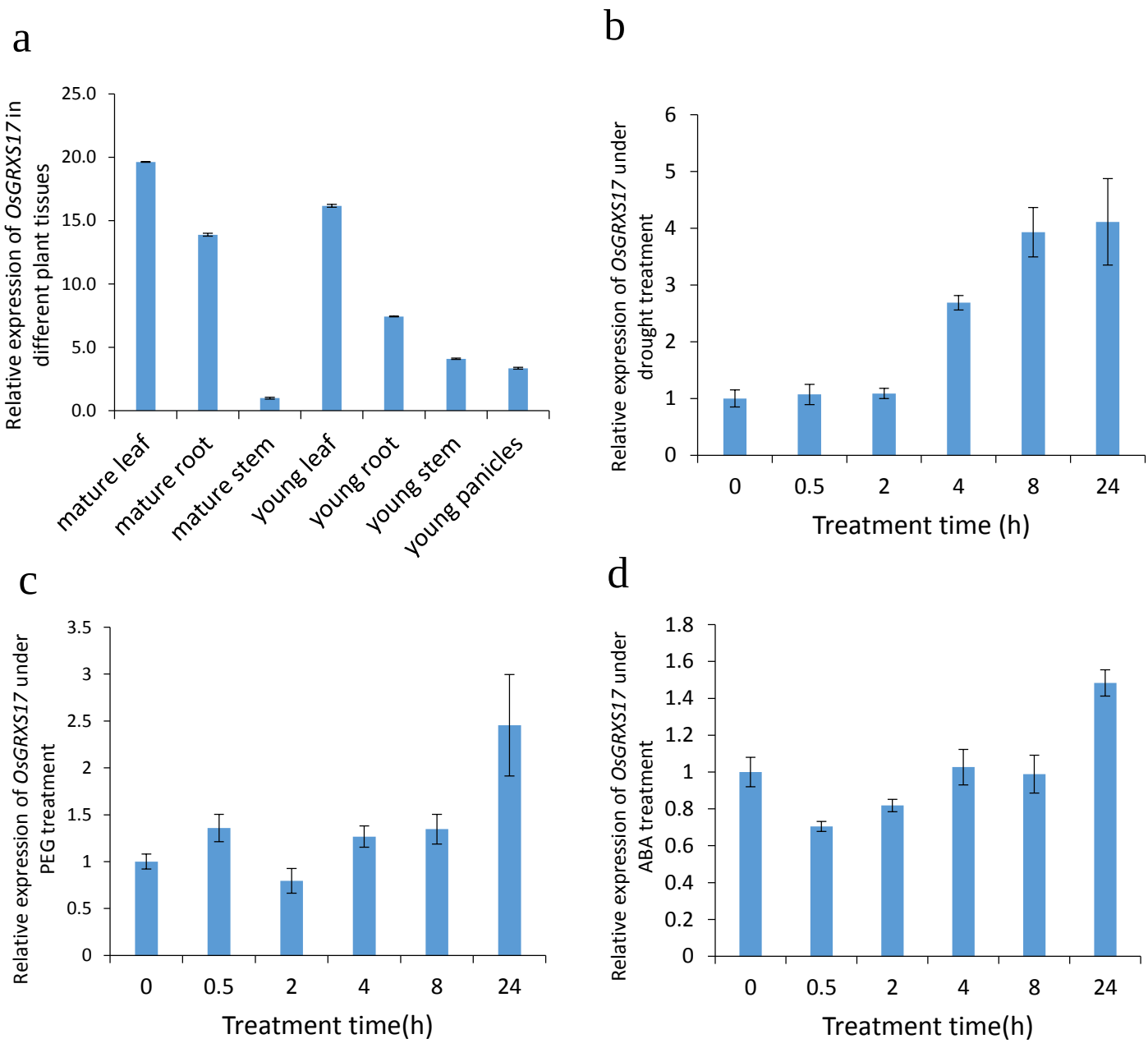
FIGURES


a
Relative expression of OsGRXS17 in different plant tissues
25.0
20.0
15.0
10.0
5.0
0.0
mature leaf
mature root
mature stem
young leaf
young root
young stem
young panicles
b
Relative expression of OsGRXS17 under drought treatment
6
5
4
3
2
1
0
0
0.5
2
4
8
24
Treatment time (h)
c
Relative expression of OsGRXS17 under PEG treatment
3.5
3
2.5
2
1.5
1
0.5
0
0
0.5
2
4
8
24
Treatment time(h)
d
Relative expression of OsGRXS17 under ABA treatment
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
0.5
2
4
8
24
Treatment time(h)

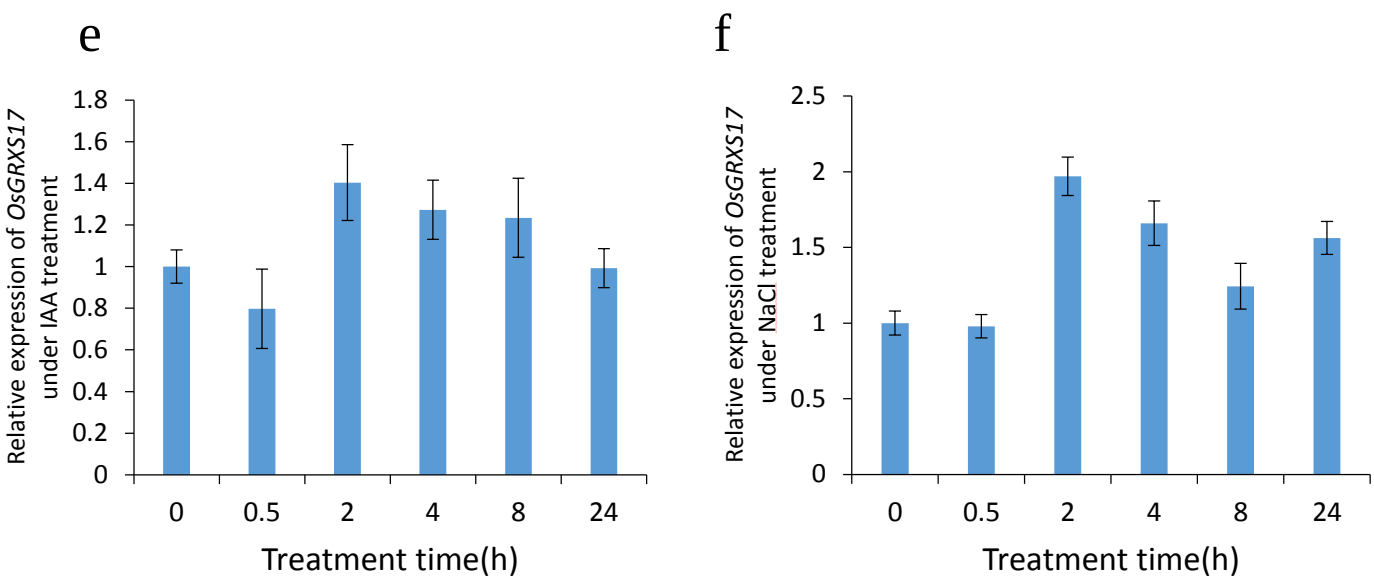


Figure 3. 1 Expression pattern of *OsGRXS17* in different tissues and under different abiotic stresses.

(a) Relative expression level of *OsGRXS17* in different tissues of two-week-old wild-type rice plants by real-time PCR analysis. Relative expression level of *OsGRXS17* by real-time PCR in the shoots of two-week-old wild-type rice plants treated with drought stress (b), PEG (c), ABA (d), IAA (e) and NaCl (f). Error bars represent the means ± SD (n = 3).

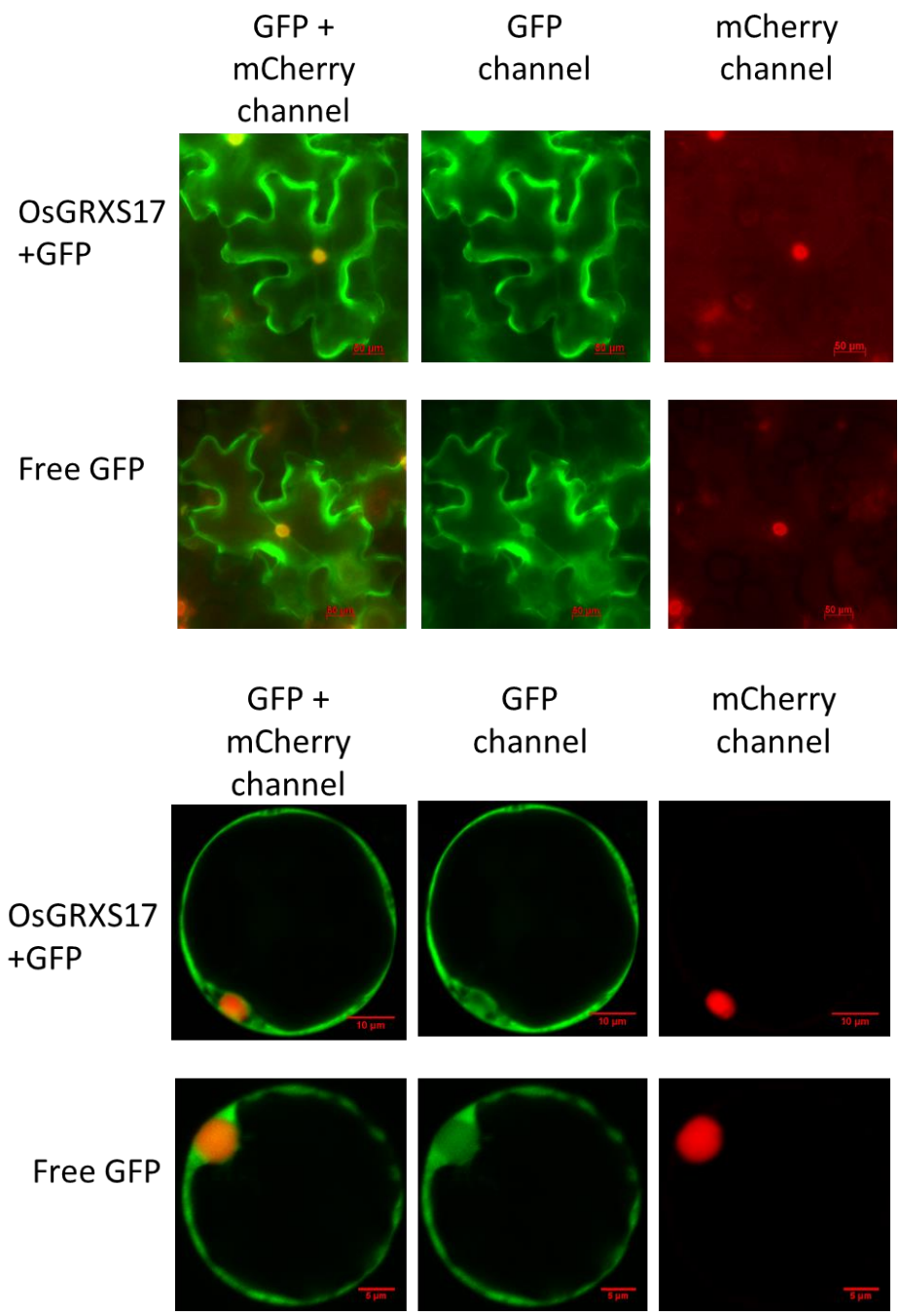


Figure 3. 2 Subcellular localization of OsGRXS17.

(a) Transient expression of GFP-OsGRXS17 and free GFP in tobacco epidermal cells. Scale bars = 50μm. The arrows highlight the nuclei. (b) Transient expression of GFP-OsGRXS17 and free GFP in rice protoplast. The arrows highlight the nuclei.

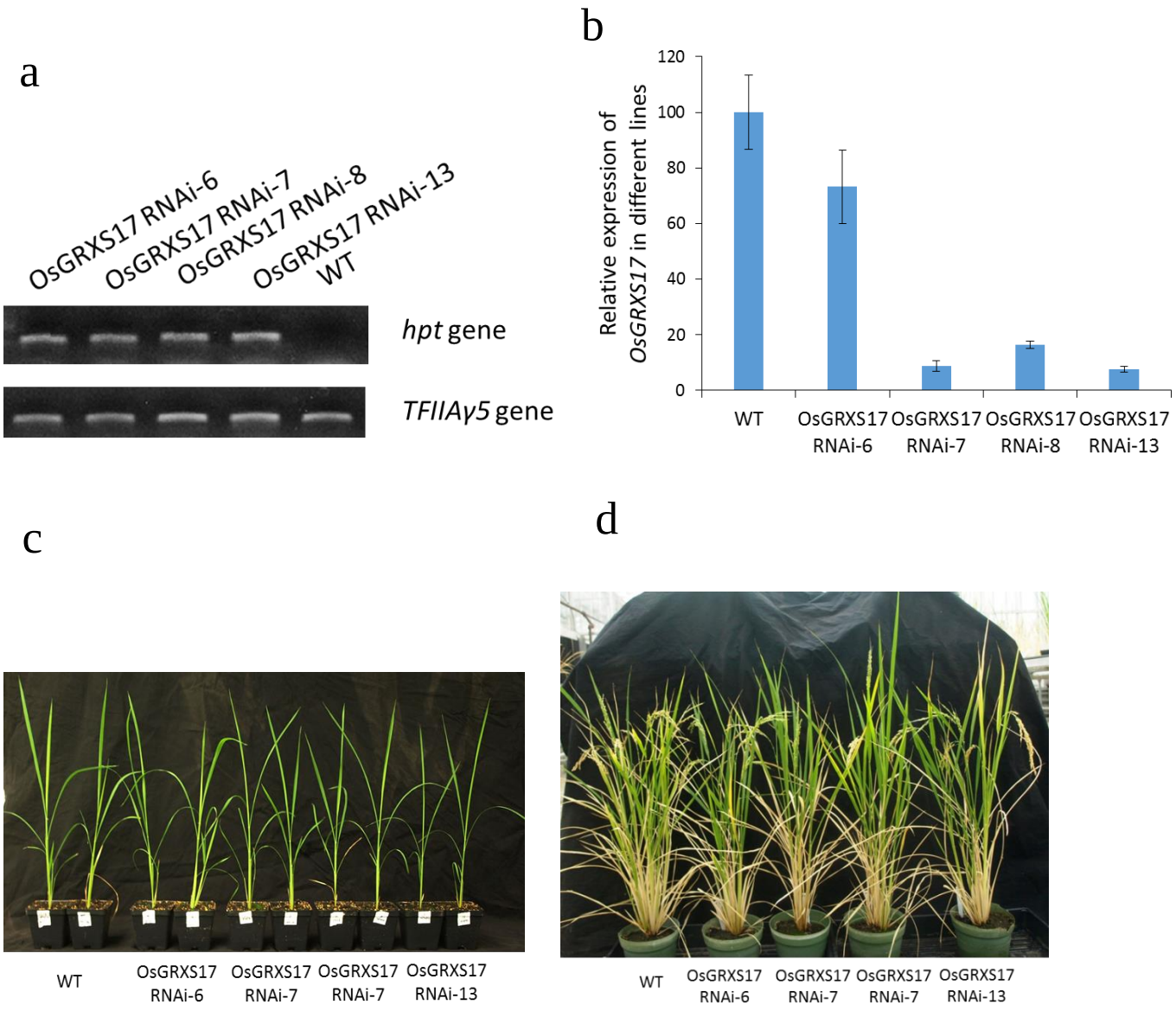
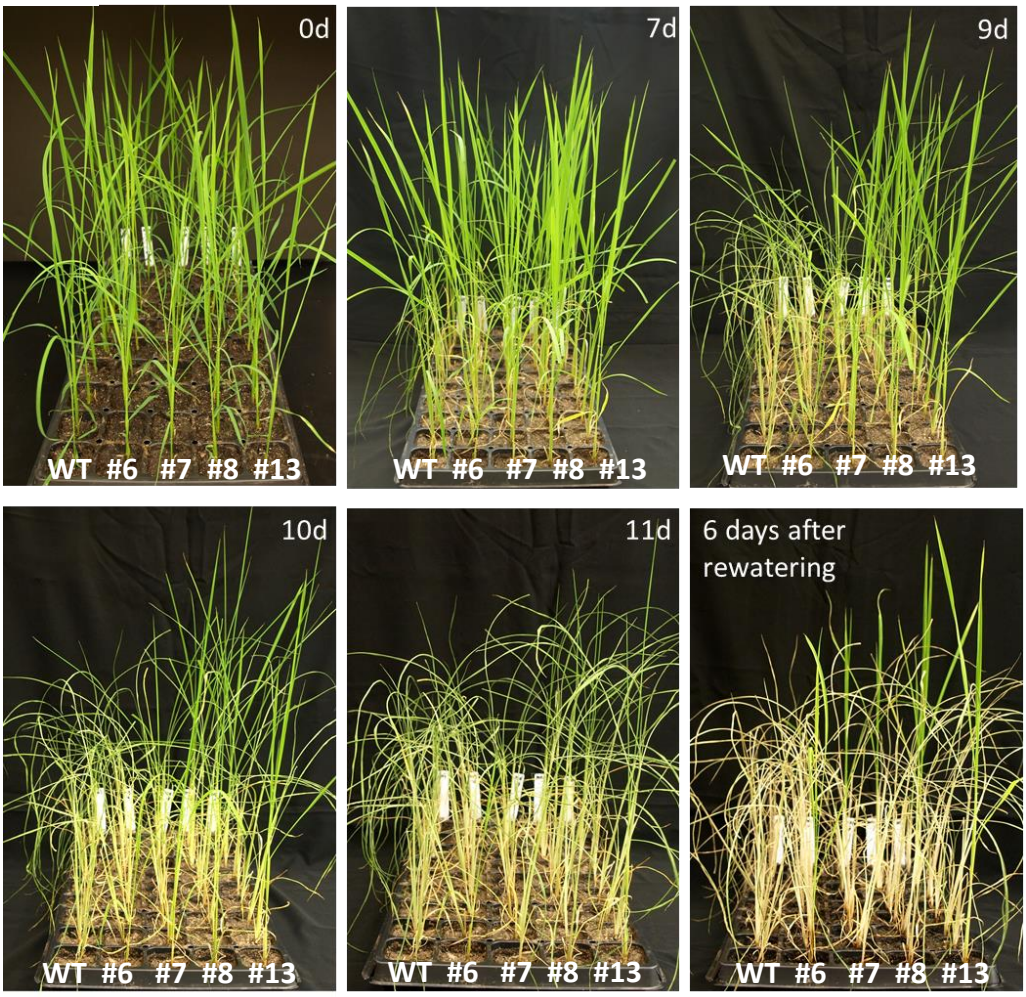


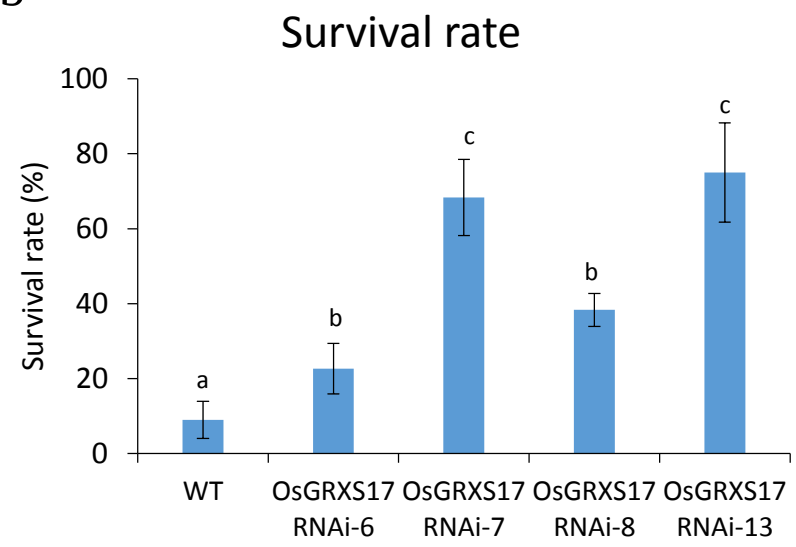
Figure 3. 3 Phenotype of four selective *OsGRXS17*-silenced rice lines (*OsGRXS17* RNAi-6, 7, 8 and 13) under normal growth condition.

(a) The expression of *OsGRXS17* was confirmed by RT-PCR in wild-type and four *OsGRXS17*-silenced rice plants. (b) The relative expression level of *OsGRXS17* was measured by real-time PCR in wild-type and four *OsGRXS17*-silenced rice plants. The phenotype of wild-type and *OsGRXS17*-silenced rice plants are indistinguishable at either seedlings stage (c) or reproductive stage (d). Error bars represent the means ± SD (n = 3).

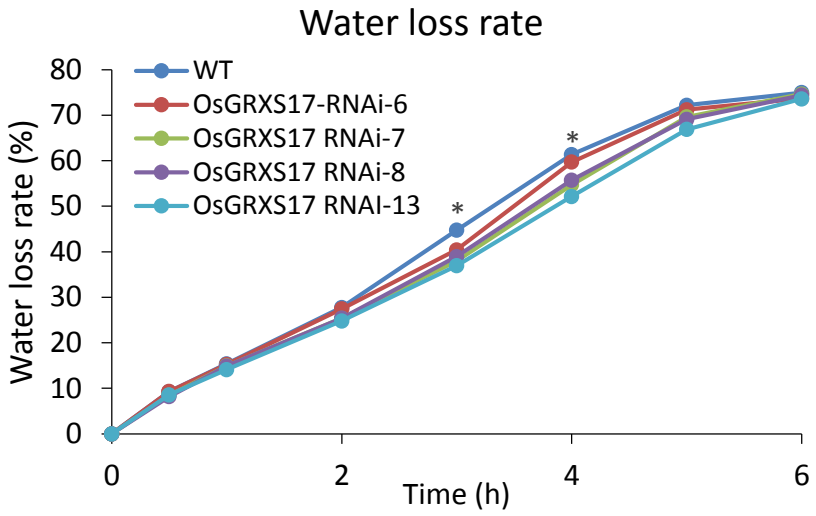
a


0d
WT #6 #7 #8 #13
7d
WT #6 #7 #8 #13
9d
WT #6 #7 #8 #13
10d
WT #6 #7 #8 #13
11d
WT #6 #7 #8 #13
6 days after rewatering
WT #6 #7 #8 #13

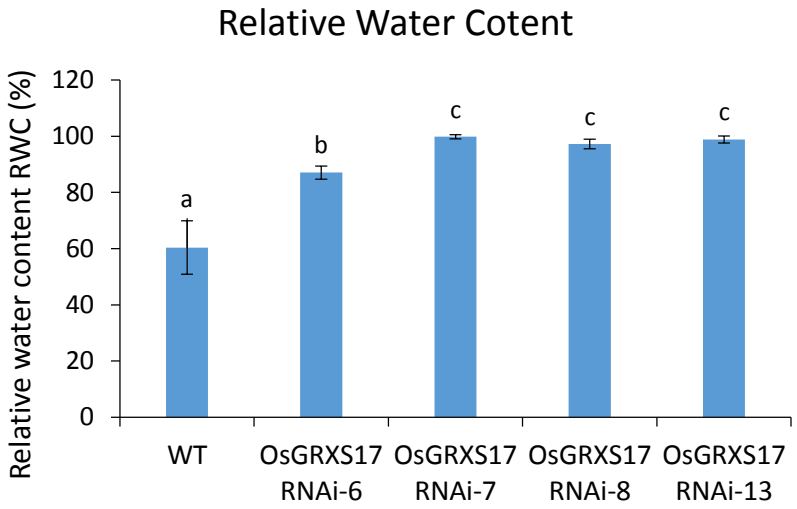
b


Survival rate
Survival rate (%)
100
80
60
40
20
0
a
b
c
b
c
WT
OsGRXS17 RNAi-6
OsGRXS17 RNAi-7
OsGRXS17 RNAi-8
OsGRXS17 RNAi-13

c


Water loss rate
WT
OsGRXS17-RNAi-6
OsGRXS17 RNAi-7
OsGRXS17 RNAi-8
OsGRXS17 RNAI-13
Water loss rate (%)
Time (h)
0
10
20
30
40
50
60
70
80
0
2
4
6
*
*

d


Relative Water Cotent
Relative water content RWC (%)
0
20
40
60
80
100
120
a
b
c
c
c
WT
OsGRXS17 RNAi-6
OsGRXS17 RNAi-7
OsGRXS17 RNAi-8
OsGRXS17 RNAi-13

e

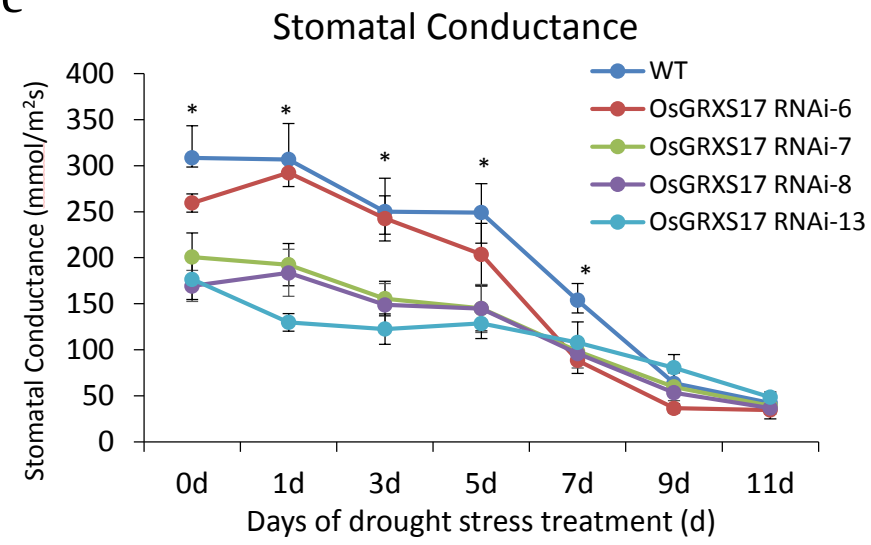
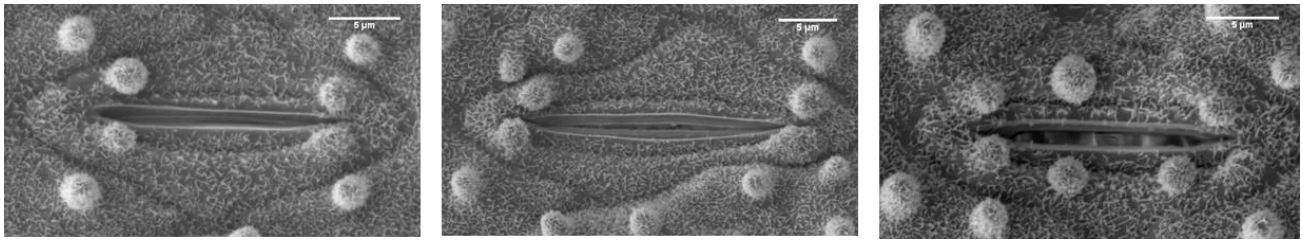

Stomatal Conductance
WT
OsGRXS17 RNAi-6
OsGRXS17 RNAi-7
OsGRXS17 RNAi-8
OsGRXS17 RNAi-13
Stomatal Conductance (mmol/m²s)
0
50
100
150
200
250
300
350
400
0d
1d
3d
5d
7d
9d
11d
Days of drought stress treatment (d)
*
*
*
*
*

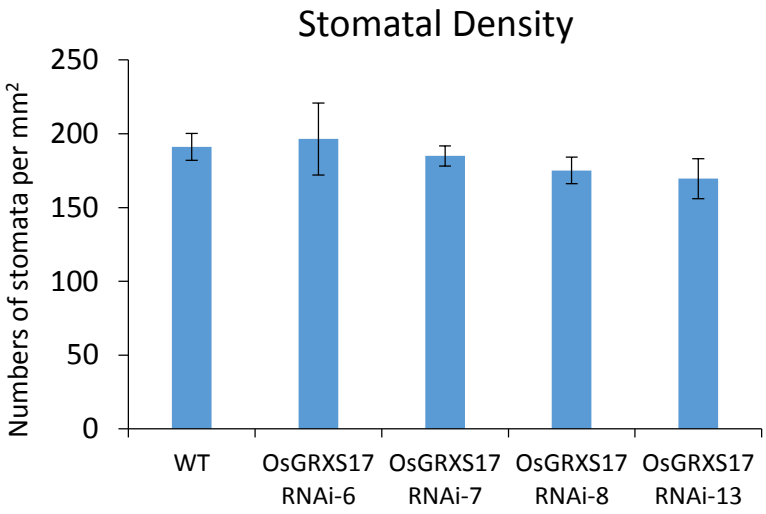
Figure 3. 4 The *OsGRXS17*-silenced rice plants show tolerance to drought stress.

(a) Drought stress treatment of wild-type and *OsGRXS17*-silenced rice plants. The four-week-old plants (0d) were treated by withholding water for 11 days and rewatered for 6 days to recover. (b) The survival rate of the wild-type and *OsGRXS17*-silenced rice plants after 11 days of drought stress treatment and 6 days of rewatering. (c) Water loss rate in the leaves cut from four-week-old wild-type and *OsGRXS17*-silenced rice plants (n = 3 repeats, 6 plants in each repeat). (d) The relative water content of four-week-old wild-type and *OsGRXS17*-silenced rice plants after 5 days of drought stress treatment (n = 3 repeats, 6 plants in each repeat). (e) Stomatal conductance of four-week-old wild-type and *OsGRXS17*-silenced rice plants under drought stress for 11 days (n = 3 repeats, 6 plants in each repeat). Error bars represent the means ± SD (n = 3). Asterisks (*, **) represent statistically significant differences between wild-type and *OsGRXS17*-expressing lines (*P < 0.05, **P < 0.01). Values with the same lowercase letter are not significantly different at the p-value < 0.05 with Turkey test.

a


Completely closed
Partially open
Completely open

b


Stomatal Density
Numbers of stomata per mm2
250
200
150
100
50
0
WT
OsGRXS17 RNAi-6
OsGRXS17 RNAi-7
OsGRXS17 RNAi-8
OsGRXS17 RNAi-13

c

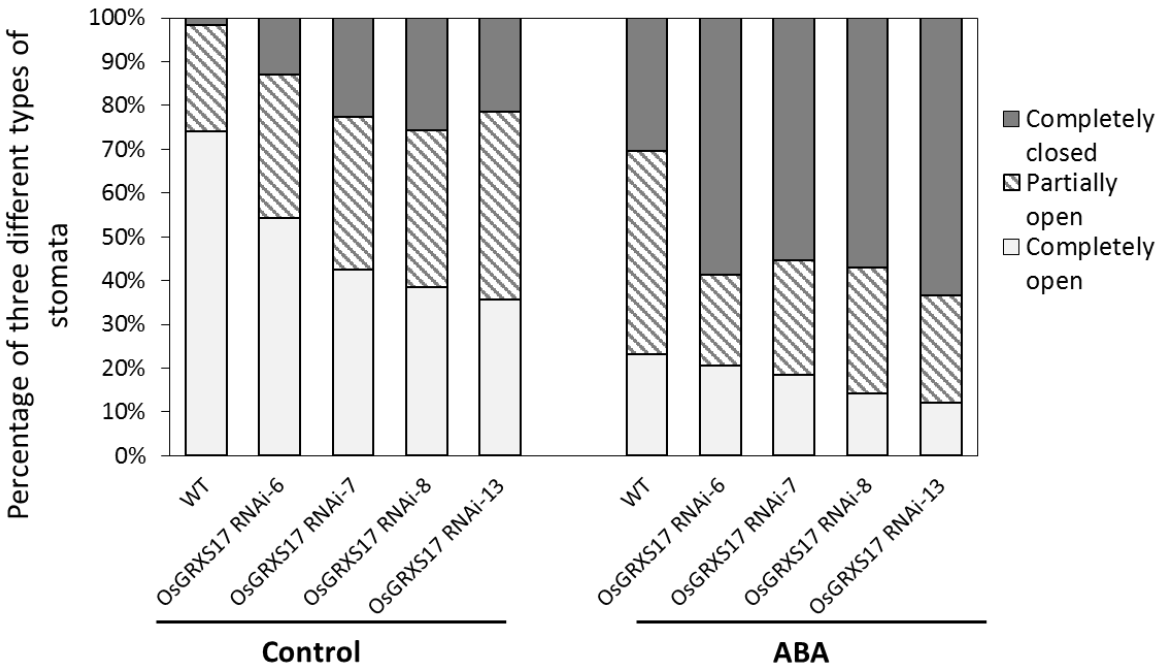
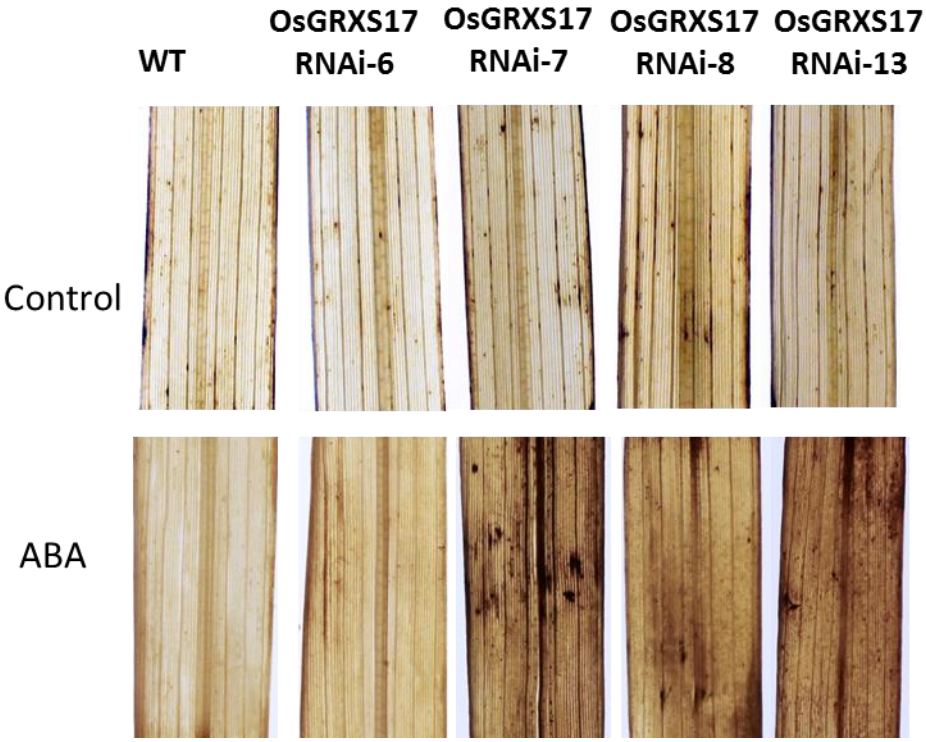

Percentage of three different types of stomata
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
WT
OsGRXS17 RNAi-6
OsGRXS17 RNAi-7
OsGRXS17 RNAi-8
OsGRXS17 RNAi-13
Control
ABA
Completely closed
Partially open
Completely open

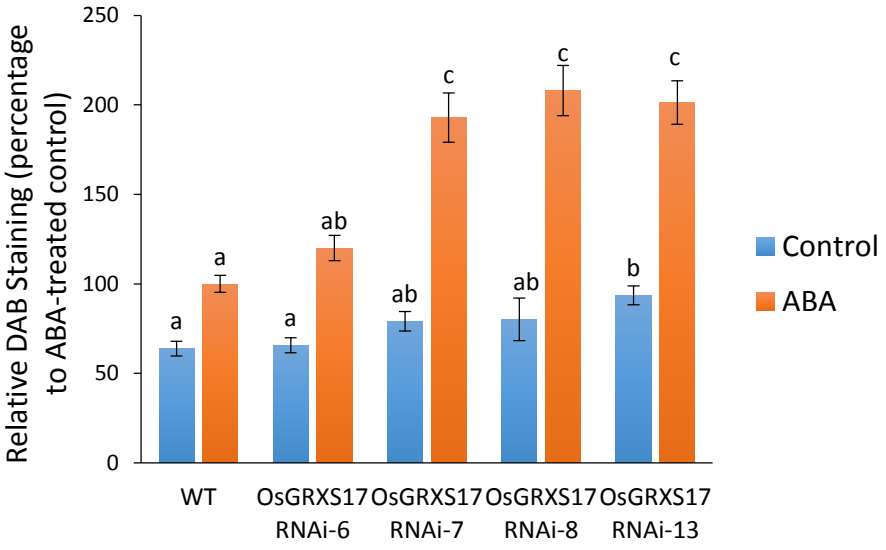
Figure 3. 5 Comparison of stomatal density and stomatal opening status between wild-type and *OsGRXS17*-silenced rice plants under ABA treatment.

(a) Scanning electron microscope images of three different statuses of rice stomata. Scale bars = 5μm. (b) Stomatal density of the first fully expanded leaves of four-week-old wild-type and *OsGRXS17*-silenced rice plants. (c) Percentage of three different types of stomata in four-week-old wild-type and *OsGRXS17*-silenced rice plants under normal growth condition or treated by 100 μM ABA (n = 62 stomata for wild-type, n = 58 stomata for *OsGRXS17* RNAi-6, n = 55 stomata for *OsGRXS17* RNAi-7, n = 65 stomata for *OsGRXS17* RNAi-8 and n = 60 stomata for *OsGRXS17* RNAi-13). Error bars represent the means ± SD (n = 3).

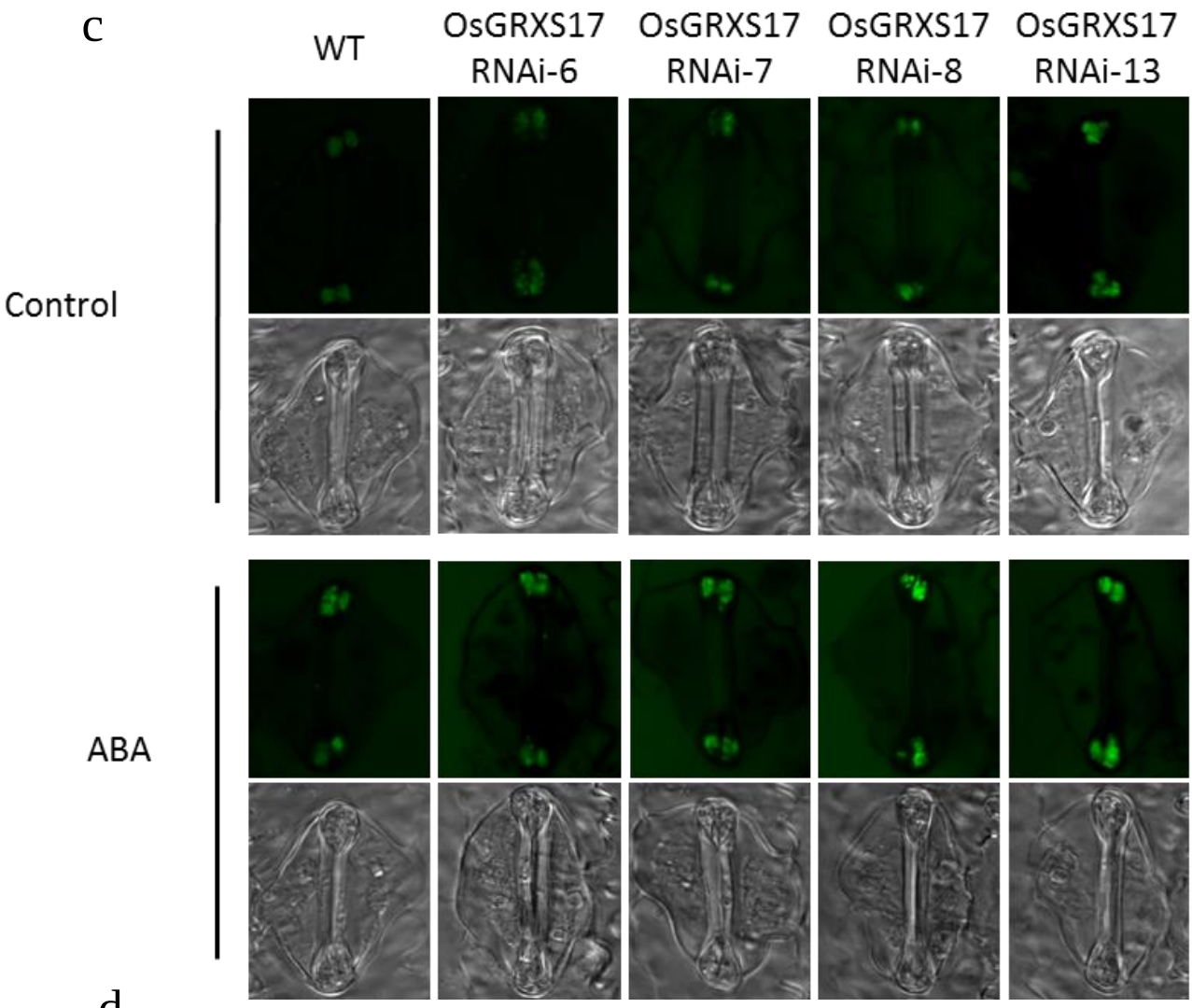
a


WT
OsGRXS17 RNAi-6
OsGRXS17 RNAi-7
OsGRXS17 RNAi-8
OsGRXS17 RNAi-13
Control
ABA

b


Relative DAB Staining (percentage to ABA-treated control)
250
200
150
100
50
0
a
a
a
ab
ab
c
ab
c
b
c
WT
OsGRXS17 RNAi-6
OsGRXS17 RNAi-7
OsGRXS17 RNAi-8
OsGRXS17 RNAi-13
Control
ABA

c


WT
OsGRXS17 RNAi-6
OsGRXS17 RNAi-7
OsGRXS17 RNAi-8
OsGRXS17 RNAi-13
Control
ABA

d

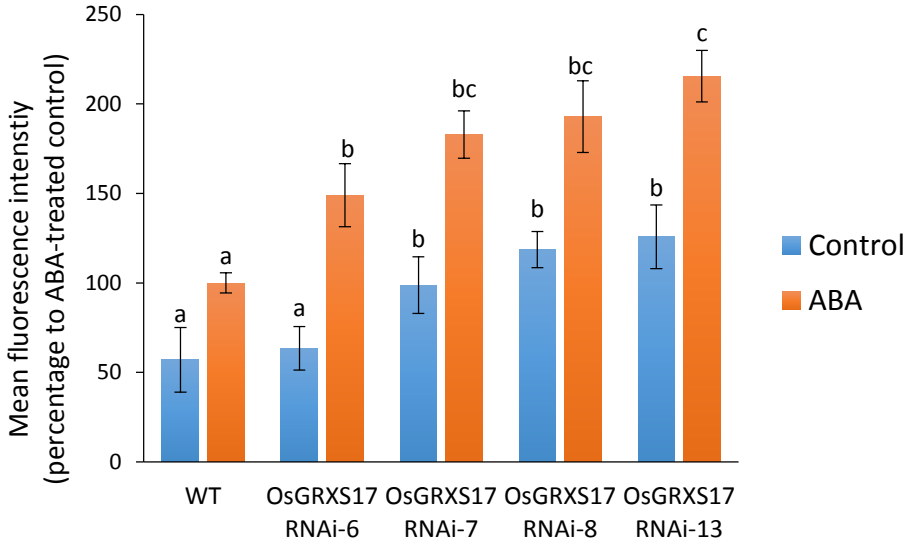
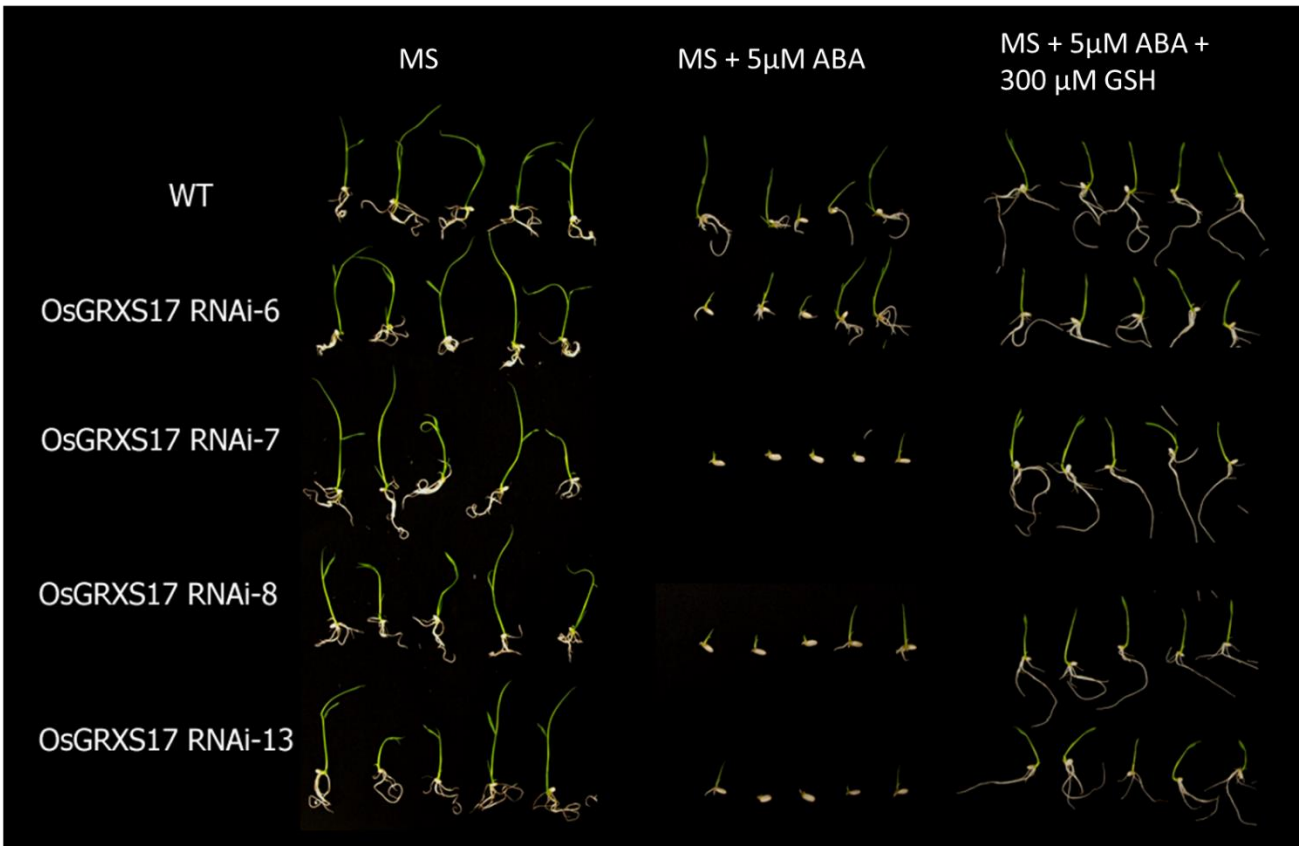

Mean fluorescence intenstiy (percentage to ABA-treated control)
250
200
150
100
50
0
a
a
a
b
b
bc
b
bc
b
c
WT
OsGRXS17 RNAi-6
OsGRXS17 RNAi-7
OsGRXS17 RNAi-8
OsGRXS17 RNAi-13
Control
ABA

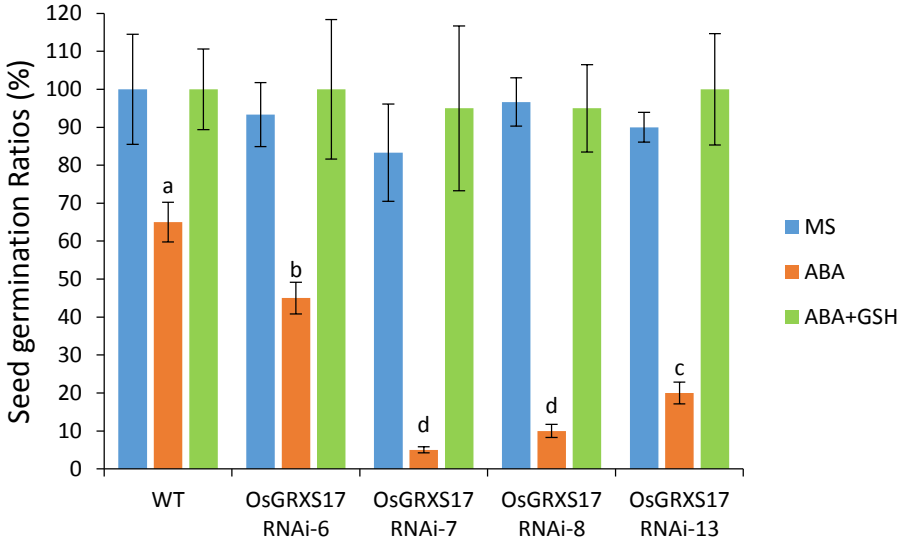
Figure 3. 6 Effect of silenced expression of *OsGRXS17* on H_2O_2 accumulation.

(a) DAB staining of middle section of first fully expanded leaf of four-week-old wild-type and *OsGRXS17*-silenced rice plants. (b) Quantitative analysis of DAB staining. The relative intensity of DAB staining leaves was analyzed after being transformed to 256 grey scale images. Data are expressed as relative values based on wild-type plants treated by 100μM ABA as reference sample set as 1.0. Error bars represent the means ± SD (n = 3). (c) H_2DCFDA staining of the guard cell of four-week-old wild-type and *OsGRXS17*-silenced rice plants. (d) Quantitative analysis of H_2DCFDA staining. Data are expressed as relative values based on wild-type plants treated by 100μM ABA as reference sample set as 1.0. Error bars represent the means ± SD (n = 3). Error bars represent the means ± SD (n = 3). Values with the same lowercase letter are not significantly different at the p-value < 0.05 with Turkey test.

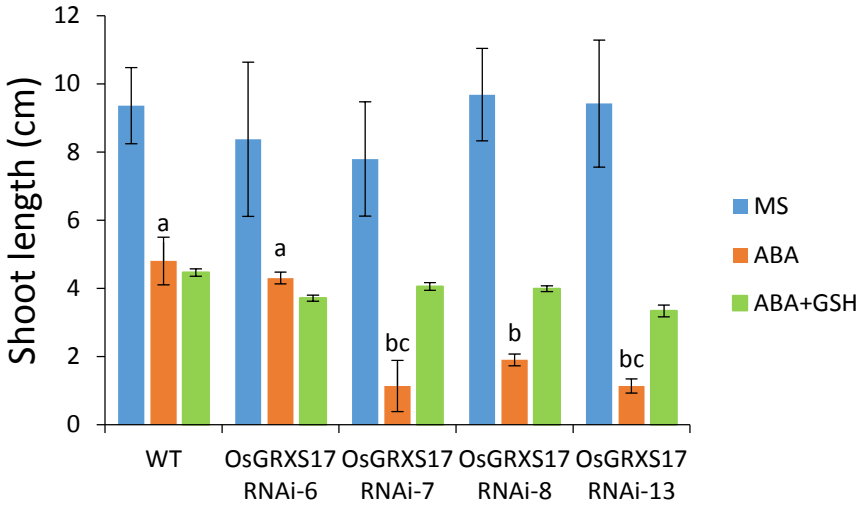
a


MS
MS + 5μM ABA
MS + 5μM ABA + 300 μM GSH
WT
OsGRXS17 RNAi-6
OsGRXS17 RNAi-7
OsGRXS17 RNAi-8
OsGRXS17 RNAi-13

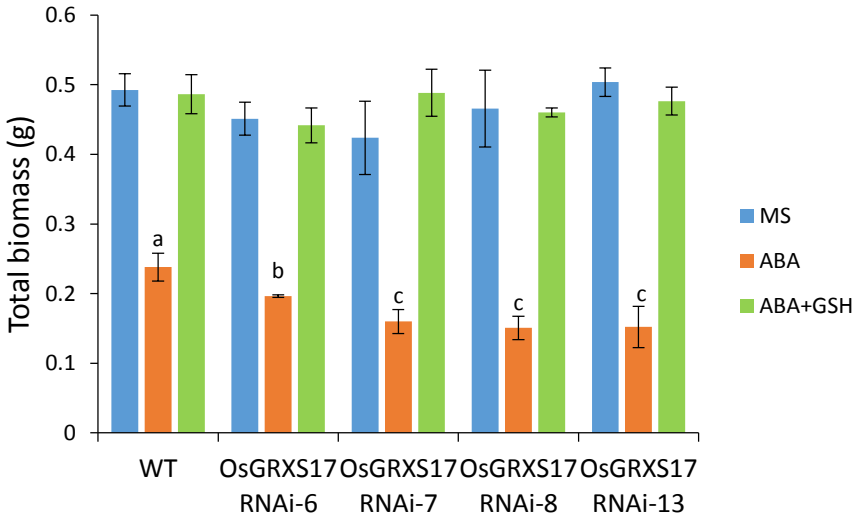
b


Seed germination Ratios (%)
120
110
100
90
80
70
60
50
40
30
20
10
0
a
b
d
d
c
WT
OsGRXS17 RNAi-6
OsGRXS17 RNAi-7
OsGRXS17 RNAi-8
OsGRXS17 RNAi-13
MS
ABA
ABA+GSH

c


Shoot length (cm)
12
10
8
6
4
2
0
a
a
bc
b
bc
WT
OsGRXS17 RNAi-6
OsGRXS17 RNAi-7
OsGRXS17 RNAi-8
OsGRXS17 RNAi-13
MS
ABA
ABA+GSH

d


Total biomass (g)
0.6
0.5
0.4
0.3
0.2
0.1
0
a
b
c
c
c
WT
OsGRXS17 RNAi-6
OsGRXS17 RNAi-7
OsGRXS17 RNAi-8
OsGRXS17 RNAi-13
MS
ABA
ABA+GSH

e

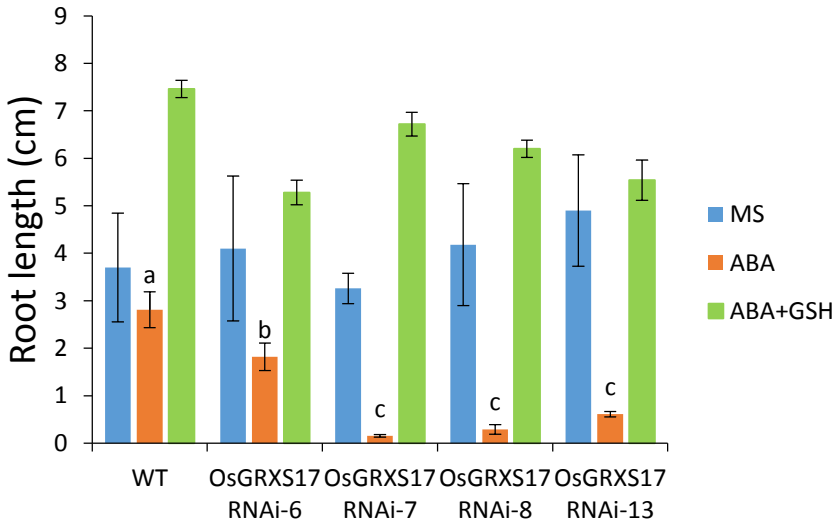
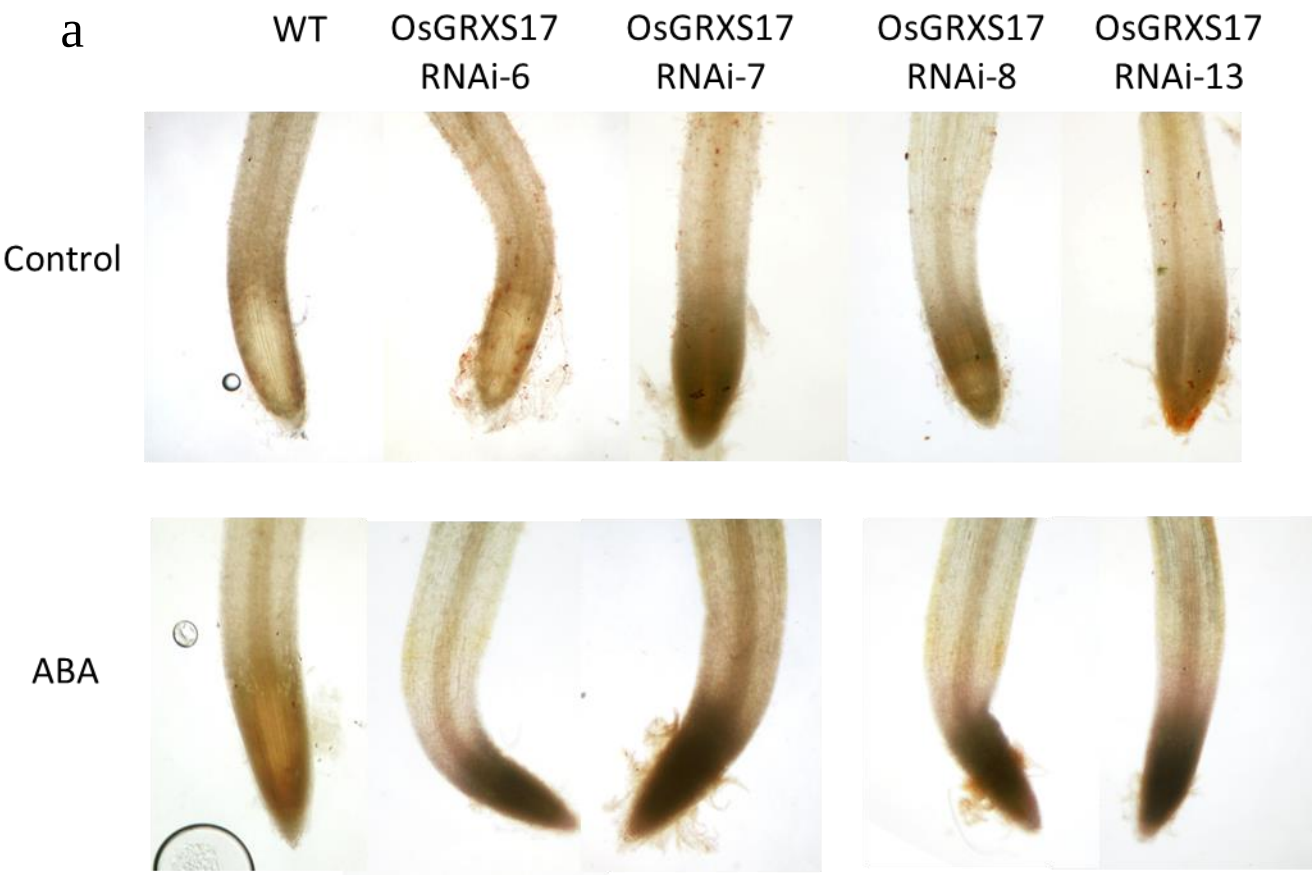

Root length (cm)
9
8
7
6
5
4
3
2
1
0
a
b
c
c
c
WT
OsGRXS17 RNAi-6
OsGRXS17 RNAi-7
OsGRXS17 RNAi-8
OsGRXS17 RNAi-13
MS
ABA
ABA+GSH

Figure 3. 7 *OsGRXS17*-silenced rice plants showed ABA hypersensitivity in seed germination and postembryogenic growth.

(a) Nine-day-old rice seedling germinated on MS, MS + 5 µM ABA and MS + 5 µM ABA + 300 µM GSH medium. (b) The seed germination ratio of wild-type and *OsGRXS17*-silenced rice plants after 4 days of germination (n = 3 repeats, 30 plants for each repeat). The shoot length (c), total biomass (d) and root length (d) was measured after 9 days of germination (n = 3 repeats, 30 plants for each repeat). Error bars represent the means ± SD (n = 3). Columns for ABA with the same lowercase letter are not significantly different at the p-value < 0.05 with Turkey test.

a


WT
OsGRXS17 RNAi-6
OsGRXS17 RNAi-7
OsGRXS17 RNAi-8
OsGRXS17 RNAi-13
Control
ABA

b

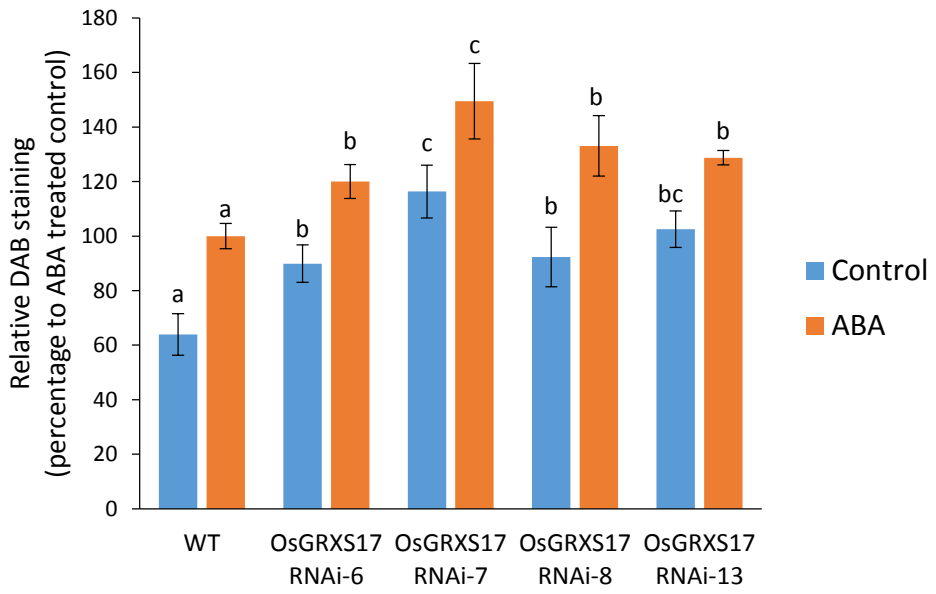

Relative DAB staining (percentage to ABA treated control)
180
160
140
120
100
80
60
40
20
0
a
a
b
b
c
c
b
b
bc
b
WT
OsGRXS17 RNAi-6
OsGRXS17 RNAi-7
OsGRXS17 RNAi-8
OsGRXS17 RNAi-13
Control
ABA

Figure 3. 8 *OsGRXS17*-silenced rice plants accumulate more H_2O_2 in the root tips.

(a) DAB staining of the root tips of nine-day-old rice seedling. (b) Quantitative analysis of DAB staining. The relative intensity of DAB staining leaves was analyzed after being transformed to 256 grey scale images. Data are expressed as relative values based on wild-type plants treated by 100μM ABA as reference sample set as 1.0. Error bars represent the means ± SD (n = 3). Values with the same lowercase letter are not significantly different at the p-value < 0.05 with Turkey test.

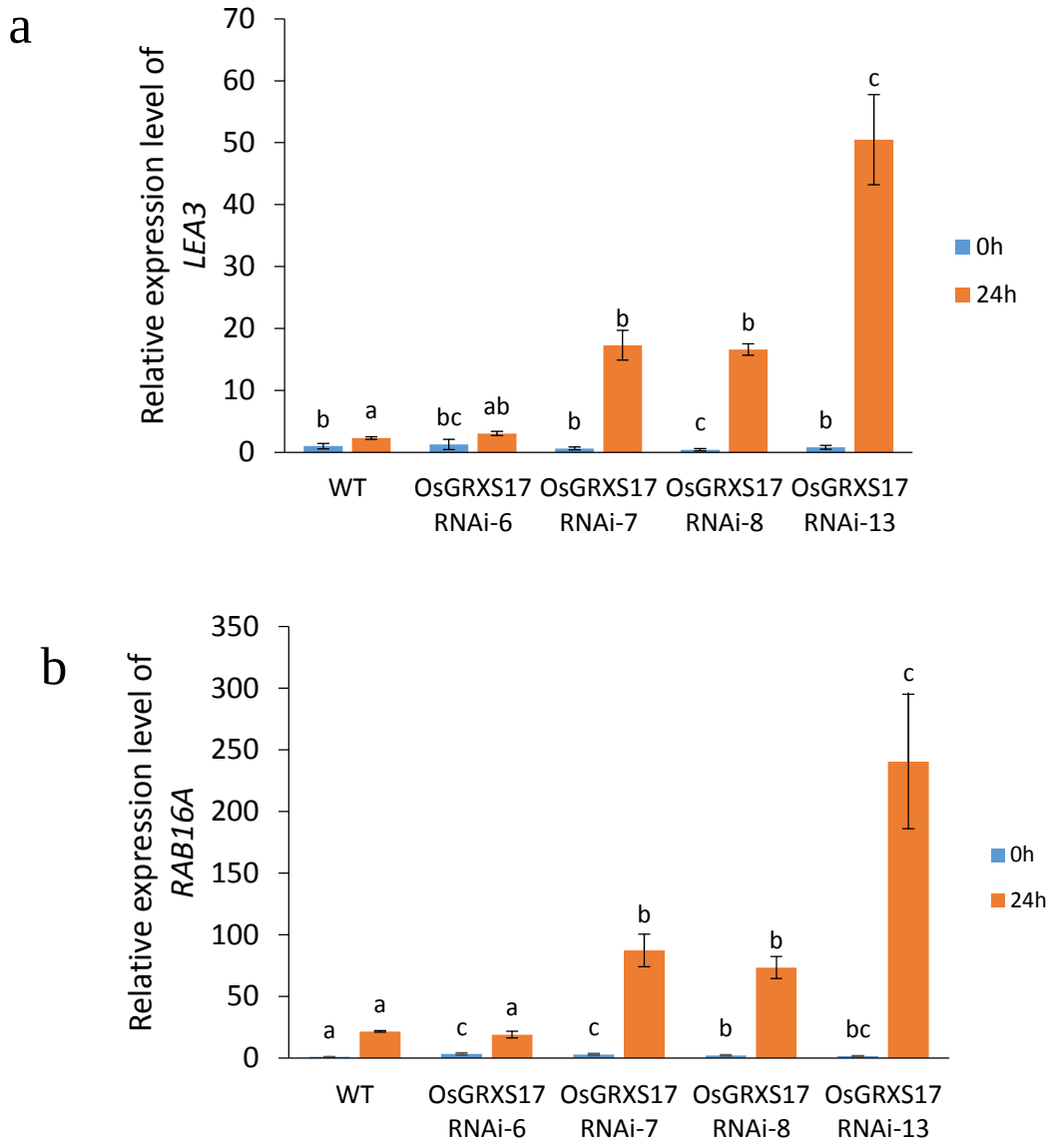
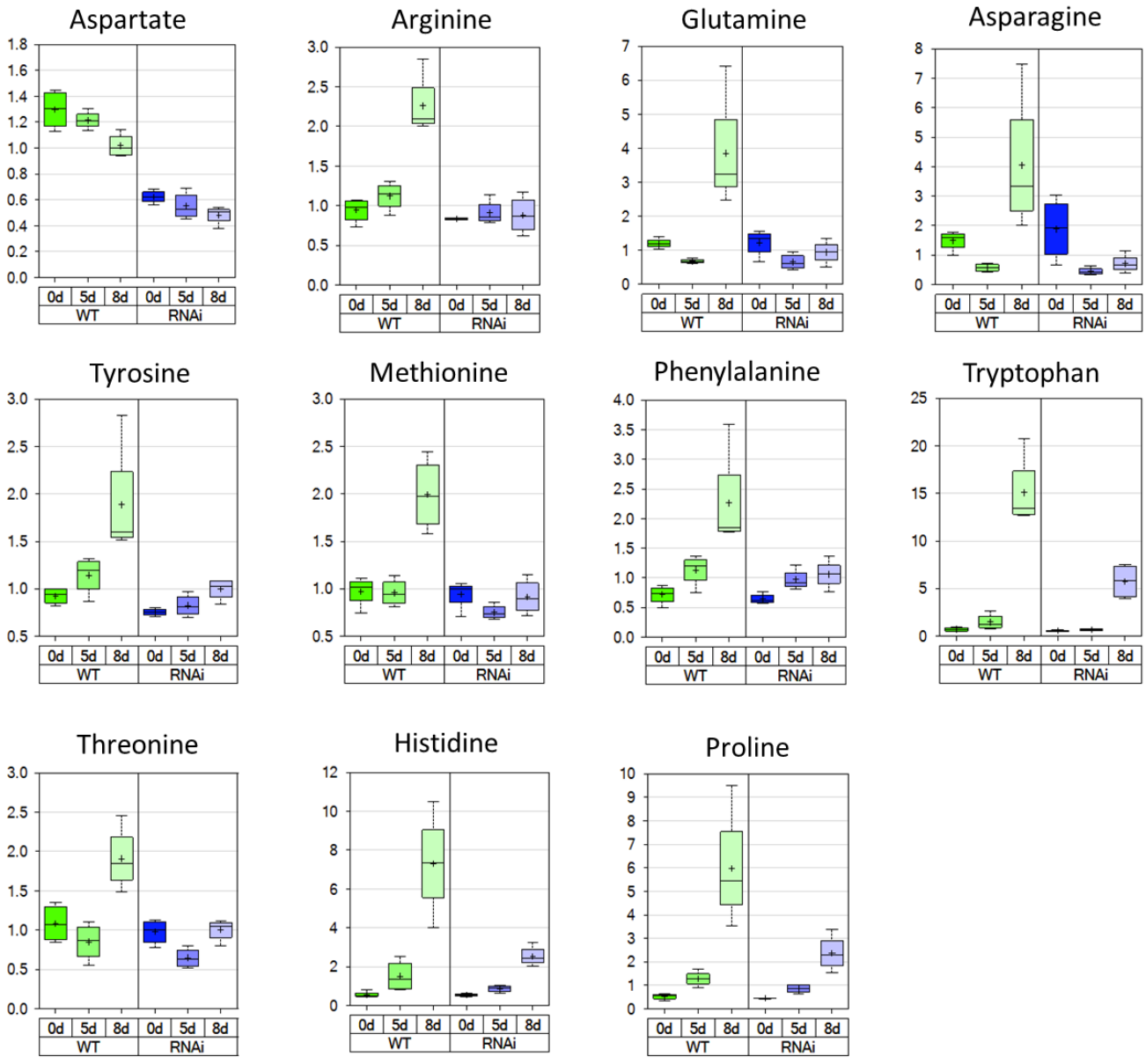


Figure 3. 9 The relative expression level of ABA responsive genes in wild-type and *OsGRXS17*-silenced rice plants.

Relative expression of *LEA3* (a) and *RAB16A* (b) in four-week-old wild-type and *OsGRXS17*-silenced rice plants were detected by real-time PCR after being treated by 100μM ABA for 0 and 24 h, respectively. Error bars represent the means ± SD (n = 3). Values with the same lowercase letter are not significantly different at the p-value < 0.05 with Turkey test.

a


Aspartate
Arginine
Glutamine
Asparagine
Tyrosine
Methionine
Phenylalanine
Tryptophan
Threonine
Histidine
Proline
0d 5d 8d 0d 5d 8d
WT RNAi

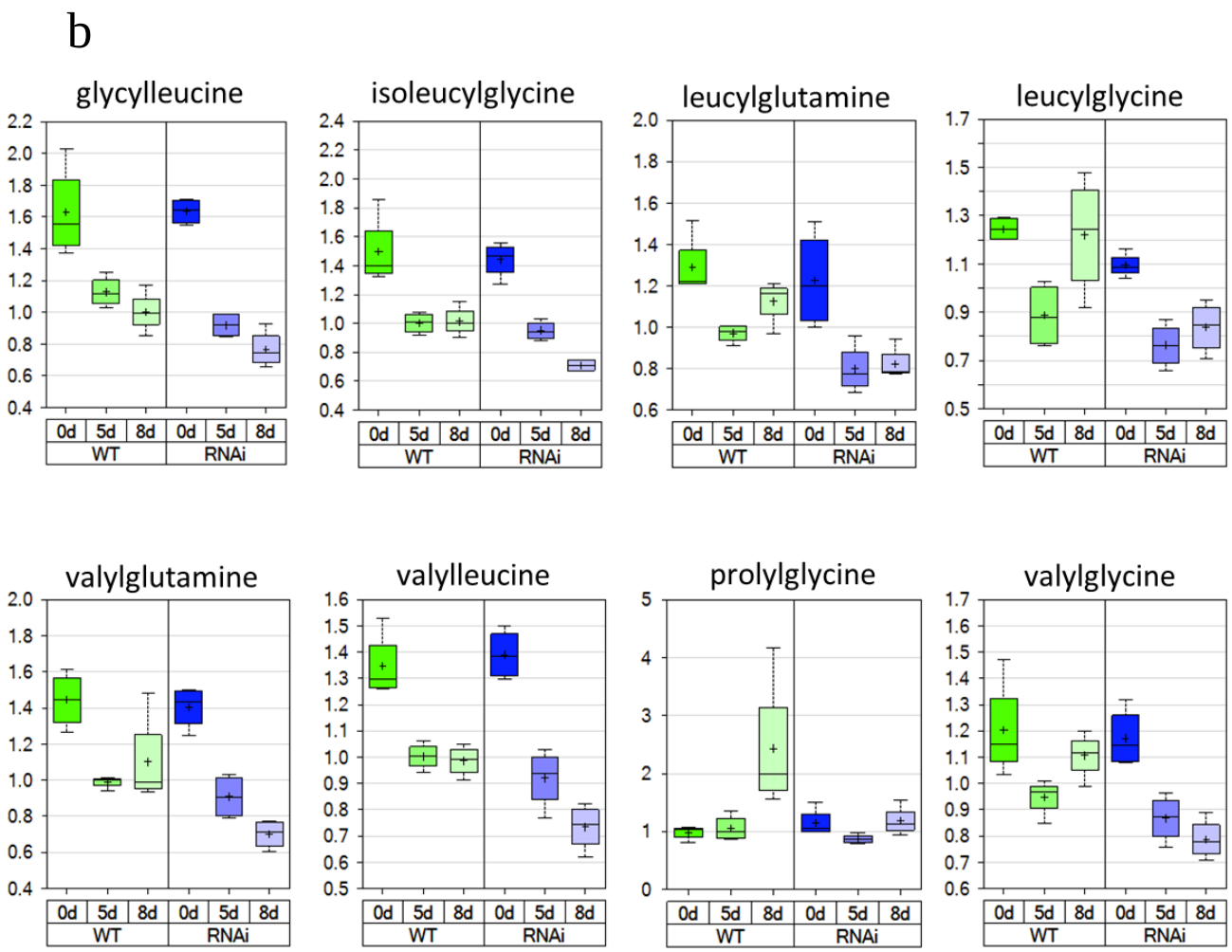


Figure 3. 10 Comparison of metabolite profiling between wild-type and *OsGRXS17*-silenced rice plants.

OsGRXS17-silenced rice plants had reduced abundance in amino acids and amino acids derivatives (a) and peptides (b) after 8 days of drought stress treatment. Error bars represent the means ± SD (n = 4).

SUPPORTING INFORMATION

SI Tables and figures

Table 3.S 1 Primers used for qRT-PCR

Gene name	Accession #	Primer sequence
OsGRXS17	AK067982.1	Forward: TTCAGTACGGTTATATTGCCAGTT Reverse: AAGCACCCTCTATTAATTGCGATA
RAB16A	NM_001074376	Forward: CCAGTTCCAGCCGATGAG Reverse: TCCTCCCTCCCATTCCAT
LEA3	NM_001062730	Forward: ACCAAGGACTCTGCCATC Reverse: GCTCTTCACCTGCTCACT
TFIIAγ5	NM_001060961	Forward: GGGTTTGCCTGGTATTTGTTAG Reverse: GTTGCTGCTGTGATATACTCTG

Table 3.S 2 Difference in metabolite abundance between wild-type and *OsGRXS17*-silenced rice plants

ANOVA Contrast	No. of metabolites ($p < 0.05$)	Percentage of metabolites ($p < 0.05$)	No. of increased metabolites vs No. decreased metabolites ($p < 0.05$)
WT_5D/WT_0D	123	35.14%	35/70
WT_8D/WT_0D	201	57.43%	112/89
RNAi_5D/RNAi_0D	89	25.43%	30/59
RNAi_8D/RNAi_0D	180	51.43%	88/100
RNAi_0D/WT_0D	27	7.71%	13/14
RNAi_5D/WT_5D	32	9.14%	9/23
RNAi_8D/WT_8D	89	25.43%	16/73

Table 3.S 3 Difference in amino acids and amino acids derivatives between wild-type and *OsGRXS17*-silenced rice plants

ANOVA Contrast	**No. of amino acids and derivatives ($p < 0.05$)**	**No. of increased metabolites vs No. decreased metabolites ($p < 0.05$)**
WT_5D/WT_0D	51	30/21
WT_8D/WT_0D	81	62/19
RNAi_5D/RNAi_0D	39	20/19
RNAi_8D/RNAi_0D	61	40/21
RNAi_0D/WT_0D	12	6/6
RNAi_5D/WT_5D	15	5/10
RNAi_8D/WT_8D	41	1/40

Table 3.S 4 Difference in peptide abundance between wild-type and *OsGRXS17*-silenced rice plants

ANOVA Contrast	**No. of peptide ($p < 0.05$)**	**No. of increased metabolites vs No. decreased metabolites ($p < 0.05$)**
WT_5D/WT_0D	18	8/10
WT_8D/WT_0D	17	10/7
RNAi_5D/RNAi_0D	15	3/12
RNAi_8D/RNAi_0D	18	7/11
RNAi_0D/WT_0D	1	1/0
RNAi_5D/WT_5D	4	1/3
RNAi_8D/WT_8D	12	0/12

Table 3.S 5 Difference in phospholipids abundance between wild-type and *OsGRXS17*-silenced rice plants

ANOVA Contrast	**No. of phospholipids ($p < 0.05$)**	**No. of increased metabolites vs No. decreased metabolites ($p < 0.05$)**
WT_5D/WT_0D	8	1/7
WT_8D/WT_0D	18	5/13
RNAi_5D/RNAi_0D	1	1/0
RNAi_8D/RNAi_0D	21	8/13
RNAi_0D/WT_0D	3	0/3
RNAi_5D/WT_5D	0	0/0
RNAi_8D/WT_8D	6	6/0

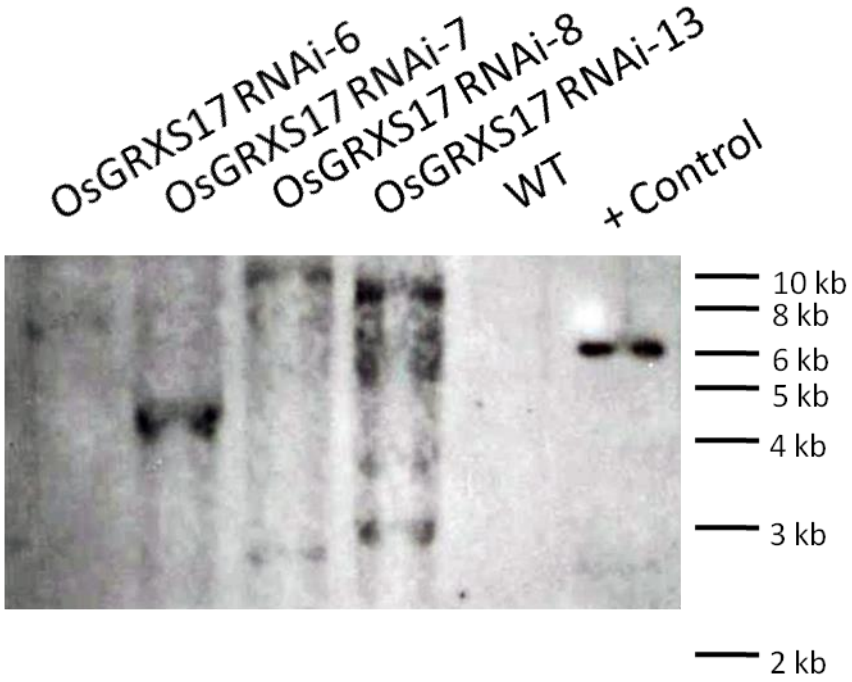
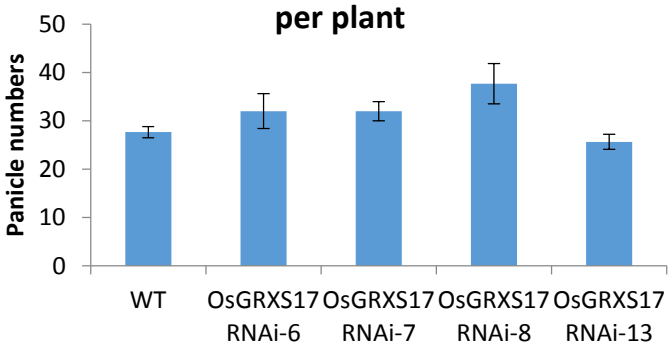
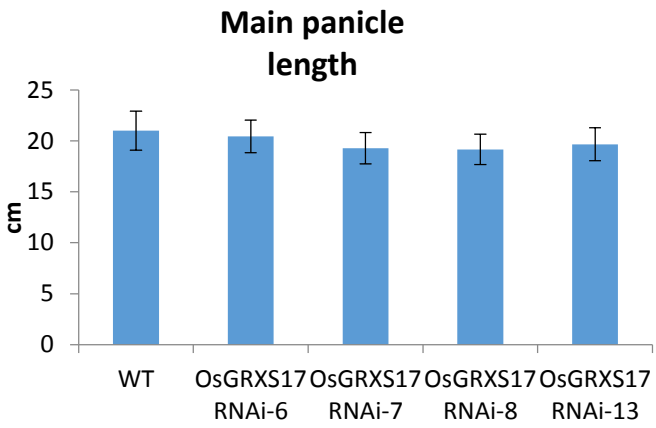


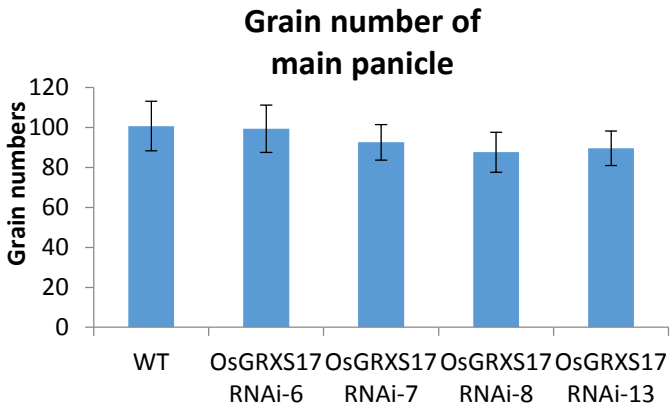
Figure 3. S. 1 Southern-blot analysis of T-DNA copy number of the *OsGRXS17* transgene.

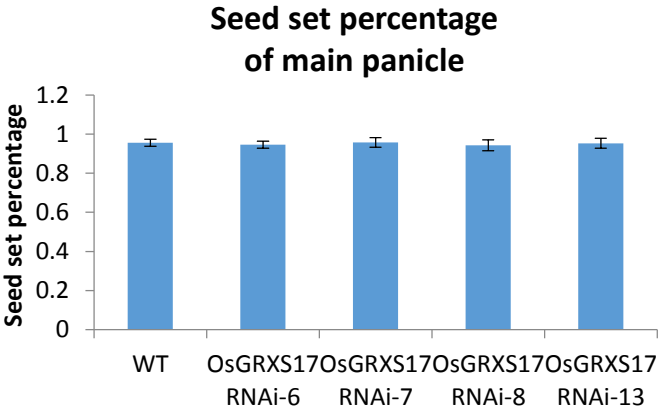
DNA probe of a hygromycin phosphotransferase gene was used for hybridization.

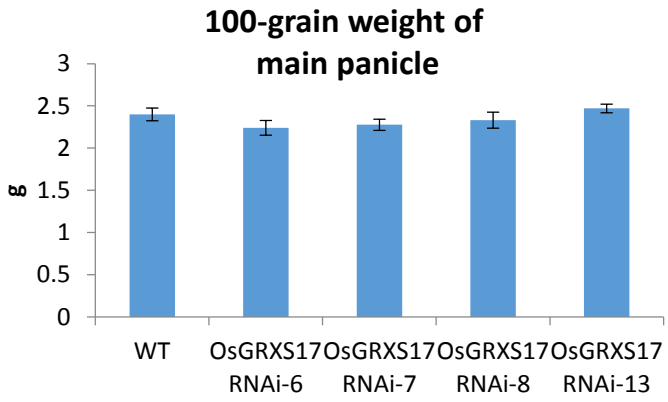
a

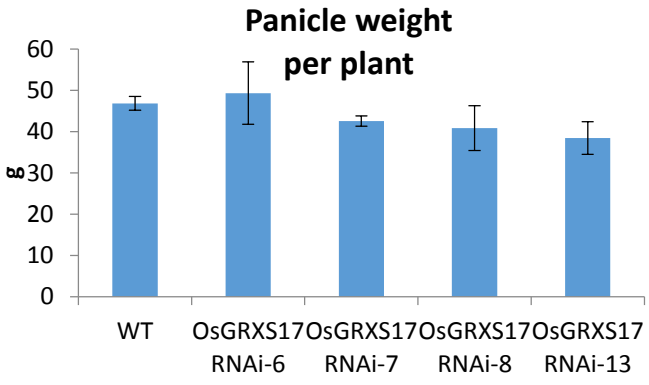

Panicle number per plant
Panicle numbers
50
40
30
20
10
0
WT
OsGRXS17 RNAi-6
OsGRXS17 RNAi-7
OsGRXS17 RNAi-8
OsGRXS17 RNAi-13


Main panicle length
cm
25
20
15
10
5
0
WT
OsGRXS17 RNAi-6
OsGRXS17 RNAi-7
OsGRXS17 RNAi-8
OsGRXS17 RNAi-13


Grain number of main panicle
Grain numbers
120
100
80
60
40
20
0
WT
OsGRXS17 RNAi-6
OsGRXS17 RNAi-7
OsGRXS17 RNAi-8
OsGRXS17 RNAi-13


Seed set percentage of main panicle
Seed set percentage
1.2
1
0.8
0.6
0.4
0.2
0
WT
OsGRXS17 RNAi-6
OsGRXS17 RNAi-7
OsGRXS17 RNAi-8
OsGRXS17 RNAi-13


100-grain weight of main panicle
g
3
2.5
2
1.5
1
0.5
0
WT
OsGRXS17 RNAi-6
OsGRXS17 RNAi-7
OsGRXS17 RNAi-8
OsGRXS17 RNAi-13


Panicle weight per plant
g
60
50
40
30
20
10
0
WT
OsGRXS17 RNAi-6
OsGRXS17 RNAi-7
OsGRXS17 RNAi-8
OsGRXS17 RNAi-13

b

Figure 3. S. 2 Comparison of agronomic traits between wild-type and *OsGRXS17*-silenced rice plants.

(a) Comparison of yield parameters between wild-type and *OsGRXS17*-silenced rice plants. (b) Total panicles collected from three plants per line.

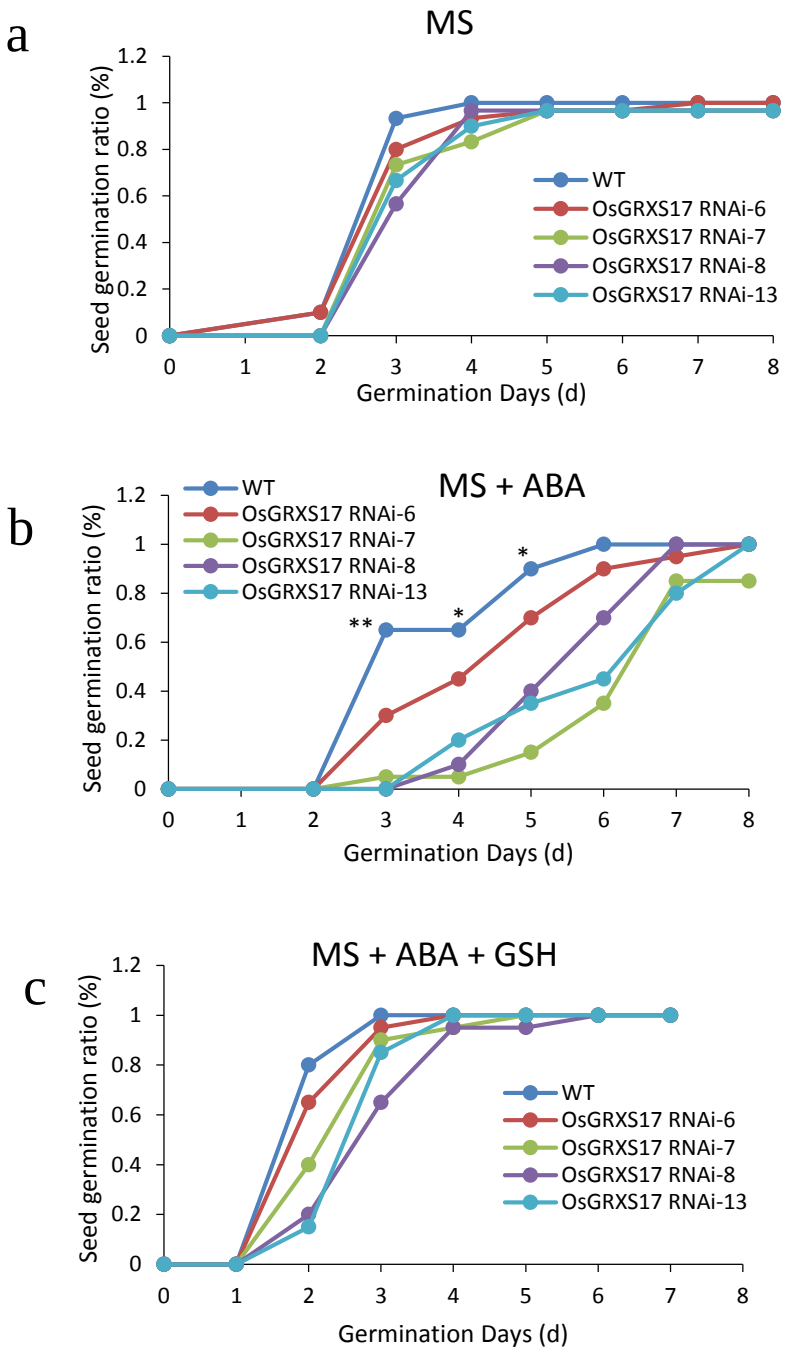


Figure 3. S. 3 Comparison of seed germination ratio between wild-type and *OsGRXS17*-silenced rice plants.

The seed germination ratio of wild-type and *OsGRXS17*-silenced rice plants on MS media (a), MS + ABA media (b) and MS + ABA + GSH media (c) in first 9 days of germination (n = 3 repeats, 30 plants for each repeat). Data were analyzed using student t test. Asterisks (*, **) represent statistically significant differences between wild-type and *OsGRXS17*-expressing lines (*$P < 0.05$, **$P < 0.01$).

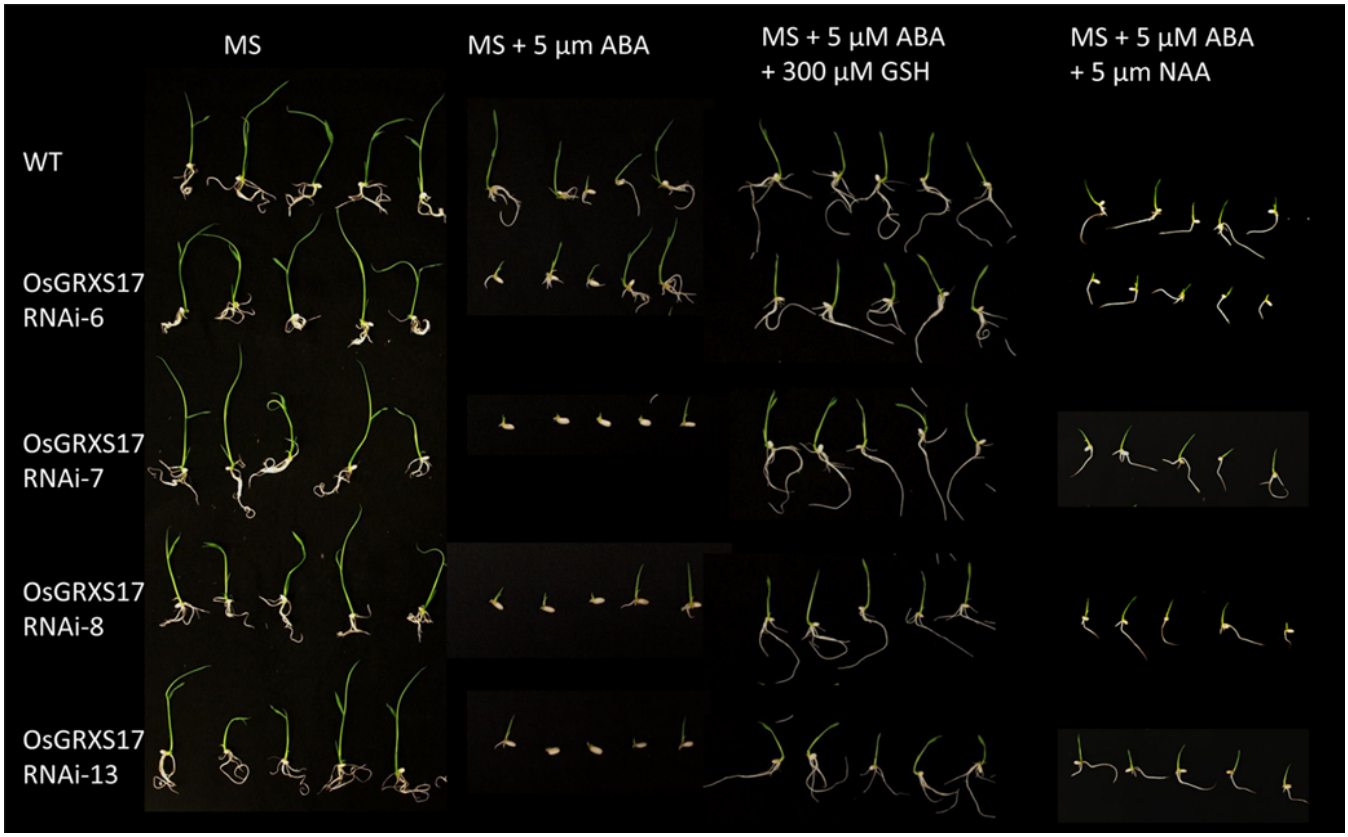


Figure 3. S. 4 *OsGRXS17*-silenced rice plants showed ABA hypersensitivity in seed germination and post-embryonic growth compared with wild-type that can be recovered by GSH but not NAA.

Nine-day-old rice seedlings germinated on MS, MS + 5 µM ABA, MS + 5 µM ABA + 300 µM GSH and MS + 5 µM ABA +5 nM NAA medium.

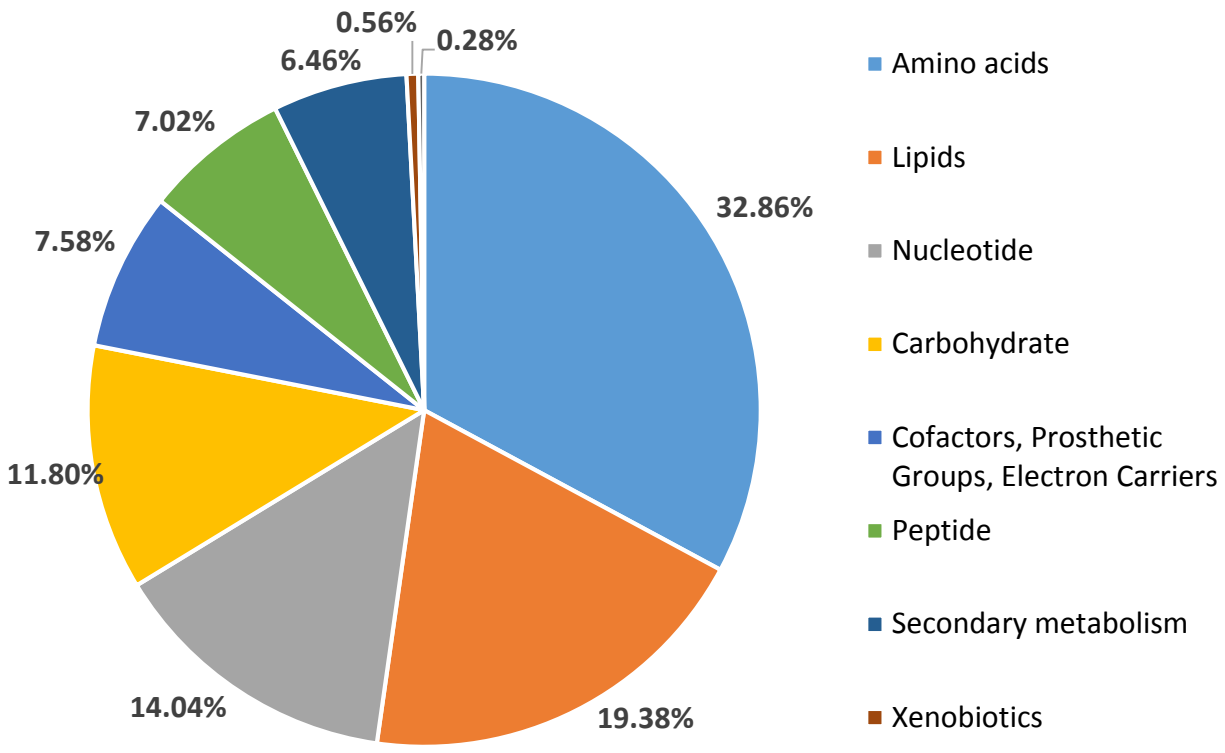


Figure 3. S. 5 Functional categorization of metabolites detected in wild-type and *OsGRXS17*-silenced rice plants at 0, 5 and 8 days of drought stress treatment.

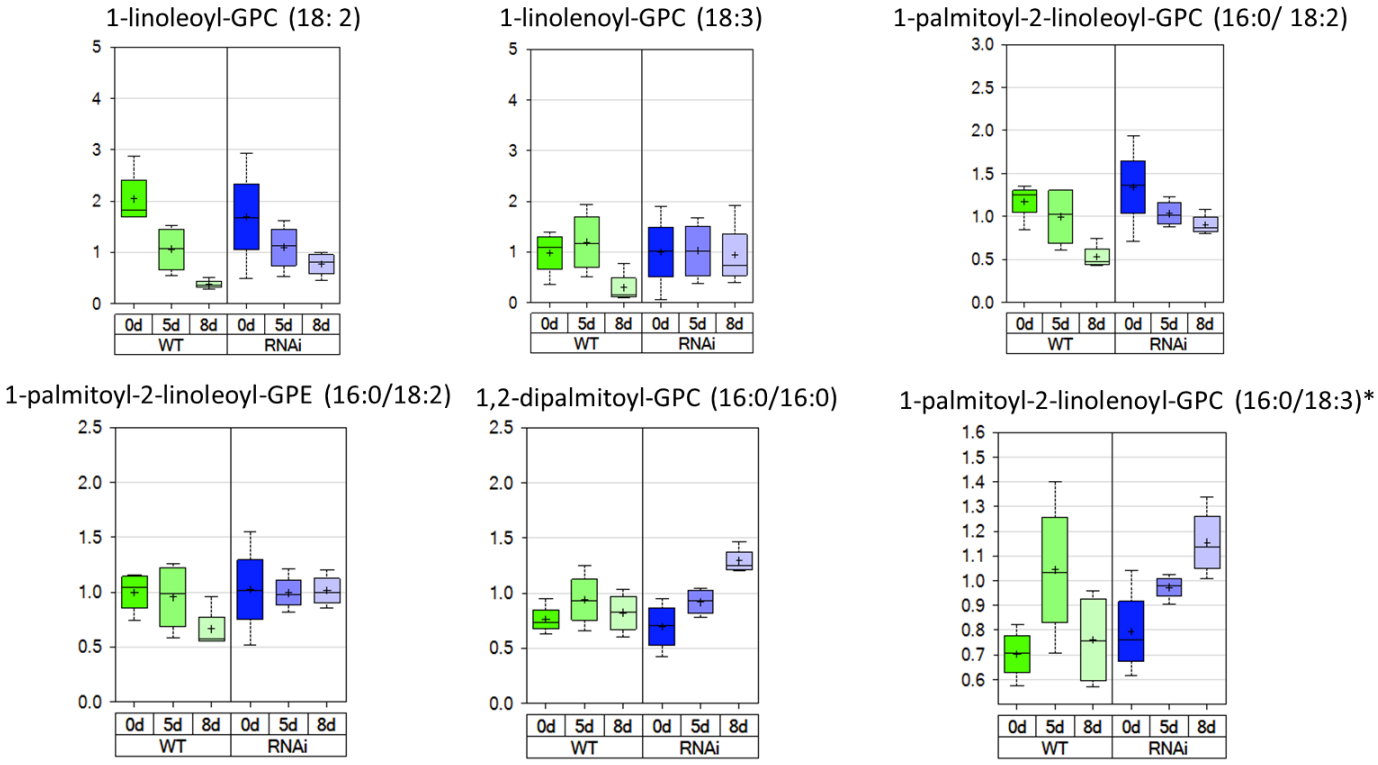


Figure 3. S. 6 Comparison of metabolite profiling between wild-type and *OsGRXS17*-silenced rice plants.

OsGRXS17-silenced rice plants had reduced abundance in phospholipids after 8 days of drought stress treatment. Error bars represent the means ± SD (n = 4).

Chapter 4 - Conclusion and Prospect

Plants are sessile organisms that have adapted dynamic responses to various abiotic stresses at physiological, biochemical, and molecular levels. Therefore, it is very important to understand the mechanisms of plant adaption under abiotic stresses and provide genetic engineering approach for improving abiotic stress tolerance in crops.

In this dissertation, I showed that *AtGRXS17*-expressing tomato plants are chilling tolerant compared with wild-type. First, I treated wild-type and *AtGRXS17*-expressing tomato plants in a walk-in growth chamber at 4 ˚C for three weeks and found that all of them showed grey-green wilting leaves and inhibited growth. After a two-week recovery under normal growth conditions, *AtGRXS17*-expressing tomato plants appeared more vigorous growth compared with wild-type. Then, I compared the membrane damage between wild-type and *AtGRXS17*-expressing tomato plants under chilling stress. My results indicated that *AtGRXS17*-expressing tomato plants had lower electrolyte leakage (indicative of reduced disruption of cell membrane) and higher Fv/Fm ratio (the maximum quantum efficiency of Photosystem II) compared with wild-type. Under chilling stress, many studies demonstrated that wilting of the aerial organs of plants are the results of excessive transpiration over slow supply of water by roots (McWilliam et al. 1982; Vernieri et al. 2001). Soluble sugar are considered as osmoprotectants to prevent cellular membrane damage caused by dehydration (Anchordoguy et al. 1987). I found that *AtGRXS17*-expressing lines showed higher sugar content as compared with wild-type tomato plants, exhibiting 2- to 3-fold elevated content after 5 days of chilling treatment. Accumulation of ROS is inevitable consequence of chilling stress and could result in oxidative damage to plant cells at phytotoxic level. We found that *AtGRXS17*-expressing tomato plants had lower H_2O_2 accumulation and higher activities of other antioxidant enzyme compared with wild-type under chilling stress. In addition, migration of

AtGRXS17 from cytoplasm to nucleus during chilling stress indicated that AtGRXS17 plays a possible role in nuclear signaling of chilling stress responses. Although the roles of GRXs in protecting of plants from abiotic stresses have been studied in many plant species, to our knowledge, this work first presents the role of GRXs in protection of plants from chilling stress and provides a helpful genetic engineering approach to improve the chilling tolerance in plants. Further research will focus on studying if the over-expression of AtGRXS17 can improve chilling stress in other crops, such as *Arabidopsis*, rice, maize and soybean. Considering the major concerns among the general public is the safety of genetically modified organism (GMO), cisgenic plants are more likely to be acceptable to the public than transgenic plants. In the future, we will focus on generating cisgenic plants which have the genetic materials from the plant own or other crossable plant species.

To further test the role of GRXs in protection of plants from abiotic stresses, we use RNA interference technology to silence the expression of a rice glutaredoxin gene, *OsGRXS17*, to test our hypothesis if silenced expression of *OsGRXS17* can confer drought susceptibility due to increased accumulation of ROS in rice. Interestingly, *OsGRXS17*-silenced rice plants showed less water loss rate, reduced stomatal conductance and higher relative water content under drought stress, indicating *OsGRXS17*-silenced rice plants are drought tolerant instead of drought sensitive compared with wild-type. Based on those physiological results, we need propose a new hypothesis to explain why *OsGRXS17*-silenced rice plants showed reduced water loss and maintain higher water content compared with wild-type under drought stress. ABA-mediated stomatal closure is a very important protection mechanism that plant adapt to drought stress conditions (Schroeder et al. 2001). Recently, many studies demonstrated that H_2O_2 acts as secondary messenger in ABA signaling to induce the stomatal closure (McAinsh et al. 1996; Pei et al. 2000a). My results

suggested that *OsGRXS17*-silenced rice plants had higher H_2O_2 content in the guard cells and showed ABA sensitivity in stomatal closure. In addition, *OsGRXS17*-silenced rice plants exhibited ABA hypersensitivity in seed germination and postembryonic growth compared with wild-type. Moreover, the transcripts of *LEA3* and *RAB16A*, two LEA (late embryonic abundance)-encoding genes, accumulated to higher level in *OsGRXS17*-silenced rice plants compared with wild-type under ABA treatment. Collectively, my results indicated that the silenced expression of *OsGRXS17* gave rise to H_2O_2 accumulation in the guard cells and promoted ABA-mediated stomatal closure, resulted in reduced water loss and higher water content, and finally enhanced drought tolerance. Considering transpiration is an important way to reduce the plant leaf temperature, it may be desirable to use a drought inducible promoter to drive silenced expression of *OsGRXS17* to minimize the side effect of decreased transpiration resulted from more closed stomata in *OsGRXS17*-silenced rice plants. Having established that silenced expression of *OsGRXS17* conferred improved drought tolerance in rice, the next step is to investigate if *OsGRXS17* orthologs in other plant species, such as wheat (*Triticum aestivum*) and maize (*Zea mays* L. ssp. *mays*), would function similarly and could be silenced to improve the drought tolerance.

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