Arabidopsis Root-Abundant Cytosolic Methionine Sulfoxide Reductase B Genes MsrB7 and MsrB8 are Involved in Tolerance to Oxidative Stress

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Excess reactive oxygen species (ROS) accumulation under various environmental stresses can damage intracellular polysaccharides, DNA, lipids and proteins. Methionine sulfoxide reductase (MSR) participates in a protein repair system that is one of the defensive mechanisms that diminishes oxidative destruction. In Arabidopsis, cytosolic MsrB7 and MsrB8 are oxidative stress-inducible protein repair enzymes that are abundant in the root. Here methyl viologen (MV) treatment was demonstrated to increase greatly the accumulation of H2O2 in MsrB7-knockdown, MsrB8-knockdown and wild-type Arabidopsis, but not in transgenic plants overexpressing MsrB7 or MsrB8. The reduction in H2O2 level under MV treatment in these overexpressing plants coincided with increased activity of glutathione S-transferase (GST), a herbicide-detoxifying enzyme. MsrB7 and MsrB8 are suggested to play an important role in defense against oxidative stress. Transgenic plants overexpressing MsrB7 or MsrB8 were viable and survived after MV and H2O2 treatment. Ectopic expression of specific cytosolic MsrB genes may be useful for application in crop improvement.

Keywords: Arabidopsis thaliana • Glutathione S-transferase (GST) • Methionine sulfoxide reductase B (MsrB) • Methyl viologen (MV).

Introduction

Global climate change is exposing organisms to more extreme environmental stresses. Environmental stresses including abiotic and biotic stresses provoke cellular redox imbalances. All living cells incur injury as a result of reactive oxygen species (ROS) such as hydroxyl radicals and superoxide ions generated as by-products of cellular metabolism in response to different environmental stresses and/or pathogen attacks (Gechev et al. 2006). To maintain redox balance and to protect against environmental stresses, most organisms, including microorganisms, plants and animals, have evolved a ROS-scavenging system to eliminate excess ROS, including non-enzymatic antioxidants, such as ascorbic acid, glutathione and carotenoids, and ROS-removing enzymes. In addition, surface-exposed sulfur-containing amino acid residues act as scavengers of a variety of oxidants (Levine et al. 1996). ROS can destroy the integrity of amino acids by chemically modifying their side chains. Both cysteine and methionine residues contain sulfur in their side chains and are especially susceptible to oxidation (Vogt 1995). The oxidation of methionine to methionine sulfoxide (MetO), which alters the activity and conformation of various proteins, can be reversed by methionine sulfoxide reductases (MSRs), which are present in most organisms including bacteria, yeast, mammals and plants (Vieira Dos Santos et al. 2005). MSRs repair oxidized proteins and protect against damage caused by oxidative stress (Moskovitz 2005, Cabreiro et al.
There are two types of MSR, MSRA and MSRB, that are specific for the S- and R-diastereoisomers of MetO, respectively. In mammals, only one Msra gene, peptide sulfoxide reductase (PMSR), is present in the genome and has been investigated extensively (Oien and Moskovitz 2008). Plants, in contrast, have more than one Msra gene. Extensive research has been devoted to the study of plant Msra genes, but little effort has been directed toward understanding the functional roles of cytosolic MSRBs in plants (Rouhier et al. 2006). There are nine Msrb genes (Msrb1–Msrb9) in the Arabidopsis genome. Among their translation products, Msrb1 and Msrb2 are predicted to target to the chloroplast; Msrb3 targets to the endoplasmic reticulum (ER) of pollen grains (Kwon et al. 2007), and the other six MSRBs are probably located in the cytosol (Rouhier et al. 2006). Microarray-based gene expression studies of Arabidopsis have revealed that Msrb1, Msrb2 and Msrb6 mRNA transcripts are relatively abundant in leaves, whereas Msrb5, Msrb7, Msrb8 and Msrb9 transcripts are prevalent in roots (Rouhier et al. 2006).

MSR enzymes function in protection against environmental stresses, diseases and aging. Very few proteins have been found to be the targets of MSR in plants. Tarrago et al. (2012b) utilized affinity chromatography to isolate potential substrates of plastidial AtMsrb1. The biological functions of the identified plastidial MSR substrates are involved in photosynthesis, translation, metabolism of sugar and amino acids, and protection against oxidative stress. These putative substrates are statistically proportional to methionine content and/or have surface-exposed methionine residues (Tarrago et al. 2012b). The most commonly identified substrates of plastidial MSR are heat shock protein 21 (Hsp21) (Gustavsson et al. 2002) and chloroplast signal recognition particles (cspSRPs) (Laujier et al. 2010). Presently, no substrate of cytosolic MSRB has been validated in plants.

In yeast, overexpression of yeast Msra led to increased resistance to toxic concentrations of H₂O₂ (Moskovitz et al. 1998). In addition, overexpression of either rice OsMsraA4.1 or OsMsrbB1.1 in yeast also enhanced cellular resistance to oxidative stress and exhibited increased viability to salt stress in OsmsraA4.1 transgenic rice (Guo et al. 2009). Methyl viologen (MV, paraquat) is one of the most widely used herbicides in agriculture. It rapidly enters leaves and then chloroplasts, where it disrupts PSI electron transport, reducing oxygen to ROS. When plant cells cannot remove excess ROS promptly, leaves become pale and necrotic. In Arabidopsis, plastidial PMSR4 (AtMsraA4) plays a role in protection from ROS induced by MV in chloroplasts (Romero et al. 2004). Arabidopsis plants that underexpress PMSR4 are more susceptible to MV-induced oxidative damage. Overexpression of ER-located AtMsrb3 in Arabidopsis enhanced tolerance not only to cold stress but also to MV; consistently, the msrb3 mutant showed much more susceptibility to stresses than the wild type (Kwon et al. 2007). In this study, we investigated whether cytosolic MSRBs play roles in response to oxidative stress. We investigated whether overexpression of particular cytosolic MSRBs can efficiently confer tolerance to oxidative stress induced by MV and H₂O₂, and whether they have potential for use in crop improvement.

**Results**

**Cytosolic Msrb7 and Msrb8 are relatively abundantly expressed in the root and can be induced by oxidative stress**

Among the nine Msrb genes in Arabidopsis, Msrb5, Msrb7, Msrb8 and Msrb9 are prevalently expressed at higher levels in roots (Rouhier et al. 2006). Msrb7 and Msrb8 genes are located close to each other on chromosome 4, and their amino acid sequences share high sequence identity (95.1%). Cauliflower mosaic virus 35S (CaMV 35S) promoter-driven green fluorescent protein (GFP)–Msrb7/8 fusion genes (GFP:Msrb8) as well as GFP were transiently expressed in Arabidopsis protoplasts. Subcellular localization studies indicated that Msrb7 and Msrb8 are localized in the cytoplasm (Fig. 1A). Real-time reverse transcription–PCR (RT–PCR) results indicated that Msrb7, Msrb8 and Msrb5 were abundantly expressed in roots, moderately expressed in the stalks and expressed at low levels in leaves and flowers (Fig. 1B, C, Supplementary Fig. S1A). However, Msrb6 was abundantly expressed in leaves, moderately expressed in roots and expressed at low levels in flowers (Supplementary Fig. S1B). In order to clarify the potential function of these cytosolic MSRBs in response to oxidative stress, the expression pattern of the Msrb genes in Arabidopsis seedlings was analyzed under time-course MV treatment (20 µM) using real-time PCR. Msrb7 and Msrb8 mRNA rapidly accumulated within 30 min, and peaked after 12 or 24 h under MV treatment, respectively (Fig. 1D, E). Expression of Msrb5 was also inducible by oxidative stress after MV treatment for 4 h which was later than that of Msrb7 and Msrb8 (Supplementary Fig. S1C), and peaked after 24 h. Nevertheless, expression of Msrb6 showed no significant difference within 8 h of MV treatment, and was further down-regulated after MV treatment for 12 h (Supplementary Fig. S1D). These results suggest that Msrb7, Msrb8 and Msrb5, but not Msrb6, are stress inducible and may play important roles in stress defense responses.

**Transgenic Arabidopsis plants overexpressing Msrb7/B8 have shorter roots**

No Msrb7 and Msrb8 knockout lines are available from the Arabidopsis Biological Resource Center (Columbus, OH, USA); therefore, to investigate the biological function of Msrb7/8 genes in stress response, we generated overexpression (B70x and B80x) and RNA interference (RNAi)-silenced knockdown expression (B7i and B8i) transgenic Arabidopsis lines (Fig. 2; Supplementary Fig. S2). Since Msrb7 and Msrb8 share extremely high sequence identity, we used their 5′ region [including the 5′-untranslated region (5′-UTR) and partial coding sequence] for RNAi (Supplementary Fig. S2C) and designed Msrb-specific primers for the 3′-UTR to distinguish the
expression of each gene by RT–PCR or real-time PCR (Supple-
mentary Table S1). The MsrB7 and B8 transgenes were
abundantly expressed in the overexpression lines (Fig. 2A, B).
Under normal growth conditions, some endogenous MsrB7
and MsrB8 transcripts were present in 10-day-old wild-type
seedlings, and could be detected by real-time PCR
(Fig. 2C). In contrast, expression of endogenous MsrB7
and B8 was reduced in the knockdown plants (Fig. 2C).

Fig. 1 Arabidopsis cytosolic MsrB7 and MsrB8 are abundantly expressed in the roots and were induced by oxidative stress. (A) MSRB7 and MSRB8 are localized to the cytoplasm. Arabidopsis protoplasts transiently expressing p35S-GFP::MsrB7, p35S-GFP::MsrB8 and p35S-GFP (control) were visualized using a confocal microscope. Scale bars, 10 µm. (B) Spatial expression of MsrB7 was determined by real-time PCR. (C) Spatial expression of MsrB8 in Arabidopsis. (D) Expression pattern of MsrB7 under MV treatment. (E) Expression pattern of MsrB8 under MV treatment. The relative expression was normalized to the PP2A or Actin2 gene.
Hygromycin-resistant homozygous T₃ lines, including overexpression and knockdown lines, then underwent the following treatments. Ten-day-old B7Ox, B8Ox, B7i, B8i and wild-type (WT) seedlings were grown in Murashige and Skoog (MS) agar medium, and the primary root lengths of these plants were measured (Fig. 2D, E). The growth phenotype indicated that MsrB7 knockdown lines had longer primary roots, while both MsrB7 and MsrB8 overexpression lines had shorter primary roots as compared with wild-type seedlings.

Cytosolic MsrB7 and MsrB8 knockdown lines are sensitive to oxidative stress, whereas overexpression lines exhibit tolerance

Ten-day-old B7Ox, B8Ox, B7i, B8i and wild-type seedlings were grown in the presence or absence of 20 mM H₂O₂ or 20 μM MV for 3 d (Fig. 3A, D). Subsequently, the Chl content and ion leakage levels were measured. The H₂O₂- or MV-treated RNAi lines and wild-type seedlings showed a severe bleaching phenotype with drastically reduced (>60%) Chl content, but the MsrB Ox plants showed only a minor alteration in phenotype (10–40% reduction) (Fig. 3B, E). Furthermore, the Chl content declined much more sharply in the B7i and B8i lines than in wild-type plants. We also examined membrane integrity by monitoring electrolyte leakage in H₂O₂- or MV-treated MsrB Ox plants. H₂O₂ or MV treatment caused severe ion leakage in wild-type plants (60%) and in RNAi lines (75%); in contrast, only mild to moderate ion leakage was detected (30–50%) in MsrB Ox plants (Fig. 3C, F). These results indicate that cytosolic MsrB overexpression in Arabidopsis protects cells from both MV- and H₂O₂-induced oxidative stresses.
MsrB7 and MsrB8 transgenic plants cultivated in soil exhibit resistance to MV spraying

Two lines each of B7Ox and B8Ox homozygous T3 seeds were directly sown in soil. MV (20 μM) was applied to 3-week-old seedlings by spraying directly onto the leaves. Two days after MV spraying, the wild-type plants showed severe wilting, while the transgenic plants remained relatively healthy (Fig. 4A).

Fresh weight and ion leakage were measured in both the wild-type plants and the transgenic lines. In agreement with the observed phenotype, the B7Ox and B8Ox transgenic plants showed significantly less reduction in fresh weight and ion leakage after MV treatment than the wild-type plants (Fig. 4B, C). These results indicate that MsrB Ox plants also conferred tolerance to MV under soil-based culture conditions.

Fig. 3 MsrB7- and MsrB8-overexpressing Arabidopsis seedlings confer tolerance to oxidative stress, while knockdown plants are more sensitive.
(A) Ten-day-old wild-type and homozygous MsrB7/B8-overexpressing (B7Ox and B8Ox, two lines each) and knockdown T3 seedlings were grown in half-strength MS agar media with or without (Control) 20 mM H2O2 for 3 d, and then the Chl content (B) and the ion leakage rate (%) (C) were measured. (D) Ten-day-old wild-type, B7/8Ox and B7/8i seedlings were grown in MS agar media with or without (control) 20 μM MV for 3 d, and the Chl content (E) and ion leakage ratio (F) were measured. Data are means ± SD (n = 6) of three independent experiments.
**Fig. 4** MsrB7- and MsrB8-overexpressing transgenic plants cultivated in soil displayed resistance to spraying with the herbicide paraquat, MV. (A) Three-week-old seedlings of B7Ox and B8Ox (two lines each) were sprayed with 20 μM MV, and a photograph was taken 2 d later. (B) The MV-sprayed seedlings were collected and the FW was measured. (C) The ion leakage rate (%) of MsrB-overexpressing and wild-type plants was measured 2 d after MV spraying. Data are means ± SD (n = 10) of three independent experiments. P-values ≤ 0.01 were considered significant (*).**

**Minimal inhibitory concentrations of MV for Arabidopsis**

To verify whether all of the cytosolic MsrB genes constitutively expressed in plants can confer tolerance to oxidative stress, we generated transgenic plants overexpressing cytosolic MsrB5, MsrB6, MsrB7, and MsrB9, with plastidial MsrB2 as a control. Seeds of the wild type, B5Ox, B6Ox, B7Ox and the MsrB5 and B6 double overexpression (B5B6Ox) transgenic plants were germinated on medium supplemented with various concentrations of MV (0, 100, 200 and 300 nM) for 10 d (Supplementary Fig. S3). Initially, the wild-type and transgenic plant seeds germinated well. However, after 10 d, wild-type seedlings ceased to grow on media containing 200 and 300 nM MV. The growth of B6Ox seedlings was inhibited by MV at ≥ 100 nM. The development of true leaves on B5Ox seeds was not affected by MV at < 300 nM. Transgenic B7Ox seedlings displayed the most robust growth at all concentrations (Supplementary Fig. S3A). The B5B6Ox plants grew well on medium containing 300 nM MV, but the growth of true leaves was slightly inhibited in comparison with the B7Ox phenotype. B7Ox tested at higher MV concentrations (400 and 500 nM) showed reasonably good growth (data not shown). Since B7Ox exhibited robust growth with greener leaves at 300 nM, we decided to use 300 nM for further experiments.

To confirm the germination and growth of other MsrB transgenic plants, including cytosolic MsrB genes (B5B6Ox, B7Ox, B8Ox, and B9Ox), with plastidial MsrB (B2Ox) as a control, their seeds along with those of wild-type plants were sown on media with or without MV (300 nM). B2Ox, B8Ox and B9Ox showed a germination pattern similar to that of B7Ox (Supplementary Fig. S3B). The growth of B5B6Ox was similar to or better than that of the wild type at 300 nM MV; however, B5B6Ox did not grow as well as B7Ox, B8Ox and B9Ox at 300 nM MV (Supplementary Fig. S3). In conclusion, transgenic plants overexpressing cytosolic MsrB7, MsrB8, MsrB9 and plastidial MsrB2 showed more tolerance to MV compared with MsrB5, MsrB6 overexpression lines and wild-type plants.

**MsrB8Ox plants accumulate less H₂O₂ and exhibit higher glutathione S-transferase activity under MV-induced oxidative stress**

In situ measurements by 3,3′-diaminobenzidine (DAB) staining showed that there was less H₂O₂ accumulation in B7Ox and B8Ox transgenic plants than in knockdown (B7i and B8i) and wild-type plants (Fig. 5A). To elucidate the possible molecular mechanism underlying the protection from oxidative stress conferred by expression of various MsrB genes, we tested the activity of various ROS-scavenging enzymes such as catalase (CAT), glutathione S-transferase (GST) and peroxidase (PX). The activity of each enzyme was measured in wild-type plants and in individual MsrB transgenic lines that had undergone treatment with MV for 1 d (Fig. 5). CAT, PX and GST activities were induced in all the MV-treated plants (Fig. 5B–D). In both the presence and absence of MV, the endogenous levels of CAT and PX were lower in MsrB8Ox than in wild-type plants (Fig. 5B, C), but higher in RNAi lines. GST activity displayed no significant difference in any of the treated plants under control conditions, whereas it was elevated in MsrB8Ox plants in comparison with RNAi and wild-type plants upon MV treatment (Fig. 5D). Taken together, CAT and PX activities were higher in the MsrB RNAi lines under oxidative stress, whereas GST activity remained higher in MsrB overexpression lines than in wild-type Arabidopsis.

**Transgenic tomato plants overexpressing AtMsrB7 show tolerance to oxidative stress**

Transgene integration in the putative transgenic tomato (variety MicroTom) plants was initially confirmed by PCR. Southern hybridization was used to confirm transgene integration in the putative transgenic plants (Fig. 6). Genomic DNA from three randomly picked MsrB7 transgenic tomato plants was digested with HindIII and probed with digoxigenin (DIG)-labeled Hpt (hygromycin phosphotransferase) (Fig. 6A). Multiple transgene insertions of Hpt were observed.

The transgenic tomato plants were further tested for transgene expression by RT–PCR using MsrB genes, Hpt and tomato Actin1 (SlAct1) primers, or by real-time PCR using MsrB7/F forward primer (MsrB7/8-qF2) and Nos terminator reverse primer (Nos3’R), and SlUBI3 primers (Supplementary Table S1). The
AtMsrB7 transgene (B7-nos) and Hpt were highly expressed in individually tested transgenic plants (Fig. 6B, C). A non-transformed wild-type sample that showed no transgene amplification signal was used as the control. The housekeeping gene SlAct1 or SlUBI3 served to normalize sample-to-sample variation in amounts of RNA.

The emerged, fully expanded leaves of 2-month-old B7Ox and wild-type tomato plants were detached and treated with 20 μM MV for 3 d (Fig. 6D). Subsequently, H$_2$O$_2$ accumulation and Chl content were measured. DAB staining in situ showed less H$_2$O$_2$ accumulation in three independent lines of B7Ox transgenic tomato plants than in wild-type plants (Fig. 6E). In addition, the Chl content remained higher in MV-treated B7Ox lines than in MV-treated wild-type leaflets (Fig. 6F). These results indicate that overexpression of MsrB7 can protect tomato plants from MV-induced oxidative stress by reducing H$_2$O$_2$ accumulation.

**AtMsrB7-overexpressing tomato plants show normal phenotype**

To verify whether AtMsrB7 overexpression had a negative influence on growth, development and yield, we also generated AtMsrB7-overexpressing CL5915 transgenic tomato (Supplementary Fig. S4), and calculated the fruit number (FN) per plant, seed number (SN) per fruit and fresh weight (FW) of the T1 progeny of transgenic and wild-type tomato plants (Table 1). Transgenic plants exhibited normal phenotype and no yield drag compared with wild-type plants.

**Discussion**

**Overexpression of cytosolic MsrB genes confers tolerance to oxidative stress**

To deal with oxidative stress, plants have evolved a ubiquitous protein repair system that employs MSRA and MSRB to reduce MetO and restore methionine. MsrA and MsrB are thus thought of as part of a plant surveillance system against oxidative stress (Moskovitz 2005). In this study, we examined the effects of cytosolic MsrB genes on oxidative stress using gain-of-function and loss-of-function strategies. Suppression of two MsrB genes, MsrB7 and MsrB8, rendered plants more sensitive to oxidative stress, while overexpression of these two MsrB genes enhanced tolerance to oxidative stress (Figs. 3, 4, 6).
However, not all the cytosolic MsrB genes that are constitutively expressed in Arabidopsis plants conferred tolerance to oxidative stress (Supplementary Fig. S3). Although MsrB5, which like MsrB7 and MsrB8 is also most highly expressed in roots, was also inducible by oxidative stress, transgenic plants overexpressing MsrB5 showed little tolerance to MV treatment (Supplementary Fig. S3A). The cytosolic MsrB genes are highly expressed in roots without stress; however, our present results suggest that when cytosolic MsrB genes are ectopically overexpressed in the whole seedling mimicking their induction by oxidative stress, they may confer further enhanced ROS destruction (Fig. 1; Supplementary Fig. S1). In contrast, the leaf-abundant MsrB6 was down-regulated by oxidative stress, and ectopic expression of MsrB6 in the whole seedling did not enhance tolerance to MV (Supplementary Fig. S3A). These results suggest that although the MSRBs share similar protein structure and the same enzyme properties at the molecular level, their enzyme specific activity and stability, and substrate/target specificity may not be totally identical. Furthermore, the spatial partition and distinctive stress responses may result in different physiological roles for MSRBs. However, more experimental evidence is needed to prove these assumptions.

Fig. 6 Characterization of AtMsrB7-overexpressing Microtom tomato plants. (A) Southern blot to confirm AtMsrB7 transgene integration in B7Ox5, B7Ox8, B7Ox9 and the wild type (WT). A DIG-labeled DNA fragment of the partial Hpt cDNA was used as probe. (B) RT–PCR was performed to determine the expression levels of MsrB7 and Hpt in the transgenic plants. Tomato Actin1 (SlAct1) served as a housekeeping gene. (C) The relative expression level of the MsrB7 transgene in transgenic (B7Ox) and WT tomato plants was determined by real-time PCR. The relative expression was normalized to the SlUBI3 gene. (D) MV-induced oxidative stress tolerance test. Transgenic and wild-type leaves were treated with 20 μM MV and photographed 3 d later. (E) DAB staining of MV-treated and untreated control leaflets of WT and B7Ox lines. (F) Chl contents of MV-treated and untreated control leaflets of WT and B7Ox lines. Data are means ± SD (n = 6) of three independent experiments. P-values ≤ 0.01 were considered significant (*).
MSRs play important roles in redox homeostasis

MSRs are known to participate in tolerance to oxidative damage; however, little is known about the role of MSRs during growth and development in plants. It is known that ROS are important secondary messengers in plant hormone signaling cascades that manipulate plant growth and development. Foreman et al. (2003) found that NADPH oxidase mutants (rd2) produced less ROS and had stunted roots in Arabidopsis. They provided evidence that ROS are required for root elongation by regulating plant cell expansion through the activation of Ca^{2+} channels (Foreman et al. 2003). We suggest that the shorter primary roots in MsrB7-overexpressing lines and the longer primary roots in MsrB7 knockout plants (Fig. 2D, E) may be correlated to lower and higher levels of H_{2}O_{2}, respectively (Fig. 5A). Our observations suggest that MSRB enzymes play important roles in redox homeostasis.

GST activity is enhanced and ROS accumulation is reduced in transgenic MsrB plants

In Arabidopsis, mutation in ER-localized MsrB3 (msrb3) leads to the loss of chilling tolerance following cold acclimation. The detached leaves of msrb3 plants were more sensitive than those of wild-type or 35S::MsrB3 plants to MV-induced oxidative stress. When exposed to low temperature, msrb3 plants exhibited a larger increase in H_{2}O_{2} content and cell damage (Kwon et al. 2007). Here, we revealed that knockdown of cytosolic MsrB7 and MsrB8 increased H_{2}O_{2} content and reduced tolerance to oxidative stresses (Figs. 3, 5). These results imply that MsrB7 and MsrB8 play significant roles in the plant response to oxidative stress by eliminating ROS.

The H_{2}O_{2} level is elevated under various conditions of stress. Excess H_{2}O_{2} may cross the phospholipid bilayer via water channels (aquaporins) and then target different organelles (Henzler and Steudle 2000). ROS may be important in signaling to protect cells against various environmental stresses. However, excess ROS are toxic to cells. Plants have evolved several strategies to remove excess ROS, including non-enzymatic antioxidants and various ROS-scavenging enzymes. Non-enzymatic antioxidants such as ascorbate, glutathione and thioredoxin that participate in ROS removal can also pass through transporters into different parts of the cells to remove ROS (Mittler et al. 2004, Moskovitz 2005). In addition, it has been hypothesized that methionine residues exposed on the surfaces of folded proteins serve as endogenous antioxidants (Levine et al. 1996). Methionine residues can scavenge excess ROS and provide protection for intercellular components, the oxidation of which would lead to irreversible consequences. MSRs are now known to interact with some methionine-rich proteins in animals, and some of their endogenous substrates have recently been identified (Alamuri and Maier 2006, Oien and Moskovitz 2008). However, only a few putative endogenous MSR substrates have been identified in plants, one of which is Hsp21. The N-terminus of Hsp21 is rich in methionine residues and has been verified as a substrate of MSRA (PMSR). PMSR can reduce methionine sulfoxidation in Hsp21 and protect the chaperone-like activity of the reduced form of this protein (Gustavsson et al. 2002). Furthermore, oxidized cpSRPs are substrates for the two plastidial MSRBs in Arabidopsis. Plastidial MSRBs play a role in preserving photosynthetic antennae by repairing oxidized cpSRP to maintain vegetative growth during environmental constraints (Laugier et al. 2010). Tarrago et al. (2012b) identified some potential substrates of plastidial MSRB1 in Arabidopsis, such as some proteins that play roles in protection against oxidative stress (i.e. heat shock cognate 70 kDa protein 3, CAT2 and CAT3), by using an affinity chromatography strategy. However, the detailed interaction properties of these putative substrates or targets and MSRB1 remain to be further investigated. The substrates of plant cytosolic MSRBs have yet to be identified. Tarrago et al. (2012a) used oxidized Schistosoma japonicum GST recombinant protein as a model substrate for yeast MSRA and MSRB in vitro assay and discovered that both MSR types were more efficient in reducing MetO in unfolded than in folded proteins. In the present report, the results of the GST activity assay revealed that constitutive expression of Arabidopsis MsrB7/MsrB8 raised GST activity under oxidative stress (Fig. 5D). From these findings, we suggest that oxidized GST may be the target of cytosolic MSRB under oxidative stress in plants.

Because MsrB7/8-overexpressing plants accumulated less H_{2}O_{2} (Fig. 5), we speculate that either MV is rapidly removed or decomposed before it enters the chloroplasts, or MV-induced ROS are scavenged efficiently in MsrB7-overexpressing plants. GST activity is elevated in MsrB-overexpressing plants (Fig. 5D). It has been reported that GST plays a role in catalyzing the conjugation and detoxification of herbicides (Dixon et al. 2010). We propose that cytosolic GST may conjugate or detoxify MV and prevent it from moving to the chloroplasts. ROS-scavenging enzymes may also participate in removing

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**Table 1** Growth characterization of wild-type and transgenic tomato plants

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<thead>
<tr>
<th>Characteristics</th>
<th>Wild type</th>
<th>14</th>
<th>18</th>
<th>22</th>
<th>24</th>
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<tr>
<td>FN</td>
<td>25.6 ± 4.5</td>
<td>26.0 ± 4.1</td>
<td>23.8 ± 3.2</td>
<td>24.5 ± 3.0</td>
<td>26.0 ± 5.0</td>
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<td>SN</td>
<td>48.7 ± 3.5</td>
<td>47.7 ± 3.2</td>
<td>48.4 ± 3.0</td>
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<tr>
<td>FW</td>
<td>132.4 ± 7.1</td>
<td>140.4 ± 7.1</td>
<td>133.4 ± 7.8</td>
<td>138.8 ± 6.6</td>
<td>130.6 ± 5.7</td>
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Data shown in each column, from top to bottom, are fruit number (FN) per plant, seed number (SN) per fruit, and FW (g) per plant. WT, wild type tomato CL5915 cultivar; 14, 18, 22 and 24, AtMsrB7 overexpression lines.

Each value is the mean ± SD (n = 5 individual plants). The measured plants were 3 months old.
excess ROS. MV-induced CAT and PX activities were higher in wild-type and Msrb7/8-silenced plants than in Msrb7/8-overexpressing plants (Fig. 5). It is possible that GST removes more MV in Msrb7/8OX than in wild-type plants, causing less ROS accumulation and further down-regulating the accumulation of ROS-scavenging enzymes. Functional studies of Capsicum annuum plastidial Msrb2 (CaMsrb2) using gain- and loss-of-function strategies indicate that CaMsrb2-silenced pepper plants showed increased production of ROS, while CaMsrb2-transgenic tomato plants showed reduced production of H2O2 and enhanced tolerance to oxidative stress and pathogen attacks (Oh et al. 2010). Plastidial Msrb8-overexpressing plants may also increase the activity of plastidial GST to enhance tolerance to oxidative stress. However, more experimental evidence is needed to support this hypothesis. In addition, the tolerance may be related to MV uptake, and the absorption of MV may coincide with the development of the root system. We cannot exclude the possibility that the shorter primary roots of Arabidopsis seedlings overexpressing Msrb8 genes may reduce MV absorption and thus result in more tolerance to MV than wild-type and RNAi seedlings (Fig. 2D).

In conclusion, ectopic expression of Msrb genes has the potential to protect plants from oxidative stress, and has potential for application to economically important crop species without changing yields.

Materials and Methods

Vector construction
Six MSR genes, Msrb2 (At4g21860), Msrb5 (At4g04830), Msrb6 (At4g04840), Msrb7 (At4g21830), MsrbB8 (At4g21840) and Msrb9 (At4g21850), were isolated by RT–PCR before being transplanted into pots and grown in a greenhouse to obtain self-pollinated T1 progeny seeds. Plant

Transformation and regeneration of tomato
Seeds of tomato (Solanum lycopersicum L.) varieties Microtom and CL5915-93D-1-0-3 (CL5915), kindly provided by AVRDC, The World Vegetable Center, were soaked at 32°C for 16 h in the light, protoplasts were observed with a Zeiss LSM510 META laser scanning confocal microscope. Protoplasts were isolated using the Tape–Arabidopsis sandwich method and transformed using the polyethylene glycol (PEG) method (Wu et al. 2009). After incubation at room temperature for 16 h in the light, protoplasts were observed with a Zeiss LSCM META laser scanning confocal microscope.
growth conditions were 26°C with a diurnal cycle of 16 h light/8 h darkness and a light intensity of 120 μmol photons m⁻² s⁻¹ as described (Li et al. 2011).

Molecular biology

To identify positive transformants, genomic DNA was extracted from all seedlings grown on MS medium with 20 mg l⁻¹ hygromycin. The genomic DNA was digested with BamHI and probed with the Hpt gene. The Hpt cDNA fragment isolated from pCAMBIA1390 was labeled with DIG-11-dUTP (Roche) by PCR (primers shown in Supplementary Table S1) following the manufacturer’s instructions (Roche).

Total RNA was isolated from plant tissues using Trizol reagent according to the manufacturer’s instructions (Invitrogen) and then used for Northern blotting or semi-quantitative RT–PCR. For RT–PCR, the cDNA was synthesized using a first-strand cDNA synthesis kit (Promega). All primers of MsrB genes were designed to distinguish each one in the MsrB family, as shown in Supplementary Table S1. Endogenous/transgenic MsrB7, MsrB8, MsrB9 and Hpt expression in the MsrB7–9 transgenic lines were analyzed by RT–PCR followed by agarose gel electrophoresis. PCR was performed in a reaction mixture containing cDNA (from 5 ng of total RNA as described above), 0.125 mM dNTPs, 0.25 mM of each forward and reverse primer (Supplementary Table S1), 2 μl of 10× NEB Taq buffer, and 2 U of Taq DNA polymerase (NEB) in a total volume of 20 μl. The mixture was incubated at 94°C for 3 min, followed by 25 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 30 s. The Actin2 expression level was used as a quantitative control.

Real-time PCR amplification was performed using SYBR Green Master Mix [Applied Biosystems (ABI)]. Amplification was monitored in real-time utilizing the ABI 7500HT sequence detection system. Data were analyzed using ABI SDS 1.4 software. Relative transcript levels were normalized to the reference gene Actin2 or protein phosphatase 2A (PP2A) for Arabidopsis (Czechowski et al. 2005), and SlUBI3 for tomato using the comparative CT method.

Hydrogen peroxide staining

Accumulation of H₂O₂ was detected by staining Arabidopsis seedlings with DAB (Sigma-Aldrich) as described previously (Chiang et al. 2006). Whole plants from 10-day-old wild-type and transgenic Arabidopsis grown in the medium were subjected to 20 μM MV for 3 d before being immersed in 1 mg ml⁻¹ DAB in 15 ml test tubes overnight under dim light. The stained seedlings were briefly rinsed twice in deionized water and then soaked in 95% ethanol. The tubes were incubated in a boiling water bath until the Chl was completely removed from the leaves. The stained seedlings were then stored in 95% ethanol until photographs were taken.

Determination of Chl content

Leaf Chl was extracted from fresh plant material with N,N-dimethylformamide and the content was determined using a spectrophotometer as described (Li et al. 2011).

Ion leakage rate

The ion leakage percentage of wild-type and MsrB transgenic plants treated with MV or H₂O₂ was measured as previously described (Hsieh et al. 2002).

Catalase activity

At the end of 24 h treatment with 20 μM MV, 100 mg of tissue was harvested from 10-day-old wild-type and MsrB transgenic Arabidopsis and homogenized in 0.5 ml of chilled potassium phosphate buffer (50 mM, pH 7.4, with 1 mM EDTA). After centrifugation at 10,000×g for 15 min at 4°C, the soluble protein fractions were quantified by the Bradford method (Bradford 1976). CAT activity was determined and defined according to the manufacturer’s manual (Cayman Chemical Company).

Peroxidase activity

For PX activity, each assay mixture (1 ml) consisted of 33.3 μl of soluble protein extract, potassium phosphate buffer (16.7 mM, pH 5.8), 7.2 mM guaiacol and 11.7 mM H₂O₂. Absorbance was measured once per minute at 470 nm with a spectrophotometer. The PX activity (U g⁻¹) was calculated using the equation: ΔA₄₇₀ / dilution factor / Δt (min) / FW (g). The unit was defined as 1 μmol tetraguaiacol produced per minute (Macadam et al. 1992).

GST activity

Ten-day-old wild-type and AtMsrB transgenic Arabidopsis were treated with 20 μM MV for 24 h. Plant tissue (300 mg) was homogenized in 0.6 ml of chilled 0.1 M phosphate-buffered saline (pH 6.5) containing protease inhibitor cocktail (Sigma P9599). The GST activity was measured by 1-chloro-2,4-dinitrobenzene (CDNB) assay as described previously (Habig et al. 1974).

Statistical analysis

Data are presented as mean values ± SD. Differences were evaluated for significance by Duncan’s multiple range test or Student’s t-test, with statistical significance defined as P ≤ 0.01 or P ≤ 0.05.

Supplementary data

Supplementary data are available at PCP online.

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References


