Quinoa (Chenopodium quinoa) Methionine Sulfoxide Reductase MSRA5.1 Interacts with Glutathione Synthase 2 to Improve Osmotic Stress Resistance in Arabidopsis Thaliana

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Abstract  Reactive oxygen species (ROS) produced by abiotic stress in plant cells can lead to the oxidation of methionine (Met). Met sulfoxide reductases (MSRs) reduce oxidized Met and protect plants from oxidative damage. However, the function of the MSR gene family in quinoa remains unknown. In the present study, a set of eight full-length Chenopodium quinoa MSR (CqMSR) complementary DNAs was isolated. And their responses to salt, drought, heat and cold stress were analyzed. To the best of our knowledge, no information on function and substrates of CqMSRA5.1 has been reported in quinoa. In the present study, its constitutive overexpression in Arabidopsis (AtOE) resulted in notable increase in MSR activity, and enhanced the tolerance of seedlings to osmotic stress. The effect of active CqMSRA5.1 in Arabidopsis decreased ROS accumulation. Quinoa glutamine synthetase 2 (GSH2) a potential interaction substrate of CqMSRA5.1, was obtained by screening STRING database, further confirmed by means of yeast two-hybrid, and bimolecular fluorescence complementation assay. Under osmotic stress, the expression of AtGSH2 and the total content of GSH in AtOE lines increased significantly, and the ratio of GSH/GSSG decreased significantly compared to wild type. BSO, one inhibitor of GST, can partially impair the phenotype of the CqMSRA5.1-induced response to osmotic stress. Together, CqMSRA5.1 participates in osmotic response partially via CqGSH2.

Keywords: Chenopodium quinoa, Methionine sulfoxide reductase, Abiotic stress, Reactive oxygen species, CqMSRA5.1

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1. Introduction

The introduction should briefly place the study in a broad context and highlight why it is important. It should define the purpose of the work and its significance. The current state of the research field should be carefully reviewed and key publications cited. Please highlight controversial and diverging hypotheses when necessary. Finally, briefly mention the main aim of the work and highlight the principal conclusions. As far as possible, please keep the introduction comprehensible to scientists outside your particular field of research. References should be numbered in order of appearance and indicated by a numeral or numerals in square brackets—e.g., [1] or [2,3], or [4,5,6]. See the end of the document for further details on references.

Drought stress, which causes a decline in quality and quantity of crop yields, has become more accentuated these days due to climatic change. It causes many physiological and biochemical changes in plants [1]. Reactive oxygen species (ROS) are produced in plant cells challenged by biotic and abiotic stresses and, if allowed to accumulate excessively, can induce oxidative damage of cells [2]. Oxidative stress, which damages cellular components such as membrane lipids, proteins, and nucleic acids, results in metabolic dysfunction [3]. Methionine (Met), including the free and protein-bound forms, can be easily oxidized in the presence of excess ROS under abiotic stress conditions to form Met sulfoxide (MetO) [4]. Plants possess repair enzymes, known as Met
sulfoxide reductases (MSRs), to reduce MetO to Met [5]. Based on the pair of MetO diastereomers, MSRs have been classified as MSRAs, which show specificity toward Met-S-O, and MSRBs, which interact with Met-R-O [6].

Many MSR genes have been characterized and cloned in various species [6-14]. Plant MSRs are expressed in the leaves, stem, root, and seed, and can be found in the cytosol and chloroplast, or can be secreted from cells [6,10,14]. Much research has been performed to determine the physiological context of plant MSRs in mutant and transgenic plants in response to diverse external stimuli, including pathogen attack [15], high light intensity [16], cadmium stress [17], oxidative stress [10,18], cold stress [19], salt stress [20,12,14], and osmotic stress [8,14]. All MSR proteins are good antioxidants candidates because cyclic oxidation and reduction of Met residues can serve as an efficient pathway to scavenge ROS in cells [6,21]. Unfortunately, most research on plant MSR function was implemented as an efficient pathway to scavenge ROS in cells [6].

Identity of the substrates of plant MSRs will allow to gain further insight into physiological roles of these thiol-dependent reductases. Several specific substrates to different plant MSRs were confirmed. Heat-shock protein 21 (HSP21) was the first specific substrate of plastidial MSR identified in plants [22]. AtMSRB7 interacts with glutathione transferases [10]. Lychee (Litchi chinensis) enzymes LcMSRA1 and LcMSRB1 use CaM as their substrate [23]. Wheat enzyme TaMSRA4.1 can reduce oxidative TaH01 [14]. TaGSTF3 was the substrate of wheat enzyme TaMSRB5.2 [24]. However, no substrate of the MSRA5 has been identified to date much less in quinoa.

Quinoa (Chenopodium quinoa) Willd. 2n = 4x = 36 was a nutritious crop adapted to thrive in a range of agroecosystems. The plant was presumably first domesticated more than 7,000 years ago by pre-Columbian cultures, and its seed was known as the ‘mother grain’ of the Inca Empire that arose in the 13th century [25]. Because quinoa has high genetic diversity and can adapt to harsh conditions (e.g., the highlands of the Andes), it can be grown on marginal soils and is resilient to frost, drought, salinity, and large day-night temperature variations [26,27,28]. Quinoa produces nutritious and gluten-free grains with a fine balance between carbohydrates, essential amino acids, oils, minerals, vitamins, and dietary fibers [29,30]. The draft of the C. quinoa genome [25] has provided insights into the mechanisms underlying agronomically important traits of quinoa and has laid the foundation for accelerating the genetic improvement of other crops [31].

The objectives of this study were to isolate a set of quinoa MSR genes and to characterize the expression profile of the gene family under normal conditions and in response to salt, drought, heat, and cold stresses. We further identified CqGSH2 as a substrate of CqMSRA5.1, investigated CqMSRA5.1 overexpression in plants, the co-expression and co-localisation of the two proteins, and demonstrated their protective roles in ROS accumulation under osmotic stress. This study thus elucidates the possible roles of MSR genes in plant abiotic stress response.

2. Materials and Methods

2.1. Plant Materials, Abiotic Stress Treatments, and Gene Expression Analyses

Quinoa plants were grown in the greenhouse. Seedlings were established under controlled conditions of 60-70% relative humidity, 14-h light, and an average temperature of 23°C until 2 weeks. They were then irrigated with 300 mM NaCl, 25% (w/v) polyethylene glycol (PEG 6000, Sigma-Aldrich, St. Louis, MO, USA) in soil homogeneously, or treated at 4°C and 40°C. Whole plant was randomly collected from five individuals at six time points (0, 3, 6, 12, 24, and 48 h) during the treatments. Three replicates were performed from different batches of treatment. Total RNA was isolated from collected samples using TRIzol Up (TransGen Biotech Co., Ltd., Beijing, China) following the manufacturer’s instructions. RNA concentrations were measured using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE, USA). For each sample, 1 µg DNaseI-treated RNA was reverse-transcribed using TransScript First-Strand Complementary DNA (cDNA) Synthesis SuperMix (Transgen). Quantitative polymerase chain reaction (qPCR) was performed in a 20-µL reaction mixture using the TransScript Tip Green qPCR SuperMix Kit (Transgen) following the manufacturer’s instructions. The quinoa elongation factor 1a [32,33] and the Arabidopsis AtActin (At3g18780) gene were amplified in parallel as positive controls and reference genes, with three independent replicates per experiment.

2.1.1. Cloning and Sequence Analysis

A BLAST search for MSR gene sequences was performed on both Phytozome v13 (https://phytozome-next.jgi.doe.gov/) and NCBI (blast.ncbi.nlm.nih.gov/) databases using the terms “Oryza sativa MSR complementary DNA (cDNA) sequences” and “A. thaliana MSR complementary DNA (cDNA) sequences”. CqMSR-specific primers were designed with Primer Premier 5.0 software based on their cDNA and genomic DNA sequences. The amplicons were cloned using the primers and inserted into the pEASY-T1 Simple Cloning Vector (TransGen) for sequencing by Sangon Biotech Co., Ltd. (Shanghai, China). Clustal X2 software [34] was used to align predicted amino acid sequences. Gene locations were inferred by a BLAST search of the quinoa sequence survey database (https://phytozome-next.jgi.doe.gov/info/Cquinoa_v1.0) with full-length cDNAs cloned in this paper. The protein isoelectric point (pI) and size were calculated using the Compute pI/Mw tool (web.expasy.org/), and protein subcellular localization was predicted using Target P software (www.cbs.dtu.dk/services/SignalP/). Sequence phylogeny was inferred using the neighbor-joining method with 1000 bootstrap replicates implemented in MEGA 6.0 software [35]. GenBank accession numbers are provided in Supplementary material. The PlantCARE database (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) was used to find cis-elements in the CqMSR upstream sequence. CqMSRA5.1 and CqGSH2 protein
structures were predicted using SWISS-MODEL (https://swissmodel.expasy.org/). HADDOCK 2.4 (https://wenmr.science.uu.nl/haddock2.4/) was used to the modeling of CqMSRA5.1-CqGSH2 complexes.

2.1.2. Subcellular Localization of CqMSRA5.1

The coding region of CqMSRA5.1 was amplified by the primer pairs CqMSRA5.1L-F/R, and introduced into the same digested sites of the p35S::GFP plasmid. This p35S::CqMSRA5.1-GFP transgene construct was introduced into tobacco cells. After overnight incubation in the dark and then in the light for 2 days, green fluorescence and chlorophyll autofluorescence were detected by confocal microscopy using excitation wavelengths of 488 nm and 647 nm, respectively.

2.1.3. Determination of Enzyme Activity

The recombinant vector was transformed into E. coli BL21 as described by Chen et al. [36]. The protein was purified from sonicated cells using a Ni2+-affinity column and assessed by SDS-PAGE. Total protein and enzyme activity of 10-day-old Arabidopsis seedlings were evaluated according to Sun et al. [12] and Ding et al. [14]. To distinguish between diastereomers, 10 μg purified CqMSRA5.1 recombinant protein was used. The activity of the ROS scavenging enzymes superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT) in leaves was quantified using kits (Beyotime Biotechnology, York City, NY, USA) to obtain a constitutive expression construct driven by the cauliflower mosaic virus (CaMV). The construct was then transformed into Arabidopsis seeds were surface-sterilized by immersion in 0.1% (w/v) HgCl2 and plated in 1% agar medium supplemented with half-strength [38] Murashige and Skoog nutrients and 3% (w/v) sucrose kept in the dark at 4°C for 3 days to break dormancy. The plates were then transferred to conditions of a 16-h photoperiod, 22°C, 4°C for 3 days to break dormancy. The plates were then transferred to conditions of a 16-h photoperiod, 22°C, 70% relative humidity, and 170-200 μmol m⁻²s⁻¹ light intensity. Uniform one-week-old seedlings were transferred to fresh medium containing various concentrations of mannitol, 1.5 mM H2O2, 300 μM L-buthionine sulfoximine (BSO) or 200 mM mannitol + 300 μM BSO for 2 weeks. Experiments were performed in triplicate.

2.1.6. Osmotic Stress Conditions

Four-week-old soil-grown Col-0, CqMSRA5.1 transgenic A. thaliana, and msra5 (SALK_201557C) plants were exposed to drought stress by withholding water for 2 weeks. Water content was measured to ensure that all genotypes were exposed to equal stress severity. The same amount of water was used for each group. After drought stress treatment, plants were watered and allowed to recover for 3 days before their survival rates were calculated. Each treatment was replicated three times.

2.1.7. Measurement of ROS Levels

ROS levels of Arabidopsis by 3,3′-diaminobenzidine (DAB) staining [39] and nitroblue tetrazolium (NBT) staining [40], in which the samples were immersed in 0.5 mg/mL NBT, dissolved in 10 mM KH2PO4/K2HPO4 (pH 7.6) at 28°C in dark, after which chlorophyll was removed by steeping in 95% ethanol in a boiling water bath.

2.1.8. Yeast-two-hybrid (Y2H) and Bimolecular Fluorescence Complementation (BiFC)

For Y2H, either CqMSRA5.1 or CqGSH2 was cloned into pGBKKT7 (Invitrogen) as the bait, and CqMSRA5.1 or CqGSH2 into pGADT7 (Invitrogen) as the prey. The bait and prey plasmids were co-transformed into yeast strain Y2H GOLD (Clontech), following the manufacturer protocol. Positive yeast transformants were selected on the basis of their ability to grow on SD minimal (-Leu-Trp) and (-Leu-Trp-His) medium and LacZ reporter. The controls chosen were P53-pGBKKT7 + Larget T-pGADT7 (positive interaction), pGBKKT7 + pGADT7 (no interaction).

The BiFC protocol closely followed that [41]. The corresponding expression vectors were introduced into tobacco cells. After an overnight incubation in the dark and then in the light for two days, the YFP signal and chlorophyll autofluorescence were detected by confocal microscopy, using excitation wavelengths of 488 nm and 647 nm, respectively. The OST1-SLAC1 interaction was used as the positive control [42].

2.1.9. Root Length Measurement

The root length of treated and untreated control seedlings was measured using winRHIZO (Régent Instruments Inc., Québec, Canada).

2.1.10. Statistical Analysis

Quantitative data are presented as means ± SE. Means were compared by Student’s two-tailed t-test.

3. Results

3.1. Transcription Profiling of CqMSR Genes

A qRT-PCR analysis showed that all eight CqMSR genes were transcribed in the root, stem, and leaf of quinoa (Figure 1a). Except for CqMSRA5.1 and CqMSRA5.2, the most abundant transcript levels were
present in the stem. Most genes were inducible by abiotic stresses, implying a role in stress response. When plants were exposed to salinity, except for $CqMSRA1.1$, $CqMSRB1.2$, and $CqMSRB2.2$, transcription was enhanced over a 6-12 h period and decreased after 24 h (Figure 1b). With the exception of $CqMSRB1.2$ and $CqMSRB2.2$, drought stress induced genes (Figure 1c). When plants were exposed to heat stress, except for $CqMSRA1$s and $CqMSRB1$s, all genes showed a significant upregulated expression trend (Figure 1d). Cold stress caused significant upregulation of $CqMSRA1.2$, $CqMSRA5.1$, and $CqMSRB1.1$ (Figure 1e).

3.2. Characteristics of $CqMSRA5.1$

To determine where $CqMSRA5.1$ functions in the cell, the gene was transiently expressed with a GFP tag under the control of a 35S promoter in tobacco cells. The green fluorescence of the $CqMSRA5.1$-GFP fusion protein and chlorophyll autofluorescence overlapped, indicating that $CqMSRA5.1$ was expressed in the chloroplast (Figure 2a). To detect in vitro MSR activity, the $CqMSRA5.1$ recombinant protein was expressed and purified. The results of in vitro enzyme activity experiments showed that $CqMSRA5.1$ could reduce Met-S-O to Met (Figure 2b, c). Taken together, the data indicated that $CqMSRA5.1$ is an MSRA gene with MSRA activity.

![Figure 1](https://example.com/figure1.png)

**Figure 1.** qRT-PCR analysis of $CqMSR$ gene expression in different plant organs (a). Expression profile of $CqMSR$ genes under salt (b), drought (c), heat (d), and cold (e) stresses.
3.3. Constitutively Expressed \textit{CqMSRA5.1} Enhanced \textit{A. thaliana} Tolerance to Osmotic Stress

The under normal conditions, no phenotypic difference was observed between Col-0, AtOE lines, and \textit{msra5} plants either \textit{in vitro} (Figure 3a) or in soil (Figure 3d). However, under osmotic stress (100 mM mannitol or 200 mM mannitol) \textit{in vitro} compromised Col-0 plant growth more than AtOE plant growth, whereas \textit{msra5} plant growth was severely inhibited, especially root growth (Figure 3b, c). In soil-based experiments, dehydration affected AtOE lines less than Col-0 or \textit{msra5} plants. Survival rates of Col-0, \textit{msra5}, AtOE1, and AtOE2 plants were ~45.2%, 35.6%, 84.2%, and 75.6%, respectively (Figure 3d, f). Overall, it showed that \textit{CqMSRA5.1} overexpression increases osmotic stress tolerance in \textit{A. thaliana}.

3.4. Heterologous Expression of \textit{CqMSRA5.1} Enhances ROS Scavenging Ability

Resistance to drought stress is frequently associated with resistance to oxidative stress, and the \textit{CqMSRA5.1} function of reducing MetO to Met indicated that it may play a role in oxidative stress resistance. Under controlled conditions, the growth and phenotype of the different plants were indistinguishable (Figure 4a). However, the AtOE lines outperformed Col-0 when exposed to 1.5 mM H$_2$O$_2$ for 14 days (Figure 4b). Primary roots were longer in AtOE lines than in the \textit{msra5} mutant (Figure 4d). Under normal conditions, less H$_2$O$_2$ accumulated in AtOE leaves than in Col-0 leaves, whereas more H$_2$O$_2$ accumulated in \textit{msra5} leaves (Figure 4c). The results of H$_2$O$_2$ content were consistent with those of ROS staining (Figure 4e). In the absence of stress, CAT (Figure 4f) and SOD (Figure 4g) activities were the highest in AtOE leaves and the lowest in \textit{msra5} leaves. Thus, the data indicate that the ectopic expression of \textit{CqMSRA5.1} leads to enhanced ROS-scavenging ability.
Figure 3. Effect of salt or osmotic stress on A. thaliana plants constitutively expressing CqMSRA5.1. Phenotypes of one-week-old transgenic and msra5 mutant A. thaliana seedlings compared with the Col-0 strain grown under conditions of control (a) and 100 mM mannitol (b) or 200 mM mannitol (c). (d) Col-0, transgenic, and mutant A. thaliana seedlings grown in soil under controlled and drought stress conditions. (e) Root lengths of A. thaliana seedlings in a-c. (f) Survival rates of plants after drought stress. Survivor plants were those able to grow after stress removal. Each experimental unit was composed of 20 plants. Data are presented as means ± SE (n = 3). *P < 0.05, **P < 0.01 vs Col-0, by Student’s t-test.

Figure 4. Characterization of A. thaliana strains treated with H$_2$O$_2$. One-week-old A. thaliana seedlings grown under conditions of no stress (a) and 1.5 mM H$_2$O$_2$ (b). (c) Leaves of four-week-old A. thaliana seedlings grown under controlled conditions were stained with DAB (upper panel) or NBT (lower panel). (d) Root lengths of one-week-old seedlings grown under conditions of no stress or treated with 1.5 mM H$_2$O$_2$ for 14 days. H$_2$O$_2$ content (e), CAT activity (f), and SOD activity (g) of seedlings grown under controlled conditions. Data are presented as means ± SE (n = 3). *P < 0.05, **P < 0.01 vs Col-0, by Student’s t-test. DAB, diaminobenzidine tetrahydrochloride; FW, fresh weight; MSR, methionine sulfoxide reductase; NBT, nitro blue tetrazolium.
3.5. *CqMSRA5.1* Overexpression Promotes the Expression of *CqGSH2*, Total GSH Content Increase and GSH/GSSG Ratio Decrease under Osmotic Stress, and Its Protein Interacted with *CqGSH2*

The expression of *AtGSH2* was elevated in AtOE seedlings compared to WT under no stress as well as treated by 200 mM mannitol (Figure 5a). Consistently, the content of GSH and GSSG of AtOE lines was significantly higher than that of WT seedlings under control as well as 200 mM mannitol treatment (Figure 5b) and *msra5* has a significantly lowest GSH and GSSG content. In the present study, the GSH/GSSG ratio assayed in AtOE lines was markedly decreased under stress (compared with control conditions), but still higher than in the WT (Figure 5c). These findings indicate that the expression of *CqMSRA5.1* gene can be regulated via *CqMSRA5.1*-alternations in cellular redox state or a redox signaling pathway in which this protein is involved.

Based on above clues, it inferred that *CqGSH2* was a candidate interactor of *CqMSRA5.1*. To test this hypothesis, Y2H and BIFC assays were performed. As shown in Figure 5d and e, *CqMSRA5.1* and *CqGSH2* interacted in Y2H and BIFC assays, suggesting that *CqMSRA5.1* and *CqGSH2* may function together. In order to more clearly analyze the interaction between the two proteins, we dock the two proteins together, as the results shown in Figure 5f and g. *CqGSH2* protein has thirteen Met residues, three of which are distributed on the surface. Interestingly, Met193 in *CqMSRA5.1* was found located in the junction of the *CqMSRA5.1*-*CqGSH2* complex and highly close to the spatial position of two active Cys residues (Cys70 and Cys227) of *CqMSRA5.1* (Figure 5g).

**Figure 5.** Confirmation of the *CqMSRA5.1*-*CqGSH2* protein-protein interaction. (a) Relative expression level of *AtGSH2* in Col-0, *msra5* and *CqMSRA5.1* transgenic seedlings under control or 200 mM mannitol. (b) GSH and GSSG content of Col-0, *msra5* and *CqMSRA5.1* transgenic seedlings under control or 200 mM mannitol treatment. (c) GSH/GSSG ratio of Col-0, *msra5* and *CqMSRA5.1* transgenic seedlings under control or 200 mM mannitol treatment. (d) Images from a yeast two-hybrid assay in which protein interactions were monitored in SD/-L-T and SD/-L-T-H media. The numbers 1, 10, 100 and 1000 indicate different dilution. PGBK7T-53+pGADT7-Larget T used as positive control and pGBK7T- pGADT7 was negative control. (e) Bimolecular fluorescence complementation assay in tobacco leaves co-transformed with *CqMSRA5.1*-YFPN and *CqGSH2*-YFPC. Scale bar: 20 μm. OST1-SLAC1 interaction was used as the positive control. (f) Three-dimensional visualization of *CqMSRA5.1*-*CqGSH2* complex shown by surface mode. (g) Three-dimensional visualization of *CqMSRA5.1*-*CqGSH2* complex shown by sticks mode. The *CqMSRA5.1* protein was shown in yellow, the *CqGSH2* protein shown in pink, Met residues in *CqGSH2* protein were shown in red and the potential redox Cys residues in TaMSRB5.2 were shown in green. Data are presented as the mean ±SE (n=3). *P<0.05 and **P<0.01 vs Col-0, as analyzed using Student’s t-test. GSH, glutathione; GSSG, oxidized glutathione.
3.6. CqMSRA5.1 Confers Osmotic Stress Tolerance Partly Depends on Its Interaction with CqGSH2

Glutathione is involved in numerous cellular processes including redox homeostasis and redox sensing, which protect cells against oxidative stress caused by reactive oxygen species (ROS) and other free radicals [43]. Therefore, the mechanism of CqMSRA5.1 in the response to osmotic stress was further investigated by using the GSH synthesis inhibitor BSO. When 300 mM BSO was added alone, no significant differences were observed of Arabidopsis plants (Figure 6a). When it was applied in the presence of osmotic stress (200 mM mannitol), the value differences of both the root length between the AtOE lines and the WT were smaller than they were under conditions of 200 mM mannitol treatment alone (Figure 6c), indicating that osmotic tolerance in the AtOE plants was repressed due to the addition of the GSH synthesis inhibitor (Figure 6c). Together, the data infer that the beneficial effect of CqMSRA5.1 on the tolerance of Arabidopsis to osmotic stress depends partly on the modification of GSH by GSH2.

4. Discussion

Plant MSRs play a supporting role in defense against oxidative stress triggered by multiple stresses, including drought stresses, but the functionality in quinoa of members of this family has yet to be ascertained. [6,7,10,12,14,22,41,44,45]. And the function of quinoa MSRA5.1 and its partner together involved in osmotic stress remains unclear. In this paper, we identified CqGSH2 as an interacting partner of CqMSRA5.1, which sheds some light on the mechanism of CqMSRA5.1 gene in quinoa in response to osmotic stress.

4.1. Quinoa MSR Gene Family Responds to Abiotic Stress

Plants have evolved a flexible protein/free amino acid repair system, based on the ability of MSRs to replace MetO with Met [9]. Adaptation to abiotic stress is undoubtedly a complex process involving several biochemical modifications, including the activation or upregulation of MSRs involved in redox balance that are harbored in the plant genome [46]. CqMSRs responded to four abiotic stresses in varying degrees, and almost all CqMSRs were induced by PEG and NaCl stresses (Figure 1b, c). In comparison, few CqMSRs responded to heat and cold stresses. These differential responses indicated that the genes probably belong to different MSR groups and vary in the choice of substrate of their product. CqMSR1.2 was found to be inducible by both drought and salinity stress while CqMSRA1.1 can only be induced by drought stress; CqMSRA5s were found to be inducible by salinity, drought and heat stresses. It was reported that TaMSR5 was induced by both drought and salinity stress. Despite both being members of the same MSR subgroup, there was a distinct difference between TaMSR1 and -A5 in terms of site of activity and structure, resulting in their having a distinctive stress response and physiological role. CqMSRB1.1 was found to be inducible by both drought and salinity stress while CqMSRB1.2 can only be induced by cold stress; It was reported that constitutive expression of TaMSR1.1 in A. thaliana resulted in a heightened tolerance to the stress imposed by either H2O2, MV or NaCl. The rice homolog of this gene, when expressed in yeast, has been shown to enhance resistance
to oxidative stress [8]. *CqMSRB2.1* was found to be inducible by both drought and heat stress while *CqMSRB2.2* can only be induced by heat stress. It was reported that *A. thaliana* plants constitutively expressing TaMSRB5.1, which was inducible by both drought and salinity, were better able to tolerate both these stresses. Thus, although the MSRBs clearly share similarity at the level of protein structure, their specific activity/stability and/or their substrate/target specificity may well not be identical [9]. The most responsive gene *CqMSRA5.1*, which is located in the chloroplast, displayed pronounced changes after the four abiotic stresses in leaves. Given that the chloroplast is a major source of ROS, chloroplast proteins may be more sensitive to damage. The presence of both MSRs and MSRBs implies that oxidized Met in proteins is efficiently repaired in the chloroplast [18]. Compared with *CqMSRA5.1*, *CqMSRA5.2* has a lower response to heat and cold stresses. Although both genes have high sequence similarity, their specific activity/stability and/or their substrate/target specificity may not be identical.

4.2. *CqMSRA5.1* was Involved in Osmotic Stress Resistance

Drought is one of the most detrimental types of abiotic stress in plant agriculture [47]. Adaptation to drought (or osmotic stress) is undoubtedly a highly complex process, involving numerous biochemical modifications, such as the activation or upregulation of MSRs, harbored in plant genome, involved in redox balance [47]. The quinoa MSR member *CqMSRA5.1* deduced peptide revealed a C-terminal PMSR domain, characteristic of MSRA sequences (Figure 2a) and shares a substantial level of homology with MSRA5 proteins in other plant species. The resulting protein reduced Met-S-SO_2 (Figure 3d, f). The above data imply that increased Arabidopsis tolerance to osmotic stress in soil (Figure 2 b and c), a function specific to MSRA. Furthermore, *CqMSRA5.1* overexpression enhanced the total MSR enzyme activity in Arabidopsis. It is known that two plant MSRA homologs, including TaMSRA2 and TaMSRA5, have been reported to enhance salt tolerance that two plant MSRA homologs, including TaMSRA2 and TaMSRA5, have been reported to enhance salt tolerance that two plant MSRA homologs, including TaMSRA2 and TaMSRA5, have been reported to enhance salt tolerance. The above data imply that increased Arabidopsis tolerance to osmotic stress in soil (Figure 2 b and c), a function specific to MSRA.

4.3. *CqMSRA5.1* Promotes Osmotic Stress Tolerance by Regulating ROS Content

At the cellular level, drought induces oxidative stress by overproduction of ROS [49]. Plants have developed a complex ROS scavenging system, containing a series of nonenzymatic compounds and a set of enzymes including SOD, CAT, POD, and ascorbate peroxidase (APX), in which, the latter set could be regulated by many genes including MSR family [14,47,49]. We demonstrated that the constitutive expression of *CqMSRA5.1* in Arabidopsis decreased the accumulation of ROS contents (Figure 4c, e), which could be beneficial from the enhancement of CAT and SOD activities. Interestingly, the expression of a set of *ArbOh* genes including *Rboh C, D, E* and *F* were significantly down-regulated in *ArOE* plants compared to WT. Our previous researches have been reported that overexpression *TaMSR* genes in *A. thaliana* decreased ROS content by up-regulating SOD, CAT, and POD activities, and down-regulating the expression of *Rboh* genes [14,45].

4.4. Biological Significance of the *CqMSRA5.1*/*CqGSH2* Interaction in Osmotic Stress

Oxidation of Met causes dysfunction for many proteins, and in certain cases, this can be restored by MSRs. Numerous proteins have been identified as substrates of MSR in animals and microorganisms in vitro [23]. However, few have been verified as MSR targets in higher plants: AtGST2/3 to AtMSRB7, LcCaM1 to LeMsrA1/B1 [23], TaHO1 to TaMSRA4.1 [14] (Ding et al. 2019) TaHO1 to TaMSRB3.1 [45] and TaGSTF3-A to TaMSRB5.2 [24]. Here, a candidate interactor of CqMSRA5.1, CqGSH2 was screened by STRING database, and identified as one of CqMSRA5.1 interacting partner (Figure 5d, e). Glutathione (γ-glutamylcysteinylglycine; GSH) is an intracellular low molecular-weight tri-peptide which is made up of glutamic acid, cysteine and glycine. It has multiple physiological activities, such as antioxidization, amino acid transport, detoxification and immune, etc [50]. Ascorbate (AsA) and GSH are the two major antioxidant compounds found in plant cells and are known to play a central role in redox regulation via the AsA-GSH cycle, which maintains cellular redox homeostasis [51]. In the present study, CqGSH2 protein has thirteen Met residues, three of which are distributed on the surface. It has been suggested that MSR substrates tend to be Met-rich proteins [7,22]. The Met content of CqGSH2 (2.1%) is higher than the average 1.7% Met found in proteins (1.7%) [52]. As shown in Figure 5f and g, Met193 in CqGSH2 located in the junction of the CqMSRA5.1-CqGSH2 complex and highly close to the spatial position of two active Cys residues (Cys70 and Cys227) of CqGSH2. We speculate that Met193 is beneficiary from this interaction. *CqMSRA5.1* overexpression in Arabidopsis not only increased the transcription level of *AtGSH2* (Figure 5a), but also elevated the GSH content (Figure 5b). *CqMSRA5.1* overexpression in Arabidopsis decreased the level of GSSG leading to increase the GSH/GSSG ratio (Figure 5c). The addition of BSO (a GSH synthesis inhibitor) led to partial suppression of the AtOE lines’ level of tolerance to osmotic stress (Figure 6b, c). Combination CqMSRA5.1 interacts with CqGSH2 and both localized in the chloroplast (Figure 1c) [13], their encoding genes were highest expressed in leaf (Figure 1a; [53]), induced by osmotic stress (Figure 1c), and positive role in response to abiotic stress (Figure 1, Figure 2, Figure 4; [54]), and physical interaction between them (Figure 5d, e), it suggested the osmotic tolerance conferred by CqMSRA5.1 depends partly on its interaction with CqGSH2. The discovery of substrate of CqMSRA5.1 will help to fully explain its effect on
osmotic stress tolerance and pave a way for explaining how CqMSRA5.1 acts on ROS pathway.

5. Conclusion

Our findings suggest that CqMSRA5.1, induced by osmotic stress, is involved in the response to osmotic stress by promoting the ROS scavenging pathway and decreasing ROS content. CqMSRA5.1 interacts with, and promotes its potential substrate CqGSH2, partly contributing to the stress response mechanism. How the ROS pathways in quinoa are modified by either or both of these proteins requires further investigation. The present study confirms the abiotic function and mechanism of CqMSRA5.1 in quinoa, and provides information on a gene for breeding quinoa tolerant to drought.

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