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The ectopic expression of methionine sulfoxide reductase 4 results in enhanced growth performance in arabidopsis

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ABSTRACT

In response to challenging oxidative environments, most aerobic organisms have developed cellular defense systems that either repair or degrade damaged proteins to prevent them from adversely affecting cellular reactions. For instance, oxidized methionine can be reduced to methionine by the enzyme methionine sulfoxide reductase (MSR). Previously, we reported that the *Arabidopsis* gene *MSR4* (AT4G25130) is induced by salt stress, and during our investigations into this gene, we generated a transgenic *Arabidopsis* that overexpressed a mutant form of *MSR4* (*mMSR4*). The transgenic plants exhibited enhanced sensitivity to high salt stress. Consistent with this observation, the ectopic expression of MSR4 enhanced growth performance in normal as well as in stress medium. To determine the targets of MSR4, we performed two-dimensional electrophoresis analyses and observed a significant reduction in the levels of chloroplast heat shock proteins, which may explain the enhanced salt stress sensitivity of *mMSR4* transgenic plants.

1. Introduction

In response to challenging oxidative environments, most aerobic organisms have developed cellular defense systems. Oxidative stress generates reactive oxygen species (ROS) that result in DNA strand-breakage, as well as protein and lipid oxidation. However, the role of ROS in biotic and abiotic stress signaling remains to be elucidated. ROS production mainly occurs in the plasma membrane because of the presence of NADPH oxidases. ROS production also takes place in chloroplasts, mitochondria and peroxisomes during the dysfunction of metabolic processes involved in photosynthesis and respiration [1–4]. Recently, it has been reported that chloroplast-generated reactive oxygen species result in localized cell death in plants, in part during the non-host interaction [5]. Protein oxidation results in the formation of nonfunctional proteins [6] and these damaged proteins must be repaired or degraded, since they can adversely affect cellular reactions. In human, it was shown that the antioxidant system for ROS scavenging is very efficient, especially during the first 45 years of life, leading to the degradation of ROS-modified proteins [7]. However, after 45 years, the antioxidant and degradation systems become progressively inactivated due to the higher rate of ROS influx into the cell.

The amino acid methionine (Met) is particularly prone to ROS-mediated oxidation, forming two methionine sulfoxide (MetO) stereoisomers, methionine-*S*-sulfoxide (MetO-S) and methionine-*R*-sulfoxide (MetO-R). Recently, one enzyme called a peptide methionine sulfoxide reductase (Msr) has received considerable attention, which can reduce Met(O) residues in proteins and free Met(O), back to Met [8]. The enzymes methionine sulfoxide reductase A and B (MsrA and MsrB) catalyze the reduction of the epimers (MetO-S and MetO-S, respectively) to methionine together with the reduction of thioredoxine [9,10]. After first purification from *Escherichia coli*, the *msrA* gene has now been cloned from various species including bacteria, yeast, plant, insects, and other animals [11–13].

The key role played by MSRs in cell and organism responses to ROS has been investigated using gene knockout or overexpression [14,15]. In yeast, bacteria and mice, *MsrA* knock-out mutants exhibited enhanced sensitivity to ROS, whereas overexpression resulted in ROS resistance. For instance, msrA mutants of *E. coli* were found to be much more sensitive to oxidative stress than wild-type cells [16,17]. However, the wild-type msrA gene that was isolated from either *E. coli* or *Mycobacterium tuberculosis* was able to restore the wild-type phenotype in the *E. coli* msrA mutant [18]. The authors also showed that the mutant form with an altered *E. coli* msrA gene (cysteine at position 52 was changed into serine) cannot complement the *E. coli* msrA mutant. These results implies that under certain conditions the inability to reduce Met(O) residues in proteins could

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be a lethal event. Similar results were also obtained in yeast cells [19]. Furthermore, it has been suggested that MSR function (repair of oxidized proteins) is tightly associated with increased longevity, since a marked reduction in MSR activity has been observed during rat aging [20]. Many studies have established that MSR plays a crucial role in plant oxidative stress. Currently, rice OsMSRA4.1 and OsMSRB1.1 have been demonstrated to confer enhanced cellular resistance to oxidative stress in yeast [21]. In agreement with this observation, transgenic rice with OsMSRA4.1-constitutive expression exhibited increased viability when exposed to salt stress.

It is known that virtually all organisms possess MSRA and MSRB [22–26]. *Arabidopsis thaliana* possesses multiple members of two MSR gene families, i.e., 5 *MsrA* and 9 *MsrB* genes [27]. We have previously reported that the *Arabidopsis MSR4* gene is induced by salt stress [28], and during our investigation into the cellular functions of MSR4, we generated a transgenic *Arabidopsis* line overexpressing a mutant form of the *MSR4* gene. Interestingly, this transgenic line exhibited enhanced sensitivity to high salt stress. Consistent with this observation, the ectopic expression of MSR4 enhanced growth performance in normal as well as in stress medium. Furthermore, in this study, we performed two-dimensional electrophoretic (2DE) analyses on the transgenic plants, which showed that these plants have reduced levels of chloroplast heat shock proteins. The results suggest that lower levels of these proteins may be involved in the enhanced salt stress sensitivity of the line overexpressing the mutant MSR4 protein.

2. Materials and methods

2.1. Plant materials and growth/stress treatment conditions

Transgenic and Col-0 ecotype *A. thaliana* plants were used in this study. Plants were seeded on Murashige and Skoog (MS) 0.6% agar supplemented with 2% sucrose. After 3 days of stratification at 4 °C, seeds were allowed to germinate and grow at 23 ± 1 °C under cycles of 16 h light and 8 h darkness, in 70% relative humidity before stress treatments.

2.2. Gene amplification and transformation of Arabidopsis

Full-length cDNA encoding *Arabidopsis* MSR4 that contained specific point mutations was amplified by PCR and cloned into *pBI121*, and expression was driven by the cauliflower mosaic virus (*CaMV*) 35S promoter. To obtain transgenic plants, *Agrobacterium*-mediated transformations were performed according to Lee et al. [29]. Homozygous T_4 transgenic plants were used for all experiments.

2.3. Methionine sulfoxide reductase (MSR) enzyme activity assay

MSR activity was measured as described previously by Moskovitz [14]. Reaction mixtures (100 μl) contained 20 mM Tris–Cl (pH 7.4), 10 mM $MgCl_2$, 30 mM KCl, 20 mM dithiothreitol (DTT), 1 mM dabsylated L-methionine-R, S-sulfoxide and MSR. Reactions were incubated at 37 °C for 1, 3 or 5 h. Following addition of 100 μl acetonitrile to stop the reaction, samples were centrifuged and 20 μl of supernatant was injected into a Supelcosil LC-18T (Supelco Inc.) column containing a Supelguard LC-18 pre-column (Supelco Inc.). The column was pre-equilibrated with 0.14 M sodium acetate buffer (pH 6.1) containing 0.5 ml/l triethylamine and 30% acetonitrile. A linear acetonitrile gradient (from 30 to 70%) was applied to the column and dabsylated products were analyzed using Agilent 1100 HPLC (Agilent Technologies), at a flow rate of 1 ml/min and detection at 436 nm.

2.4. Northern blot analysis

A. thaliana Col-0 (wild-type) plants were grown on standard MS 0.6% agar supplemented with 2% sucrose, in lighted growth chambers for 3 weeks. The seedlings were then treated with mannitol, ABA (100 μM) or NaCl (300 mM). Total RNA was extracted from whole seedlings (~0.3 g), as described previously [29], and then 20 μg was added to each lane of a 1.5% (w/v) agarose gel containing 38% formaldehyde and separated by electrophoresis. The RNA was then transferred to a Hybond^{TM}-XL membrane (Amersham Biosciences, NY), which was pre-hybridized at 42 °C for 1 h in a buffer containing 10× SSC, 50× Denhardt's solution, 20% (w/v) SDS and 10 mg/ml denatured salmon sperm DNA. ^{32}P-random-primed probes were hybridized separately onto the membrane at 42 °C for 18 h. Blots were washed twice at 45 °C in 1× SSC, 0.1% SDS for 30 min, and then rewashed at 45 °C in 0.1× SSC, 0.1% SDS for 30 min. For visualization of hybridization, the membrane was exposed to X-ray film and developed. cDNAs of *Cor15a* and *KIN1* were used as probes.

2.5. ROS (H_2O_2) staining and measurements

H_2O_2 detection in 7-day-old seedlings was performed as described by Thordal-Christensen et al. [30], with some modifications. The seedlings were washed several times in 50 mM potassium phosphate buffer (PBT) and incubated for 10 min in 1 ml PBT containing 0.3 mg DAB (3,3-diamionbenzidine). For color reactions, 1 μl 30% H_2O_2 was added to each tube and then each seedling was rinsed twice with PBT for 5 min. Samples were dehydrated using 100% methanol.

For reactive oxygen species (ROS) measurements, we harvested approximately 1000 seedlings. After cell lysis, supernatants were retrieved by micro-centrifugation. The contents of hydrogen peroxide were measured at 560 nm using a Bioxytech H_2O_2-560 assay kit (OXIS International Inc.). This assay relies on the oxidation of ferrous ion (Fe^{2+}) to ferric ion (Fe^{3+}) by ROS under acidic conditions. For normalization, protein concentrations were determined using a Coomassie Blue dye-binding assay [31].

2.6. Two-dimensional gel electrophoresis (2DE) and Western blot analysis

2.6.1. Protein sample preparation

Cultured bacterial cell pellets were washed twice with ice-cold PBS and sonicated for 10 s using a Sonoplus (Bandelin Electronic, Germany). Plant tissues were homogenized (PowerGen125, Fisher Scientific) in sample lysis solution containing 7 M urea, 2 M thiourea, 4% (w/v) 3-([3-cholamidopropy] dimethyammonio)-1-propanesulfonate (CHAPS), 1% (w/v) dithiothreitol (DTT), 2% (v/v) pharmalyte and 1 mM benzamidine. Proteins were extracted for 1 h at room temperature with vortexing. After centrifugation at 15,000 × g for 1 h at 15 °C, insoluble material was discarded and the soluble fraction was used for 2DE. Protein loading was normalized using the Bradford method.

2.6.2. 2DE

IPG dry strips were equilibrated for 12–16 h with 7 M urea, 2 M thiourea, 2% CHAPS, 1% DTT and 1% pharmalyte. Isoelectric focusing (IEF) of samples (200 μg) was performed at 20 °C using a Multiphor II electrophoresis unit and EPS 3500 XL power supply (Amersham Biosciences), following the manufacturer's instructions. For IEF, the voltage was increased linearly from 150 to 3500 V over 3 h during sample entry, after which a constant 3500 V was maintained until focusing completion at 96 kVh. Prior to second dimension separation, strips were incubated for 10 min in equilibration buffer (50 mM Tris–Cl [pH 6.8], 6 M urea, 2% SDS and